9-2023

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Recommended Citation

Kim, Jinha, et al. "Advances and perspectives of using stable isotope probing (SIP)-based technologies in contaminant biodegradation." Water Research X (2023): 100187. https://doi.org/10.1016/j.wroa.2023.100187

Water Research X 20 (2023) 100187

Contents lists available at ScienceDirect

Water Research X

journal homepage: www.sciencedirect.com/journal/water-research-x

Advances and perspectives of using stable isotope probing (SIP)-based technologies in contaminant biodegradation

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ARTICLE INFO

Keywords:
Stable isotope probing (SIP)
Biodegradation
Co-metabolism
RDX

ABSTRACT

Stable isotope probing (SIP) is a powerful tool to study microbial community structure and function in both nature and engineered environments. Coupling with advanced genomics and other techniques, SIP studies have generated substantial information to allow researchers to draw a clearer picture of what is occurring in complex microbial ecosystems. This review provides an overview of the advances of SIP-based technologies over time, summarizes the status of SIP applications to contaminant biodegradation, provides critical perspectives on ecological interactions within the community, and important factors (controllable and non-controllable) to be considered in SIP experimental designs and data interpretation. Current trend and perspectives of adapting SIP techniques for environmental applications are also discussed.

Introduction

In natural and engineered environments, microorganisms live together within communities where they interact with each other through competition, mutualism, amensalism, commensalism, predation, and sometimes with no interaction. Microorganisms within the communities also respond to stressors and stimuli in their surrounding environment, resulting in dynamic microbial community structure and functions which are assembled through two mechanisms: deterministic (i.e., natural selection) or stochastic (i.e., random dispersion, cell growth, cell death, immigration, and speciation.) (Pholchan et al., 2013; Faust and Raes, 2012). While information of 16S rRNA genes, RNA genes, and amino acid sequences have provided a better understanding of microbial community structures, or functions, or implicated ecological functions within engineering and natural systems (Hugerth and Andersson, 2017; Venkataraman et al., 2015; Wang et al., 2013, 2009), the linkages between the structure and their function were challenging and difficult (Aitchison, 1982; Gloor et al., 2017) until the applications of stable isotope probing (SIP) techniques.

The concept of using stable isotopes to study microbial function can be dated back many decades ago. In 1958, Meselson and Stahl reported the first study of using ^{15}N-labeled nitrogen to validate the hypothesis of the semi-conservative DNA replication rule (Meselson and Stahl, 1958). Yet, the application of stable isotopes to link microbial function to metabolically active microorganisms was realized much later. In 2000, Radajeweski and co-workers reported the first study using ^{13}C-labeled methanol and/or methane to identify the presence and diversity of methylotrophic populations in natural environments based on DNA-based SIP (Colin Murrell and Radajewski, 2000; Morris et al., 2002; Radajewski et al., 2000). Through DNA-based SIP, researchers can track the flow of the isotopes in the labeled compound to the microbial cellular components such as DNA in the metabolically active microbes within a complex community. After separation of the labeled DNAs from non-labeled DNAs through concentration gradient ultracentrifugation, labeled DNAs contributed from the metabolically active microbes are then sequenced and then used to identify the active microbes. Years later, DNA-based SIP with ^{13}C-labeled multi-carbon substrates such as glucose, phenol, caffeine, and naphthalene (Jeon et al., 2003; Padmanabhan et al., 2003; Yu and Chu, 2005) and with ^{15}N-labeled explosive (Roh et al., 2009) were applied to identify active contaminant degraders in soil, wastewater treatment, and groundwater/aquifer sediments. Recent applications of DNA-based SIP to understand biodegradation of various priority and emerging contaminants are summarized and discussed in the section of SIP applications with different isotopically labeled compounds below.

Meanwhile, researchers explored SIP with different biomarkers such as RNA, proteins, lipids, and metabolites to probe unknown metabolic reactions, unique microbial functions, and to explore unknown

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https://doi.org/10.1016/j.wroa.2023.100187
Received 12 April 2023; Received in revised form 18 May 2023; Accepted 6 June 2023
Available online 7 June 2023

interactions of active microbial populations at varying levels (Kruse et al., 2013; Manefield et al., 2002; Tsugawa et al., 2019; von Bergen et al., 2013). Based on the choice of biomarkers, SIP-based techniques have been designated as DNA-SIP (Radajewski et al., 2000), RNA-SIP (Fortunato and Huber, 2016; Manefield et al., 2002), fatty acid methyl esters (FAME)-SIP (Lerch et al., 2009, 2007), or Protein-SIP (Jehmlich et al., 2008). The above SIP-based technologies also coupled with advanced multi-omics such as metagenomics (Chen and Murrell, 2010), transcriptomics (Nuccio et al., 2021), proteomics (von Bergen et al., 2013), and metabolomics (Birkemeyer et al., 2005; Hassanpour and Aristilde, 2021; Hou et al., 2021; Tsugawa et al., 2019; Wilhelm et al., 2022), advancing our understanding of microbial structure and function in various environments. SIP with fluorescence *in situ* hybridization (FISH) (Huang et al., 2007; Orphan et al., 2001; 2002) has been utilized but it is time-consuming. This drawback is overcome by Chip-SIP (Mayali et al., 2012, 2019) which enables high-throughput processing of labeled rRNA gene sequences by using RNA microarrays. More recently, SIP has been coupled with multiple advanced techniques including meta-omics, Raman microspectroscopy, or nanoscale secondary ion mass spectrometry (NanoSIMS), collectively referred to as single-cell SIP (SC-SIP). Raman microspectroscopy can directly reveal the biochemical fingerprints of a single cell without labeling, and NanoSIMS can create nanoscale maps of element or isotope distribution of a sample. Thus, when integrating these techniques to track isotopic signals in a single cell, the SC-SIP becomes a powerful non-destructive method in studying microbial ecology particularly for a better understanding of spatial variations of cellular metabolism, phenotypic heterogeneity, and cellular interactions such as syntrophy or cross-feeding within a complex community (Berry et al., 2015; Chen and Murrell, 2010; Lee et al., 2021; Wagner, 2009). Excellent reviews on SC-SIP techniques are currently available elsewhere (Alcolombri et al., 2022; Hatzenpichler et al., 2020). To quantify the variations of the growth of functional microbes in a complex community, a quantitative SIP, called qSIP, was developed by integrating ^{18}O-H_2O and a ^{13}C-labeled substrate, and quantitative PCR (qPCR) and metagenomics based on 16S rRNA genes (Hungate et al., 2015). Particularly, qSIP has been demonstrated to be powerful in quantifying taxon-specific substrate assimilation rate or flux variations by glucose-utilizing microorganisms in soil samples (Hungate et al., 2015). Subsequently, qSIP has been applied to study various research topics including legacy carbon turnover in soil, soil bacteria involved in assimilation of fertilizer nitrogen and soil carbon, litter decomposition in freshwater, and PAH degradation (Coskun et al., 2018; Dong et al., 2022; Hayer et al., 2016; Hungate et al., 2015; Sieradzki et al., 2021).

