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# Innovative chemical biology tools for monitoring activity in complex microbiomes

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ABSTRACT

The functional analysis of complex microbiomes is hindered by their cellular heterogeneity and dynamic interactions. Conventional approaches often lack the resolution to resolve the metabolic activity of individual cells in situ. Recent advances in chemical biology have introduced powerful tools—such as bioorthogonal chemistry, stable isotope probing (SIP), and single-cell phenotyping—that enable non-destructive, high-resolution profiling of microbial activity across diverse ecosystems. These techniques bridge the gap between genotype and phenotype by targeting translational and metabolic functions in live cells, including uncultured or low-abundance taxa. This review outlines the principles, applications, and current limitations of these tools, including challenges in probe biocompatibility, throughput, and spectral or isotopic data analysis. We highlight recent innovations, including BONCAT-FACS integration, automated SIP platforms, and microfluidic Raman-activated cell sorting (RACS), which enhance analytical scalability. Emphasis is placed on the integration of chemical biology tools with multi-omics workflows to generate causal insights into microbial function. By addressing key technical and analytical barriers, these tools promise to expand our capacity to monitor and manipulate microbiomes for applications in ecology, biotechnology, and health. Their continued development will be critical for unlocking the functional potential of microbial communities across environmental and engineered systems.

## 1. Introduction

Microbiomes—complex communities of microorganisms inhabiting diverse environments such as the human gut, soil, and oceans—are fundamental to global biogeochemical cycles, human health, and industrial processes (Gilbert, 2018; Flemming and Wuertz, 2019; Sessitsch, 2023; Redeker et al., 2018; Shahi et al., 2019; Zhang, 2021). Comprising thousands of species with unique metabolic functions, these communities drive critical processes such as nutrient cycling, carbon sequestration, and host metabolism (Redeker et al., 2018; Shahi et al., 2019; Lloyd, 2018; Jansson and Hofmockel, 2020). Despite their importance, understanding the functional activity of individual microbes within these communities remains a significant challenge due to their diversity, metabolic heterogeneity, and the limitations of traditional methods (Steen, 2019; Hatzenpichler, 2020).

Traditional approaches to studying microbial activity, such as cultivation-based methods and bulk omics techniques (e.g., metagenomics and metatranscriptomics), have yielded valuable insights but fall short in capturing the dynamic and heterogeneous nature of microbiomes. Metagenomics, for instance, reveal the genetic potential of a community but not which genes are actively expressed or how functions are distributed across individual cells (Lloyd, 2018; Hatzenpichler, 2020). Cultivation-based methods are limited by the fact that the majority of environmental microbes remain unculturable, leaving their ecological roles poorly understood (Lloyd, 2018; Steen, 2019). These limitations highlight the need for innovative tools that can monitor microbial activity in situ, at high resolution, and without disrupting the native microbiome structures.

Recent advances in chemical biology have ushered in a new era of microbiome research, offering powerful tools to bridge the gap between microbial genotype and phenotype (Fig. 1). Techniques such as bioorthogonal chemistry, stable isotope probing (SIP), and single-cell phenotyping now enable researchers to track metabolic activity, identify functional roles, and study microbial interactions at unprecedented

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resolution (Hatzenpichler, 2020; Singer, 2017). Bioorthogonal non-canonical amino acid tagging (BONCAT) enables labelling and visualization of newly synthesized proteins in active cells, providing insight into translational activity within complex communities (Hatzenpichler, 2014; Hatzenpichler, 2016). Similarly, SIP coupled with Raman micro-spectroscopy can trace substrate assimilation at the single-cell level, linking metabolic activity to taxonomic identity (Huang, 2007; Wang, 2016). These non-destructive tools are compatible with downstream analyses such as single-cell genomics and fluorescence-activated cell sorting (FACS), greatly enhancing microbiome research (Müller and Nebe-von-Caron, 2010; Lee, 2019; Rubbens and Props, 2021; Tang, et al., 2024).

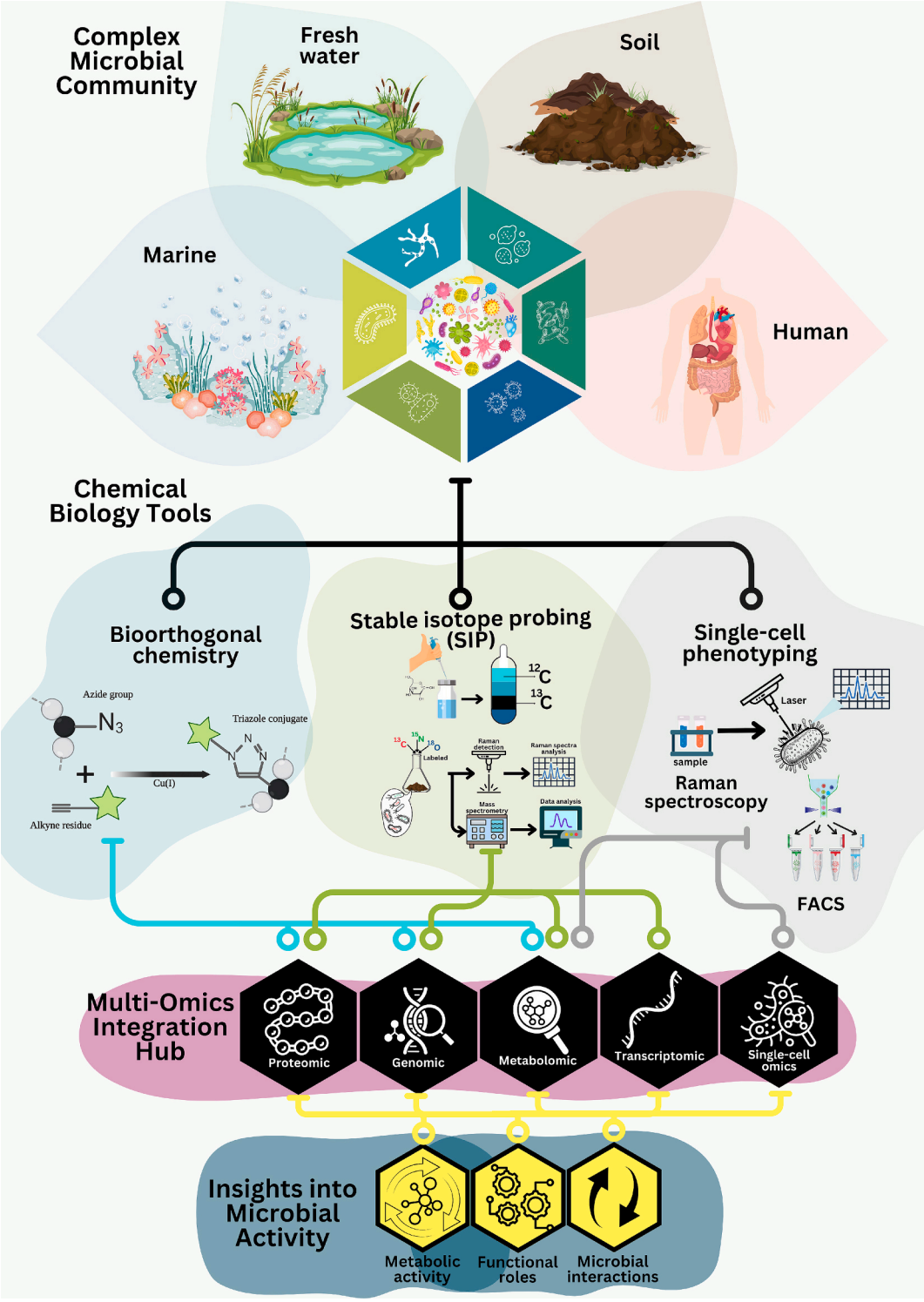


**Fig. 1. Overview of Chemical Biology Tools in Microbiome Research.** A conceptual diagram illustrating how bioorthogonal chemistry, stable isotope probing (SIP), and single-cell phenotyping integrate with multi-omics approaches to study microbial activity in complex microbiomes. These tools enable researchers to bridge the gap between microbial genotype and phenotype, providing unprecedented insights into metabolic activity, functional roles, and interactions within microbial communities.