Fig. 1 briefly illustrates timeline of the advances of SIP-based technologies with different biomarkers and advanced technologies. A comparison of selected key SIP-based technologies is briefly summarized in Table 1. Excellent reviews on those key SIP-based technologies and their applications to public health and host-microbe interactions are available (Berry and Loy, 2018; Berry et al., 2013; Bruntz et al., 2017). The advantage and disadvantage over the choice of different labeled biomarkers such as PLFA (phospholipid-derived fatty acid), RNA, DNA in SIP microbial ecology studies has been discussed in previous reviews (Manefield et al., 2004; Neufeld et al., 2007). However, many ecological aspects and technical issues that commonly complicate SIP application and data interpretation are rarely discussed.

To this end, this review discusses various ecological aspects and technical issues of SIP, with a focus on biodegradation and

Table 1
Comparisons of several key SIP-based technologies (revised from Alcolombri et al. 2022).

Techniques	Target	Comments	Refs.
DNA-SIP	DNA	• Can identify phylogenetic information of active members of a community • Can be deployed in the environment simply • Is a destructive method • Is unable to detect single-cell variations • Is unable to provide spatial information	(Jehmlich et al., 2016; Uhlik et al., 2013b)
RNA-SIP	RNA	• Is limited by low yields of retrieved mRNA • Is difficult to clone mRNA and separate them by centrifugation	(Jehmlich et al., 2016 ; Neufeld et al., 2007)
Protein-SIP	amino acid	• Could provide phylogenetic and functional information together • Requires high volume of cells ($>10^5$)	(Jehmlich et al., 2016)
Chip-SIP	RNA	• Is useful to apply to diverse systems • Can analyse stable isotope assimilation to rRNA	(Mayali et al., 2019)
FAME-SIP	lipid	• Is relatively more sensitive than DNA/RNA-SIP	(Lerch et al., 2009)
SC-SIP	Whole-single cell	With NanoSIMS • Provides information on phenotype, genotype, and spatial arrangement directly • Is a destructive method With Raman microscopy • Is available with minimal sample preparation • Is difficult to detect nitrogen stable isotopes • Can be interfered by autofluorescence • Is a non-destructive method	(Alcolombri et al., 2022)
qSIP	DNA/ RNA	• Provides quantitative assessment of assimilation rates of labeled substrates • Is a destructive method	(Hungate et al., 2015; Papp et al., 2018; 2020)
HT-SIP	DNA	• Offers automated high-throughput sample processing • Is a destructive method	(Nuccio et al., 2022)

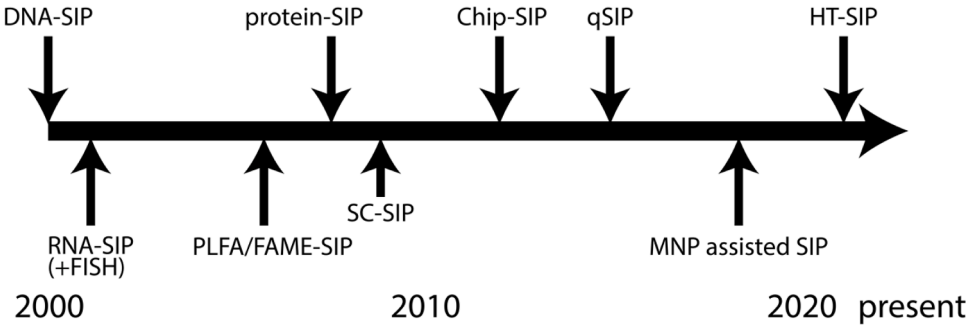

Fig. 1. Timeline of the development of SIP-based techniques.

bioremediation of environmental contaminants. Mostly, factors such as variation of catabolic mechanisms, and key technical aspects that are frequently overlooked, and consideration in interpreting data obtained from SIP-based studies for contaminant degradation processes are also reviewed. Current trends and perspective of adapting SIP techniques for studying co-metabolic biodegradation of environmental contaminants and their environmental applications are also identified and discussed.