Integration of chemical biology tools with multi-omics approaches is now at the forefront of microbiome science, enabling a more comprehensive understanding of microbial function (Fig. 1). For instance, combining SIP with metatranscriptomics has uncovered active metabolic pathways in uncultured microbes from deep-sea hydrothermal vents (Fortunato and Huber, 2016), while coupling BONCAT with single-cell genomics has identified translationally active methane-oxidizing archaea in marine sediments (Hatzenpichler, 2016). These approaches mark a shift from descriptive to mechanistic microbiome research (Gilbert, 2018; Shanahan and Hill, 2019; Walter, et al., 2020), especially in systems with high functional redundancy, where multiple taxa encode similar pathways but only a subset is active under specific conditions (Pan, 2022). For example, while many microbes possess nitrogen fixation genes, only certain taxa express them at a given time (Cui, et al., 2018). By pinpointing functionally active organisms, chemical biology tools help bridge the gap between genetic potential and realized activity in situ.

Looking ahead, the continued development of high-throughput and automated platforms, such as microfluidic devices for Raman-activated cell sorting (RACS), promise to enhance the scalability and precision of these methods (Lee, 2019; Zhang, et al., 2015; Jing, 2022; Zhang, et al., 2024; Huang et al., 2009). The synthesis of novel bioorthogonal probes and isotopic labels will expand the range of metabolic pathways and biomolecules that can be studied, offering new opportunities to explore the functional diversity of microbiomes (Devaraj, 2018; Zhang, 2020; Fu, 2024; Calles-Garcia and Dube, 2024). As these technologies evolve, they hold immense potential for advancing microbial ecology, host-microbe interactions, and biotechnological applications, ultimately enabling targeted manipulation of microbiomes for health and environmental benefits.

This review begins by outlining the key methodological and conceptual challenges in assessing microbial activity within complex communities. We then evaluate emerging chemical biology tools, detailing their principles, current applications, and limitations. A comparative framework is introduced to guide tool selection for specific research contexts. Selected case studies demonstrate real-world applications, followed by a discussion of persistent challenges and future directions for integrating chemical biology with multi-omics strategies.

## 2. Challenges in monitoring microbial activity in complex microbiomes

Monitoring microbial activity in complex microbiomes remains a major challenge due to their immense diversity, functional redundancy, and environmental heterogeneity. Microbial communities often comprise thousands of species with overlapping metabolic functions, complicating efforts to assign specific ecological roles. In environments like the human gut, microbes interact competitively and cooperatively (cross-feeding on fibre-derived oligosaccharides or co-producing short-chain fatty acids) which shape community functions and host physiology (Gilbert, 2018; Flint, 2012; Louca, 2018). Even genetically identical cells can differ in activity based on microenvironmental cues, emphasizing the need for methods that capture functional heterogeneity at the single-cell level (Hatzenpichler, 2020; Louca, 2018).

Traditional approaches, including cultivation and bulk omics, offer only limited insights. Cultivation-based methods are hindered by the fact that most environmental microbes remain unculturable under laboratory conditions (Steen, 2019). This often due to the absence of essential nutrients in artificial media, the toxic nature of some media components, or the disruption of essential microbial interactions within their native communities (Lloyd, 2018; Steen, 2019). Many microbes exist in structured biofilms where they depend on neighbouring cells for metabolites, signalling molecules, or growth factors, and removal from their native environment can render them nonviable (Wade, 2002). Bulk omics approaches like metagenomics and metatranscriptomics provide community-wide views of genetic potential and gene expression but lack spatial and single-cell resolution (Louca, 2018; Franzosa et al., 2018; Jansson and Baker, 2016). These methods average signals across entire communities, masking the contribution of low-abundance or transiently active taxa and failing to identify which cells are functionally engaged in situ (Hatzenpichler, 2020; Hrdlickova et al., 2017; Shakya et al., 2019; Ye Simon, et al., 2019).

High-resolution tools such as microautoradiography (MAR) and nano-scale secondary ion mass spectrometry (nanoSIMS) can overcome some of these limitations by offering single-cell insight (Nielsen, et al., 2003; Lee, 1999; Kopf, 2015). However, these techniques are inherently destructive via cell lysis (Hatzenpichler, 2020; Wang, 2016). MAR can localize radiolabelled substrate uptake but requires cell fixation, precluding downstream analyses such as fluorescence-activated cell sorting (FACS) or single-cell genomics (Wang, 2016; Mir, 2024). Similarly, nanoSIMS achieves nanometer-scale resolution but destroys samples during analysis, limiting its compatibility with integrative workflows (Mir, 2024).

Environmental complexity further complicates analysis. In soil, dense and opaque matrices hinder optical access and reduce nucleic acid extraction efficiency (Redeker et al., 2018; Riis et al., 1998; DeFord and Yoon, 2024). Humic substances may inhibit enzymatic reactions, compromising molecular assays (Sidstedt, 2015; Wnuk, et al., 2020). In host-associated microbiomes, non-invasive sampling is often essential to maintain tissue integrity and microbial structure (DeFord and Yoon, 2024). These conditions demand the development of non-destructive, highly sensitive tools adaptable across diverse environments.

Finally, the integration of multi-omics data remains technically and computationally demanding. High-throughput studies can generate 5 – 10 terabytes of data from metagenomics, transcriptomics, proteomics, and metabolomics which requiring harmonization of resolution, scale, and quality across platforms (Picard, et al., 2021). Without robust analytical frameworks, correlating microbial identity with real-time function remains a significant bottleneck.

Together, these challenges underscore the need for high-resolution, minimally invasive, and integrative tools that can link metabolic activity to microbial identity at the single-cell level in situ.

## 3. Innovative chemical biology tools

Microbial activity in complex environments can be investigated using advanced chemical biology techniques, which differ primarily in their labelling strategies. These strategies fall into two major categories:

1. Non-isotopic labelling — relies on synthetic probes or fluorescent tags to detect translationally active cells (e.g., BONCAT, often coupled with FACS).
2. Isotopic labelling (Stable Isotope Probing, SIP) — traces the incorporation of isotopically enriched substrates (e.g., $^{13}C$, $^{2}H$, $^{15}N$) into biomolecules.

While both approaches enable functional profiling at high resolution, they differ in temporal resolution, target molecules, and compatibility with downstream analyses. Following the labelling stage, various cell isolation and sorting methods (e.g., FACS, RACS) can be used to recover and analyse target populations. The following sections outline the principles, workflows, strengths, and limitations of representative techniques, followed by a comparative overview.

### 3.1. Non-Isotopic labelling approaches

#### 3.1.1. Bioorthogonal chemistry

Bioorthogonal chemistry offers a powerful, non-invasive way to study microbial activity within complex microbiomes. These highly specific reactions proceed without disrupting native biological processes, enabling precise labelling and tracking of biomolecules in live cells (Bird et al., 2021; Steward et al., 2020). This unique feature makes

bioorthogonal chemistry an invaluable tool for probing microbial activity in situ.

Bioorthogonal non-canonical amino acid tagging (BONCAT) is a powerful technique within bioorthogonal chemistry that enables the visualization and quantification of protein synthesis in active microbial cells. By incorporating synthetic amino acids, such as L-azidohomoalanine (AHA), into newly synthesized proteins, researchers can selectively label these proteins with fluorescent dyes via click chemistry (Fig. 2A). This approach has been instrumental in studying translationally active methane-oxidizing archaea and bacteria in marine sediments, shedding light on their roles in methane cycling (Hatzenpichler, 2016).

Recent advancements have significantly broadened the applications of BONCAT, extending its use to diverse fields. For instance, it has been employed to investigate viral-host interactions, active prokaryotes, and