Growth-linked mechanisms are the key in labeling metabolites and cellular components of metabolically active microbes

When a labeled substrate is degraded inside metabolically active microorganisms, relying on the growth-linked mechanisms of the microbes, the flow of isotopically labeled atoms in the substrate is first converted to labeled metabolites, which are further degraded or incorporated into a wide range of internal cellular components in the metabolically active microorganisms, with some secreted extracellularly (Fig. 2). Each of the labeled metabolites and cellular components such as DNA and RNA, fatty acids and lipids in cell membranes, intracellular and extracellular proteins, and secreted materials such as soluble microbial products (SMP) and extracellular polymeric substances (EPS) can be tracked individually or combined to investigate previously unknown microbial taxonomy and functions (Jehmlich et al., 2016; Kruse et al., 2013; Wang et al., 2015; Xiao et al., 2022). Biomarker DNA has been the simplest means to identify microbial taxa that linked to the metabolic active microbes; while characterization of the biomarker RNA provides direct link of microbes that are specifically active to certain trophic environments (Dumont et al., 2013; Fortunato and Huber, 2016; Radajewski et al., 2000). Profiling of labeled proteins, particularly enzymes involved in biodegradation of contaminants, allows identification of amino acid sequences of the units that directly responsible for the microbial functions (Jehmlich et al., 2008). Characterization of the assimilated FAME could fingerprint active microbial community compositions (Evershed et al., 2006) and the assimilated SMP or EPS could be used as substrates to evaluate the utilization of metabolic products by other bacteria (Wang et al., 2015; Xiao et al., 2022).

Tracking the flux of the labeled metabolites have provided valuable insights of a specific metabolic network in biological systems such as plants and the new insights have been used to guide design in metabolic engineering, biotechnology, and microbiology (Birkemeyer et al., 2005; Fan et al., 2012; Tsugawa et al., 2019). Recently, by tracking labeled metabolites (i.e., SIP-metabolomics) derived from ^{13}C-glucose that was spiked into oxic or anoxic soil, ^{13}C enrichment dynamics of metabolites under different redox conditions were revealed (Hassanpour and Aristilde, 2021). By tracking ^{13}C from nine isotopically labeled carbon sources into the water-soluble soil metabolite pool over 48 days, researcher observed that the type of carbon source amended into the soils caused the production of different metabolites, and that the fate of the carbon in the metabolites in soils were not controlled by time, but due to their differences, enhancing our understanding of soil carbon cycling (Wilhelm et al., 2022). However, unlike common carbon source compounds, studies using certain extracellular products, such as SMP and EPS, as biomarkers are challenging, and such applications have not been reported to our best knowledge.

While a vast amount of information pertaining to biological activity can be inferred from the labeled cellular components (such as RNA, proteins, lipids), mapping such information back to the taxonomy of the associated microbes in the community remain very challenging, and often impossible. Accordingly, evaluating DNA with assimilated stable isotopes (i.e., DNA-SIP) remains as the simplest and most straight forward approach to identify active microbial community in complex environmental matrices. The following sections describe the advances of DNA-based SIP to environmental science and engineering.

SIP studies with different isotopically labeled compounds (i.e., ^{13}C, ^{15}N, ^{2}H, ^{18}O, $^{33/34}S$) allow for interrogating various microbial-meditated processes in the environment

(i) ^{13}C-DNA-SIP studies have been expanded from using C1 to multi-carbon substrates

^{13}C-Labeled substrates are the most frequently used in SIP studies, as carbon is an energy and/or carbon source required for microbial growth. Since the pioneer microbial ecology work of using ^{13}C-labeled methanol and methane to identify active methylotrophic populations in soil (Morris et al., 2002; Radajewski et al., 2000), the ^{13}C-DNA-SIP technique has been quickly adapted to study biodegradation and bioremediation of many environment contaminants, including ^{13}C-labeled multi-carbon aliphatics and aromatics suh as ethane, propane, n-hexadecane, polyaromatic hydrocarbons (PAHs), benzene, toluene, ethylbenzene, xylene, methyl tert-butyl ether, bisphenol A, triclosan, pesticides, estrogen, synthetic lignin, organic solvents and stabilizers (Bao et al., 2022; Cupples, 2016; Dang and Cupples, 2021; Farhan Ul Haque et al., 2022; Hu et al., 2021; Jiang et al., 2018; Kasanke et al., 2019; Lee et al., 2014; Levy-Booth et al., 2022; Liu et al., 2019; Sathyamoorthy et al., 2018; Sun et al., 2022; Thomas et al., 2019; Wilhelm et al., 2019; Zhang et al., 2021a). Recently, ^{13}C-labeled acetate or carbonate has been used to better understand the identities of microbes involved in denitrification and anaerobic ammonia oxidation (ANAMMOX) (Bellini et al., 2017; Xing et al., 2018; Zhang et al., 2021b)

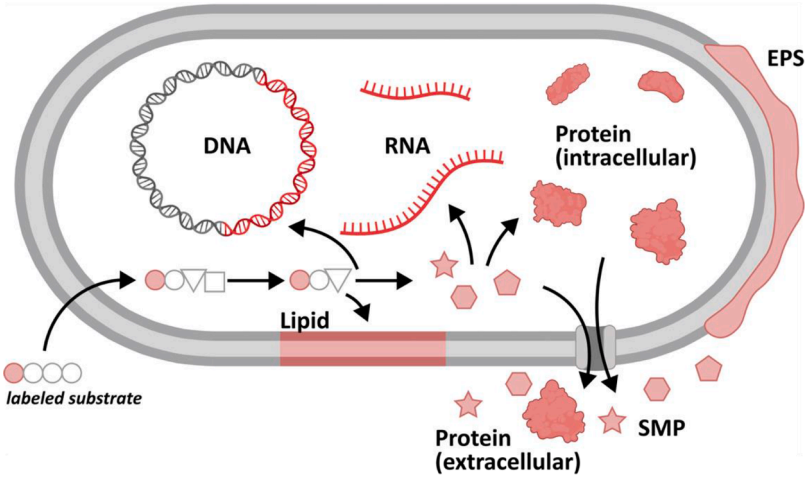

Fig. 2. The flow of labeled atoms from the growth substrate into various degradation metabolites and different cellular components such as DNA, RNA, lipids, proteins, soluble microbial products (SMP), and extracellular polymeric substances (EPS). The red color was used to show the location of labeling.

or enhanced biological phosphorus removal processes (Guo et al., 2018; Wang et al., 2020a). Using partially labeled 17β-estradiol ($^{13}C_{3,4}$-E2) have shown to identify distinct E2-assimilating bacterial species compared to its non-labeled counterpart ^{12}C-E2 (Zhang et al., 2021a). Yet, SIP studies are typically limited by the high cost of labeled contaminants and the availability of labeled contaminants with desired labeling locations and numbers.