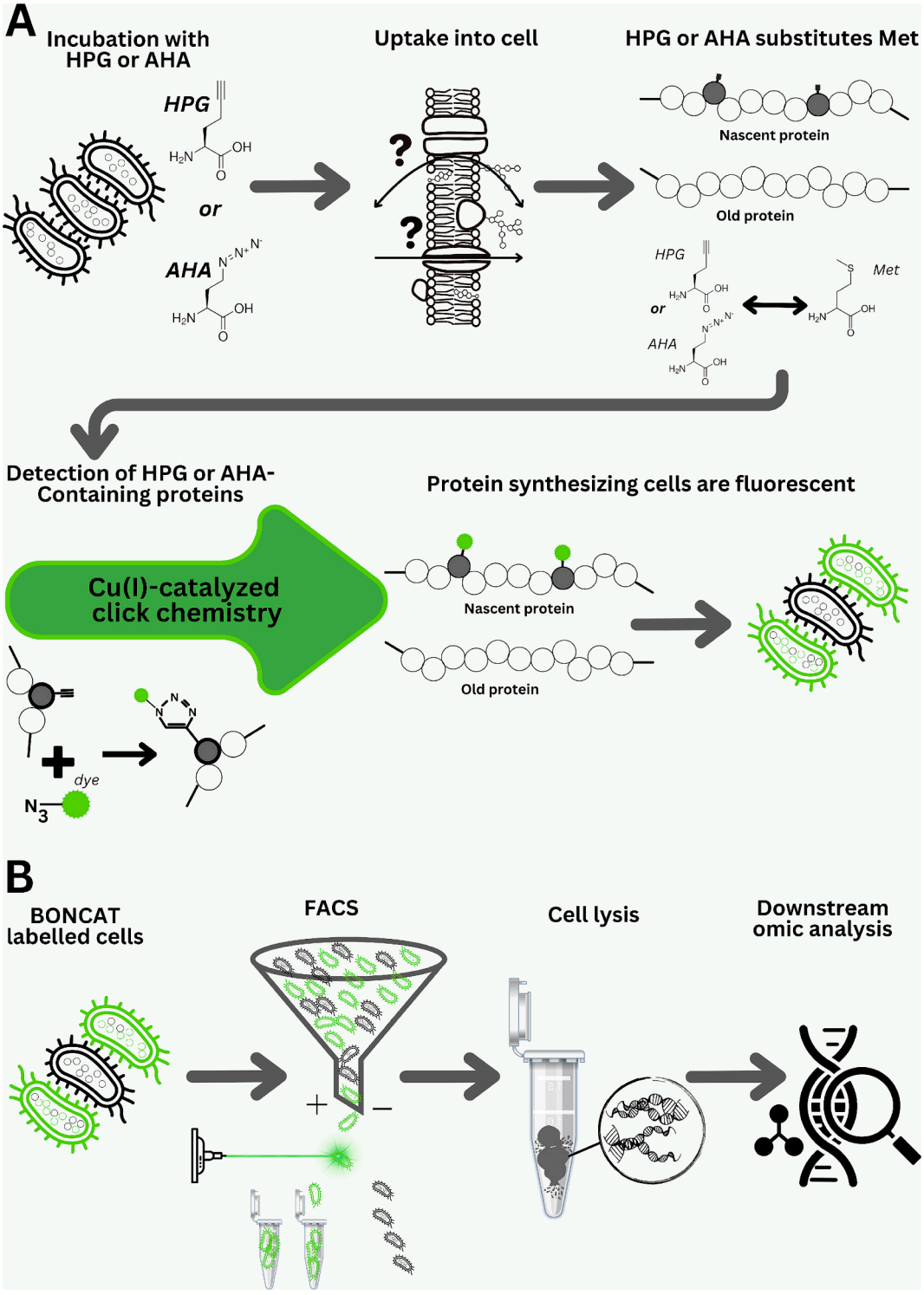


**Fig. 2.** Workflow of Bioorthogonal Non-Canonical Amino Acid Tagging (BONCAT). A step-by-step flowchart showing (A) the incorporation of synthetic amino acids (e.g., L-azidohomoalanine) into newly synthesized proteins, followed by click chemistry labelling with fluorescent dyes, and visualization or (B) sorting of active cells using fluorescence-activated cell sorting (FACS). HPG: L-homopropargylglycine; AHA: L-Azidohomoalanine.
Adapted from (Hatzenpichler, 2016).

stress responses in marine and deep ocean microbiomes (Leizeaga, 2017; Pasulka et al., 2018; Sebastián, et al., 2019; Lindivat, 2020; Michels et al., 2021; Martinez-Varela et al., 2021; Birnstiel et al., 2022). Additionally, BONCAT has been utilized to study the active fractions of soil microbiomes (Couradeau, 2019), the evolution and physiology of cable bacteria (Kjeldsen, 2019), and active subpopulations within the cystic fibrosis lung microbiota (Valentini, 2020).

The technique has also been applied to examine single-cell activity in hot spring microbial communities under varying growth substrates and physicochemical conditions (Reichart et al., 2020), the active fraction of anammox microbiota (Chen, 2021), and the human gut microbiota (Riva, et al., 2020; Taguer et al., 2021). Furthermore, BONCAT has been used to analyse sediment and activated sludge microbiomes (Marlow, et al., 2021; Du and Behrens, 2021; Madill et al., 2021) and the active fraction of biocrust communities (Trexler et al., 2023). These diverse applications underscore the versatility and broad utility of BONCAT in advancing our understanding of microbial activity across various environments.