(ii) ^{15}N-DNA-SIP studies identify microbes using nitrogen-containing pollutants as nitrogen source

Nitrogen stable isotope, ^{15}N, is the next most frequently used stable isotope in DNA-SIP to study biodegradation of nitrogen-containing pollutants such as hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) (a nitroamide explosive) (Ariyarathna et al., 2019; Cho et al., 2016, 2015; Roh et al., 2009), polyhexamethylene biguanide (PHMB) (a nitrogenous disinfectant and antibiotic) (O'Malley et al., 2007), and atrazine (a herbicide) (Shaffer et al., 2010). These studies identified microbes capable of using the contaminants as their sole nitrogen source. An interesting approach was to use ^{15}N-$NH_4H_2PO_4$ as a sole nitrogen source to label active hydrocarbon-degrading degraders in microcosms, since active hydrocarbon degraders also required ^{15}N as nitrogen source when utilizing hydrocarbon for growth (Bell et al., 2011). By using ^{15}N-DNA-SIP in partial nitrification/ANAMMOX processes, autotrophic denitrification *via* anammox pathway was found dominant in ammonia-rich wastewater and symbiotic relationships among anammox bacteria, Anaerolineae, Proteobacteria, and Planctomycetacia during the nitrogen conversion were revealed (Li et al., 2019).

(iii) ^{2}H-H_2O or ^{18}O-H_2O DNA-SIP studies assess active microbial population within a microbial community based on water availability

Unlike carbon and nitrogen that are linked to specific metabolic processes, all microorganisms ubiquitously require and utilize water for growth. Thus, the stable isotope of hydrogen (i.e., ^{2}H; deuterium) or oxygen (i.e., ^{18}O) within H_2O can be applied to assess all active microbes. This concept has inspired researchers to apply ^{18}O-H_2O-DNA-SIP to identify toluene degraders (Woods et al., 2011), and to assess rapid responding microbial populations followed of soil rewetting after significant drought (Aanderud and Lennon, 2011). A recent ^{18}O-H_2O-DNA-SIP study reported that moisture contents in soils shaped active microbial composition through potential increase of osmotic regulatory and transport pertaining genes with the implementation of metagenomics (Dai et al., 2021). Furthermore, using deuterated water (i.e., D_2O, ^{2}H-H_2O) along with Raman microspectroscopy (Berry et al., 2015) (i.e., SC-SIP) and metaproteomics had identify and differentiate active groundwater microbes that were capable of degrading veratric acid or methylamine (Taubert et al., 2018).

(iv) $^{33/34}$S -labeled SIP studies were mainly for proteomic studies

While sulfur is an essential nutrient, the requirement for sulfur is much lower compared to that for carbon or nitrogen during microbial assimilation processes. As no sulfur is present in nucleotides, applications of isotopically labeled sulfur (i.e., $^{33/34}$S) in DNA-SIP or RNA-SIP are not possible. Yet, ^{34}S has been used to evaluate proteomic shifts in *Pseudomonas fluorescens* during naphthalene oxidation, suggesting that naphthalene might exert oxidative stress responses during the degradation (Herbst et al., 2013).

SIP studies with dual or multiple isotopically labeled compounds decipher guild structures with unique degradation abilities in the microbiome

Few recent studies have applied DNA-SIP with a compound containing single or multiple isotopes with different labeling profiles, based on labeling positions and types of stable isotopes within a compound, in contaminant biodegradation (Fig. 3). Ggood examples are DNA-SIP studies on biodegradation of RDX, a widely used cyclic nitramine-based munition since World War II. RDX is toxic and commonly detected in groundwater and soil near munition manufacturing facilities, hand-grenade ranges, antitank rocket ranges, bombing ranges, artillery ranges, munitions testing sites, explosives washout lagoons, demolition areas and open burn/open detonation sites. RDX contains six nitrogen atoms with three in its C-N ring and other three in the nitro functional groups attached on the ring. To decipher the guild of microbes capable of using nitrogen or carbon from RDX, ^{15}N-nitro-, ring-, fully-labeled RDX or ^{13}C-labeled RDX were applied in DNA-SIP studies using RDX-contaminated aquifer materials (Cho et al., 2016, 2015). Different guild of microbes capable of utilizing the nitrogen from the ring and the nitro-groups, or both for growth, were identified and previously unknown guild of microbes capable of using RDX as carbon sources were observed. By comparing the guild of microbes, it was possible to decipher degraders with the ability to use RDX as both carbon and nitrogen sources, resulting in new knowledge for developing effective RDX bioremediation in the future (Cho et al., 2015).

Application of co-labeling, i.e., using a combination of ^{13}C and ^{15}N isotopes in a single compound like glyphosate (Fig. 3) has led to identification guilds of microbes capable of assimilating glyphosate as carbon and/or nitrogen source, and their relative contributions to the degradation of the respective labeled nutrients through metabolite analysis (Wang et al., 2016). This approach has led to discoveries of speciation of non-extractable glyphosate metabolites and its degradation capability as both carbon and nitrogen source within environmental systems.

In a very recent SC-SIP study (based on NanoSIMS), ^{33}S-sulfate was applied along with ^{13}C-acetate and ^{15}N-ammonium to enable identification of spatial distribution of certain groups of microbes, mainly to Gamma- and Deltaproteobacteria, in biofilm due to assimilation of differernt isotopes (i.e., ^{33}S, ^{13}C, ^{15}N) (Dawson et al., 2016). Also, as described previously, coupling ^{18}O-H_2O with ^{13}C-labeled compounds and qPCR, a quantitative SIP (qSIP) was powerful in assessing different stable isotope assimilation rates of taxa-specific groups in soil microcosms (Hungate et al., 2015).

Technical considerations in SIP-based studies

The abundance and diversity of individual microorganisms within a community are subjected to evolve and change over time, which is, in part, due to the various microbial interactions and interactions with their surrounding environment. Five different microbial interactions – competition, commensalism, syntrophy, predation, amensalism, and no interaction – occur constantly in natural environment. On the top of these interactions, the geochemical properties, available nutrients, and/or other physical constituents in their surrounding environment can also serve as stimuli and stressors that further modulate the structure and function of the microbial community. Thus, these aspects (ecological interactions and the overall responses to environmental stresses and stimuli) need to be carefully considered during SIP experimental design and data interpretation, since non-targeted microbes might be labeled due to different incubation periods, quantities of the labeled substrate applied, and characteristics of the target microbes (Whiteley et al., 2006).

(i) Factors affecting data interpretation of SIP-based studies

Several factors including cross-feeding (Mooshammer et al., 2021), G+C content (Cadisch et al., 2005; Cupples et al., 2007; Lueders et al., 2004) and degree of isotope enrichment (Neufeld et al., 2007) can result in bias in SIP data and interpretation. While several SIP-based protocols are available, there are no standardized SIP experimental designs to tackle various scientific questions in biodegradation of a wide range of

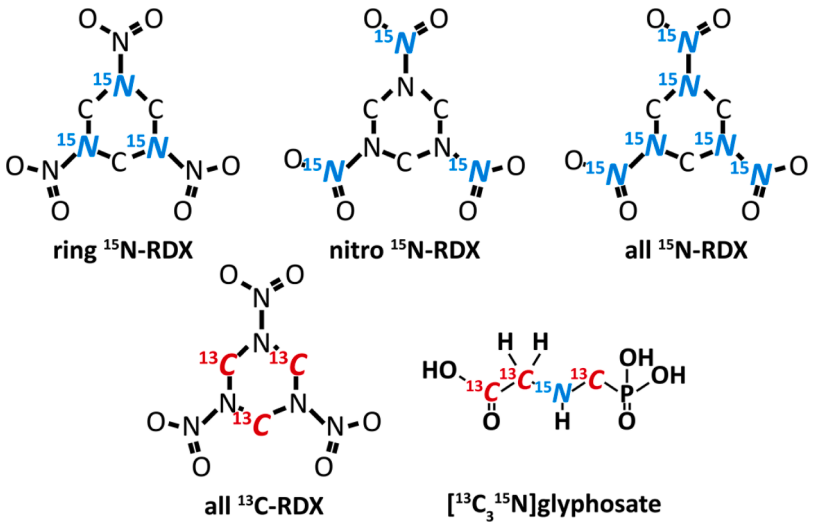

Fig. 3. Recent studies using single isotope labeled in different locations of RDX structure (Cho et al., 2016, 2015, 2013) or dual isotopes in glyphosate (Wang et al., 2016) to identify guild microbial populations responsible degrading the labeled nutrients (carbon or nitrogen, or both) for growth.

environmental contaminants. As environmental contaminants are typically multi-carbon compounds with various degradation potential under different experimental and/or environmental conditions, it is also important to consider the number, location, and type of isotopes in the labeled compounds to be used in a SIP biodegradation study and scientific questions to be answered.

Studies using single or multiple ^{13}C in a multi-carbon substrate have brought new insights into microbial community and function in various environments that were previously poorly understood. Yet, unlike ^{13}C one-carbon substrates such as methane or methanol, using single or multiple ^{13}C in a multi-carbon substrate can further complicate the interpretation of SIP experimental data, often leading to different conclusions due to difference in the labeled positions. This is because microbes degrade compounds *via* specific degradation pathways such that the differences in the bioavailability of certain positions of the labeled atoms within the compound would lead to identify different microbial populations. For example, certain dioxygenases attack C-1 and C-2 positions and others attack C-2 and C-3 positions of phenanthrene (Chemerys et al., 2014; Peng et al., 2008). Thus, using phenanthrene labeled at C1 and C2 generated different microbial community derived from using phenanthrene labeled at C2 and C3 positions. Findings of a SIP study using $^{13}C_{3,4}$-E2 (Zhang et al., 2021a) were supported by a previous study reporting that E2 degradation was preferred over the C-3 and C-4 positions (Chen et al., 2017). As the structure of contaminants being investigated becomes more complex over years, how and where and types of stable isotopes were labeled in such compounds from probing-based experiments would be important. Stable isotope positions would highly relate to tracking the fate of stable isotopes in specific biodegradation pathways and ultimately help deduce their efficacy in similar analogue degradation research. Addition of multi-substrates with varying labeled substrates enables researchers to track the assimilation of distinct carbon sources based on varying bioavailability and identify the diverse and complicated dynamics of soil microbial communities, including the assimilation, degradation, and as well as their microbial niches (Barnett et al., 2021; Verastegui et al., 2014).