Click chemistry, a cornerstone of bioorthogonal chemistry, typically involves the alkyne cycloaddition reaction, catalysed by copper(I) or strain-promoted mechanisms (e.g., using cyclooctynes) (Kiick, 2002; Dieterich, 2006), is highly specific and operates under mild conditions, making it ideal for biomolecule labelling in live cells (Devaraj, 2018; Yi, 2022). This powerful bioorthogonal chemistry approach has revolutionized the study of bacterial cell wall dynamics by enabling precise spatial and temporal visualization of peptidoglycan synthesis in intracellular pathogens such as *Listeria monocytogenes*; *Chlamydia trachomatis*, and *Helicobacter pylori* (Sloan Siegrist, et al., 2013; Liechti, 2014; Pilhofer, 2013; Taylor, 2020), as well as in diverse environmental bacterial species (Kuru et al., 2012; Teeseling and Muriel, 2015). Beyond individual species, this technique has been instrumental in studying complex microbial communities. For instance, it was used to track the fate of transplanted Gram-positive bacteria within the mouse gut microbiome. By first labelling bacteria with alkyne-D-alanine, followed by an ex vivo click reaction with an infrared dye for deep-tissue imaging, researchers successfully monitored the dynamics of transplanted microbiota within the recipient host (Wang et al., 2020). This innovative application highlights the potential of clickable analogues in unravelling microbial interactions and adaptations within complex ecosystems.

Recent studies have leveraged click chemistry to track nutrient uptake in the gut microbiome with unprecedented specificity. For instance, an azide-containing analogue of the LPS core sugar, 3-deoxy-D-*manno*-oct-2-ulosonic acid, was used to monitor its assimilation by Gram-negative bacteria in the mouse gut microbiome (Wang et al., 2017). Click chemistry has also provided a powerful tool for investigating how commensal bacteria salvage host-derived nutrients. For example, an azide-functionalized fucose (Az-Fuc) enabled the selective labelling of Bacteroidales species (Besanceney-Webler, 2011), while a bioorthogonal variant of sialic acid, N-acetyl-9-azido-9-deoxy-neuraminic acid (Sia9Az), was used to track bacterial species capable of utilizing this mammalian carbohydrate (Han, 2021). By employing a live-cell-compatible click reaction with a fluorophore, researchers were able to selectively label only those bacteria that had incorporated the tagged sialic acid. This approach, coupled with fluorescence-activated cell sorting (FACS) and 16S rRNA sequencing, led to the discovery of a previously unidentified strain of *E. coli* capable of metabolizing this glycan. These findings underscore the potential of bioorthogonal chemistry in uncovering new microbial metabolic pathways and interactions within complex microbiomes.

The use of clickable lipid analogues allowed precise tracking of lipid incorporation into the bacterial outer membrane, offering a powerful tool to study membrane dynamics and antibacterial lipid interactions in *N. meningitidis* (Becam, 2017). This approach provides an alternative to traditional isotopic labelling, enabling more targeted investigations of Gram-negative bacterial membranes within complex microbial communities (Evershed et al., 2006).

The use of clickable thymidine analogues, such as EdU, has enabled precise tracking of DNA replication in complex microbial communities, providing a powerful means to distinguish replicating from quiescent cells (Salic and Mitchison, 2008; van Kasteren and Rozen, 2023). This approach, particularly when combined with FACS-seq, has allowed researchers to quantify actively dividing bacteria in diverse environments, including marine ecosystems and the murine microbiome (Smriga, et al., 2014; Beauchemin et al., 2023). Its application has revealed critical insights, such as shifts in microbial replication following antibiotic exposure, highlighting its potential for studying microbiome dynamics under different conditions. However, challenges remain, including species-specific differences in EdU incorporation and growth rate-dependent labelling, which must be addressed to fully harness the potential of this technique for broader microbiome studies (van Kasteren and Rozen, 2023).

Bioorthogonal chemistry offers several key advantages that make it a powerful tool for studying microbial activity. First, it is non-destructive, allowing labelled cells to remain viable for downstream analyses such as fluorescence-activated cell sorting (FACS) or single-cell genomics (Fig. 2B). This feature is particularly valuable for studying live cells in their native environments. Second, bioorthogonal reactions are highly specific, minimizing background noise and enabling precise detection of target molecules even in complex microbial communities. This specificity is critical for accurately tracking metabolic activity in situ. Finally, bioorthogonal chemistry is highly versatile, compatible with a wide range of biomolecules, including proteins, lipids, and nucleic acids, making it applicable to diverse research questions. As a result, bioorthogonal chemistry has become a cornerstone of modern microbiome research, bridging the gap between microbial identity and functional activity.