(ii) Cross-feeding scenarios in SIP-based studies

Cross-feeding, conventionally defined as labeling non-target microbes, is a limitation commonly raised in SIP studies (Dumont et al., 2011; Mooshammer et al., 2021; Wang et al., 2020b). Such undesired labeling events could be minimized but might not be able to be completely avoided. Both degradation metabolites and multiple cellular components can be labeled during the degradation of a labeled substrate. These labeled metabolites and cellular components become the secondary labeling sources that can be utilized and subsequently integrated into the cellular components of non-target microbes as incubation time increased. This phenomenon can be insignificant or pronounced, depending on the characteristics of target microbes in the SIP experimental design.

The characteristics of the target microbes, through interactions with non-target microbes, can potentially affect the types and extent of non-target microbes were potentially being labeled over the course of SIP experiments, leading to observation of different microbial community structures. Considering the following two scenarios as shown in Fig. 5, *Scenario 1*: target microbes (shown as red microbes) secrete little or no labeled products during the growth of labeled substate; *Scenario 2*: Target microbes (shown as blue microbes) secrete significant amount of labeled extracellular products during the growth. Under *Scenario 1*, non-target microbes are less likely to be labeled during the short-term incubation. However, long-term incubation will lead to labeling non-target microbes due to possibility of the uptake from debris of dead target microbes. Under *Scenario 2*, if the target microbes secrete significant amounts of labeled metabolites and/or extracellular products, labeling non-target microbes is very likely even though a short-term incubation is used. Microbial interactions, particularly commensalism, syntrophy, and cooperation, can intensify the extent of labeling non-target microbes, leading to different microbial community structures that comprise of both target and non-target microbes. Yet, the amount of the initial stable isotope potentially passed to other microbes remains an interesting but unanswered scientific question, as the process might be highly depending on the extracellular labeled products exchange between target and non-target microbes over time.

(iii) Efforts for minimizing cross-feeding in SIP-based studies

A recent SC-SIP (with SIMS) could potentially minimize cross-feeding effects (Wilhelm et al., 2018). Interestingly, the cross-feeding effects have been used to understand bacterial predation (Hungate et al., 2021). To minimize cross-feeding interference in SIP experiments, a recent study used a continuously flow-through model that allows medium with isotopic nutrients to pass through a membrane, retaining bacterial cells, which take away secondary metabolites that can be utilized by non-target microbes (Mooshammer et al., 2021). However, this approach has two limitations. Firstly, the cost of isotopes, such as ^{13}C in $NaHCO_3$, may be prohibitive for other isotopically labeled compounds

or contaminants of interest. Second, the response of microbes living on the membrane may be different from those forming flocs and growing in activated sludge. Despite these limitations, they do offer a possible way to minimize the effect of cross feeding in SIP experiments. For biodegradation of contaminants in the environment, the focus of cross-feeding can be potentially shifted to understanding the whole active microbial community that could achieve complete biomineralization of the targeted contaminants.

(iv) Degree of isotope enrichment: dosage and incubation time

The degree of isotope enrichment can also cause bias in SIP data, either underestimate due to sensitive issue or overestimate due to cross-feeding or excess production of extracellular labeled compounds such as SMP and EPS. Supplying sufficient dosage of labeled substrate to ensure detectable labeled metabolites and cellular components is another consideration when implementing SIP studies. While high and frequent dosages of labeled compounds might improve the sensitivity of labeled metabolites and cellular components, it can also lead to a high degree of labeling non-target microbes. Yet, a sufficient dosage is necessary to result in efficient labeling for fractionation used in follow-up analysis.

Unlike the factors discussed above, incubation time is a controllable parameter in SIP experimental designs. For example, insufficient labeling may be due to a short incubation time or possible cross-feeding in the microbial communities due to long incubation time. A few researchers have already provided some general approaches to ensure sufficient labeling and the appropriate sampling time points. Yet, it remains challenging and impractical to establish a standardized protocol for SIP studies, due to the high variations of contaminant types, environmental metrices, and characteristics of the target microorganisms in the degradation experiments that were performed (Uhlik et al., 2013a; Whiteley et al., 2006).

Prior research has considered using a short incubation time to minimize non-target labeling that could occur through microbial interactions (Whiteley et al., 2006). For multi-carbon substrates degradation studies, time course experiments along with metabolite analysis are necessary to guide the ideal sampling time when most of the labeled substrates were potentially integrated into the DNA of active degraders. However, the most ideal state for sampling is also highly depending on the objective of the intended studies, either to identify the primary degraders responsible for the initial step of contaminant degradation, or to determine guild of microbes capable of assimilating the isotopes at a specific position of the substrate, or to understand the overall microbial community required for complete biomineralization of a contaminant with a complex structure.

Future research priorities and trend

(i) Is SIP suitable for studying co-metabolic biodegradation of contaminants?

The SIP-based technology is a method built on the assimilation of isotopically labeled nutrients into various labeled biomarkers such as DNA, RNA, proteins, and lipids. Accordingly, previous SIP-based studies have been exclusively applied to study biodegradation of contaminants that can be metabolized by microorganisms through assimilation (growth-linked) mechanisms. However, many environmental contaminants cannot be degraded *via* growth-linked mechanisms, but only *via* non-growth-linked mechanisms, also known as co-metabolism. In co-metabolic reactions, microorganisms use enzymes that are produced to degrade their primary substrates (i.e., growth substrates) to degrade contaminants (i.e., co-metabolic substrates). However, degradation of co-metabolic substrates generates no growth benefits (such as energy source or as carbon-, nitrogen-, or sulfur- source) to microbes (Fig. 4). The phenomenon was first reported in the early 1950s, the definitive studies reported that a methane-oxidizing bacterium *Pseudomonas methanica* (now known as *Methylomonas methanica*) was able to oxidize ethane but not able to utilize it as its sole carbon and energy source (Leadbetter and Foster, 1958, 1959; 1960). In 1985, Wilson and Wilson reported co-metabolic biodegradation of trichloroethylene (TCE) by methane monooxygenase expressing methane-oxidizing bacteria in soil (Wilson and Wilson, 1985). Since then, many oxygenase-expressing microorganisms have been known to co-metabolically degrade many other chlorinated compounds (Semprini, 1997; Wang and Chu, 2017), and emerging contaminants (Hand et al., 2015; Tran et al., 2013), including fluorotelomer alcohols and fluorotelomer-based compounds (Kim et al., 2012; Lewis et al., 2016; Yang et al., 2022). Unlike growth-linked degradation mechanisms of contaminants, co-metabolic degradation of contaminants can be achieved at extremely low concentration of emerging contaminants such as in ppb (parts per billion) or even ppt (parts per trillion) levels.

While researchers have applied DNA-SIP to target on assimilatory reactions in the degradative microbes using primary substrate, applications of SIP to identify microbes capable of performing co-metabolic (i.e., non-growth-linked) degradation on those interested contaminants has not been realized until recently (Shao et al., 2019). In this recent DNA-SIP study with $^{13}CH_4$ as primary growth substrate, Shao and co-researchers reported previously unknown active acidophilic methanotrophs in acidic groundwater (pH <5.5) microcosms capable of cometabolizing trichloroethene (TCE), and vinyl chloride, but not cis-dichloroethenecis (cDCE). As neutralophilic methanotrophs have been known to express methane monooxygenase (MMO) (soluble and/or particulate MMO) to oxidize methane and the expressed MMO are also responsible for co-metabolic biotransformation of various chlorinated solvents (Hwangbo et al., 2023; Oldenhuis et al., 1989), it

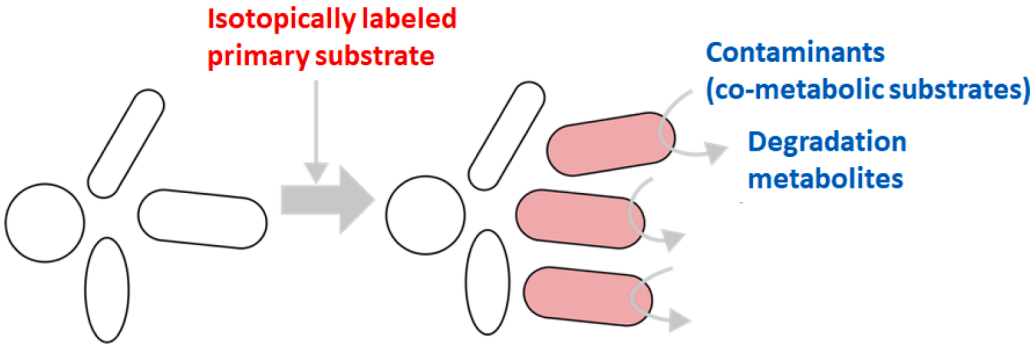

Fig. 4. Schematic of co-metabolic degradation of contaminants by microbes grown on their primary growth substrate. Degradative microorganisms expressed enzymes to oxidize isotopically labelled primary substrate for growth and the induced enzymes were able to co-metabolize the contaminant (non-growth substrate).

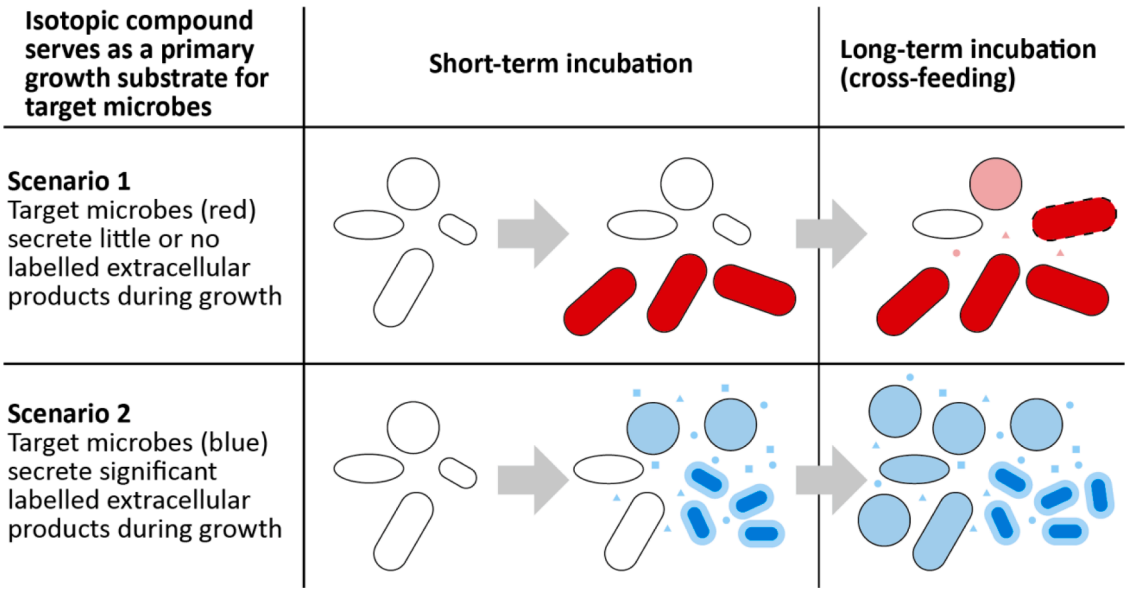

Fig. 5. Two scenarios illustrate the occurrence of labeling non-target microorganisms over time during a SIP study. Scenario 1: Target microbes (red) secrete little or no labelled extracellular products during the growth, and Scenario 2: target microbes (blue) secrete significant labelled extracellular products during the growth.

was interesting to detect novel and diverse particulate MMO and soluble MMO in the acidophilic methanotrophs (Shao et al., 2019). Under this premise, it comes of great importance in identifying and characterizing microbes and specific enzymes that co-metabolically degrade emerging contaminants in complex microbial communities. This first application of DNA-SIP in co-metabolic biodegradation of chlorinated solvents not only successfully demonstrated the power of SIP but also suggested the importance to push forward the application of SIP for studying co-metabolic biodegradation of contaminants. Only few bacteria species are recently known to co-metabolically degrade fluorotelomer-based PFAS, such as *Pseudomonas oleovorans, Pseudomonas butanovora* and *Mycobacterium vaccae* JOB5 (Kim et al., 2014), *Rhodococcus jostii* RHA1 (Yang et al., 2022), *Gordonia* NB4-1Y (Shaw et al., 2019), and *Dietzia aurantiaca* J3 (Méndez et al., 2022). While the mentioned approach is in its early stage, similar approach can be applied along with other advanced techniques (i.e., multi-omics, qSIP, SC-SIP etc.) to identify active microorganisms that are responsible for co-metabolic degradation of other contaminants within mixed microbial communities.

(ii) Integration of SIP with advanced techniques

SIP-based studies have adapted multi-omics such as metagenomics (Chen and Murrell, 2010), transcriptomics (Nuccio et al., 2021), proteomics (von Bergen et al., 2013), and metabolomics (Birkemeyer et al., 2005; Hassanpour and Aristilde, 2021; Hou et al., 2021; Tsugawa et al., 2019; Wilhelm et al., 2022) to better understand microbial structure and function in the context of various environments while tracking potential key-role players through SIP. However, only few types of labeled contaminants have been used in biodegradation studies that couples SIP and multi-omics (Bradford et al., 2018; Hou et al., 2021; Uhlik et al., 2013b; Vasileiadis et al., 2022). Future research using qSIP and SC-SIP with various types of labeled contaminants is needed to assist assessment of *in-situ* degradation kinetics that are particularly useful for formulating effective treatment and management strategies for biodegradation or bioremediation of contaminants at sites.

Furthermore, recent development of high-throughput SIP (HT-SIP) operational pipelines (Nuccio et al., 2022) and simulation of metagenomic SIP datasets (Barnett and Buckley, 2020) could be helpful in minimizing errors and labor from conventional SIP-based studies. Meanwhile the processed large data sets are significantly helpful in identifying and isolating distinct active contaminant degraders from complex microbial communities, leading to a better understanding on the key players in degradation processes.

While recent applications of SIP techniques to study contaminant biodegradation are increasing, SIP-based studies are still limited by the commercially available labeled substrates and/or by the high price of custom-made label substrates. This challenge was partially addressed by a study using magnetic nanoparticles (MNP) to assist isolation of active phenol degraders and compared to those identified through DNA-SIP application of ^{13}C-labeleld phenol (Zhang et al., 2015). Subsequent applications of MNP with SIP and cell-sorting techniques have been reported to identify active acetonitrile degraders (Sun et al., 2021) and phenanthrene degraders (Li et al., 2018, 2022). Yet, as magnetic Fe_3O_4 nanoparticles have been shown toxicity to microbes (Manke et al., 2013), this aspect needs to be thoroughly investigated to determine whether MNP can truly capture the targeted metabolically active microbes under various environmental conditions. Despite these limitations, the use of MNP itself for isolation of active degraders has advantages of being non-destructive and not requiring labeled substrates which might not be readily available.

(iii) Translation of SIP findings for environmental remediation of contaminants

Results of SIP studies have provided fundamental knowledge on the identities of metabolic active microbes and their roles in degrading environmental contaminants. One immediate translation of SIP findings is to identify unique species or signature microbial community structures as biomarkers which can be used to monitor the progress and success of active bioremediation processes, or to assess the potential of natural attenuation. Also, SIP findings can lead to the identification of unique and effective contaminant degraders and degradative enzymes that are present at sites. For example, previous SIP studies using [^{13}C] phenanthrene or [^{13}C] pyrene have identified and characterized novel polycyclic aromatic hydrocarbon dioxygenases from the labeled metagenomic bacterial DNA from contaminated soils (Chemerys et al., 2014; Li et al., 2017; Singleton et al., 2012). The SIP findings can also guide the design of biostimulation and bioaugmentation efforts or assist on the enrichment and isolation of degraders that can be used to study degradation pathways and identification of degradative enzymes.

Conclusions

- Over the past few decades, SIP-based technologies integrated along with multi-omics and spectroscopy techniques allowed researchers to gain a better understanding of the identity and roles of microorganisms in numerous microbial-mediated processes within natural and engineered environments.
- MNP-assisted SIP could potentially offer an alternative to address the challenges of the availability of labeled compounds. Yet, applications of MNP-assisted SIP might not be readily applicable in studying microorganisms that are sensitive to the toxicity of nanoparticles.
- While results of SIP-based studies may over- or under-estimate the diversity of target microbial populations, SIP studies coupled with new techniques has been continuously developed over the past decades to provide a better picture of the complexity of the microbial ecosystem during the contaminant biodegradation. Yet, many challenges still exist due to the complexity of microbial systems, co-metabolism mechanisms, and availability of isotopically labeled compounds.
- Future direction should still focus on elucidating microbial identities, gene functions, and the complicated microorganism-chemical compound interactions. Furthermore, future research efforts need to be placed on how to capitalize the knowledge derived from SIP studies to facilitate bioremediation applications.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgments

This work was partially supported by Strategic Environmental Research and Development Program (SERDP) Project ER-2531.

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