Despite its strengths, bioorthogonal chemistry has some limitations. One challenge is labelling efficiency, as the incorporation of synthetic amino acids or probes may vary between microbial species, leading to incomplete labelling (van Kasteren and Rozen, 2023). Additionally, some bioorthogonal probes, particularly at high concentrations, can be toxic to sensitive microbial cells, potentially altering their metabolic activity (Steward et al., 2020; Landor, 2023). Another limitation is the complexity of optimizing reaction conditions for different microbial systems and environments, which can be time-consuming and technically demanding. These factors must be carefully considered when designing experiments using bioorthogonal chemistry.

### 3.2. Isotopic labelling approaches (Stable isotope Probing, SIP)

Stable isotope probing (SIP) is a cultivation-independent technique for directly linking microbial identity to metabolic function in complex communities (Neufeld et al., 2007). First developed in the early 2000 s, SIP uses non-radioactive heavy isotopes such as $^{13}C$, $^{15}N$, $^{18}O$, or deuterium ($^{2}H$) to label substrates that microbes actively metabolize. Upon assimilation, these isotopes are incorporated into cellular biomolecules— including nucleic acids, proteins, and cellular biomass—which can then be analysed using molecular or spectrometric tools (Neufeld et al., 2007; Radajewski, 2000; Dumont and Colin Murrell, 2005; Kong, 2020).

A major strength of SIP lies in its ability to reveal in situ activity by tracing substrate incorporation, offering experimental evidence of "who does what" in microbial ecosystems (Neufeld et al., 2007; Dumont and Colin Murrell, 2005). This contrasts with inference-based approaches like metagenomics or metatranscriptomics, which lack direct functional validation (Kong, 2020). Over the past two decades, SIP has diversified into distinct methodological branches that differ in the molecular targets of isotope incorporation and the temporal resolution they capture (Singer, 2017). DNA-SIP links isotope assimilation to genome replication and is particularly powerful for detecting taxa that grow over extended incubation periods. RNA-SIP traces incorporation into rapidly turning-over transcripts, thereby capturing short-term transcriptional

responses to environmental fluctuations. Protein-SIP connects isotope assimilation directly to enzymatic function, allowing the identification of metabolic pathways that are actively engaged in situ. Raman-SIP, by contrast, uses vibrational microspectroscopy to detect isotope incorporation at the whole-cell level, providing single-cell resolution without the need for nucleic acid or protein extraction.

In the classical SIP workflow (Fig. 3), an environmental or engineered sample is amended with an isotopically labelled compound (e.g., $^{13}$C-glucose or $^{15}$N-ammonium) and incubated under relevant conditions. Nucleic acids are then extracted and separated by isopycnic centrifugation, where labelled (heavier) and unlabelled (lighter) DNA or RNA form distinct bands. Subsequent 16S rRNA gene sequencing, metagenomics, or qPCR of these fractions identifies taxa that have directly incorporated the labelled substrate (Radajewski, 2000). Collectively, the different branches of SIP offer complementary perspectives: DNA-SIP resolves long-term growth-linked assimilation, RNA-SIP provides snapshots of immediate activity, Protein-SIP links isotope uptake to functional enzymes, and Raman-SIP uncovers cell-to-cell heterogeneity. When used in combination, they enable a multi-layered understanding of microbial activity in natural and engineered ecosystems.

### 3.2.1. *DNA-SIP*

DNA-SIP is the most established and widely used form of SIP, targeting organism that incorporate isotopic labels into their DNA during cell division. Because the method requires genome replication for label incorporation, relatively long incubation periods—often exceeding 12 days—are needed to obtain sufficient isotopic enrichment (Kong, 2020; Morris, 2002; Gallagher, 2005; Radajewski et al., 2003; Ziels, 2018; Pepe-Ranney, 2016; Eichorst and Kuske, 2012). This makes DNA-SIP particularly effective for detecting slow-growing or dominant microbes that carry out nutrient transformations over extended timescales (Kong, 2020; Ziels, 2018; Pepe-Ranney, 2016).

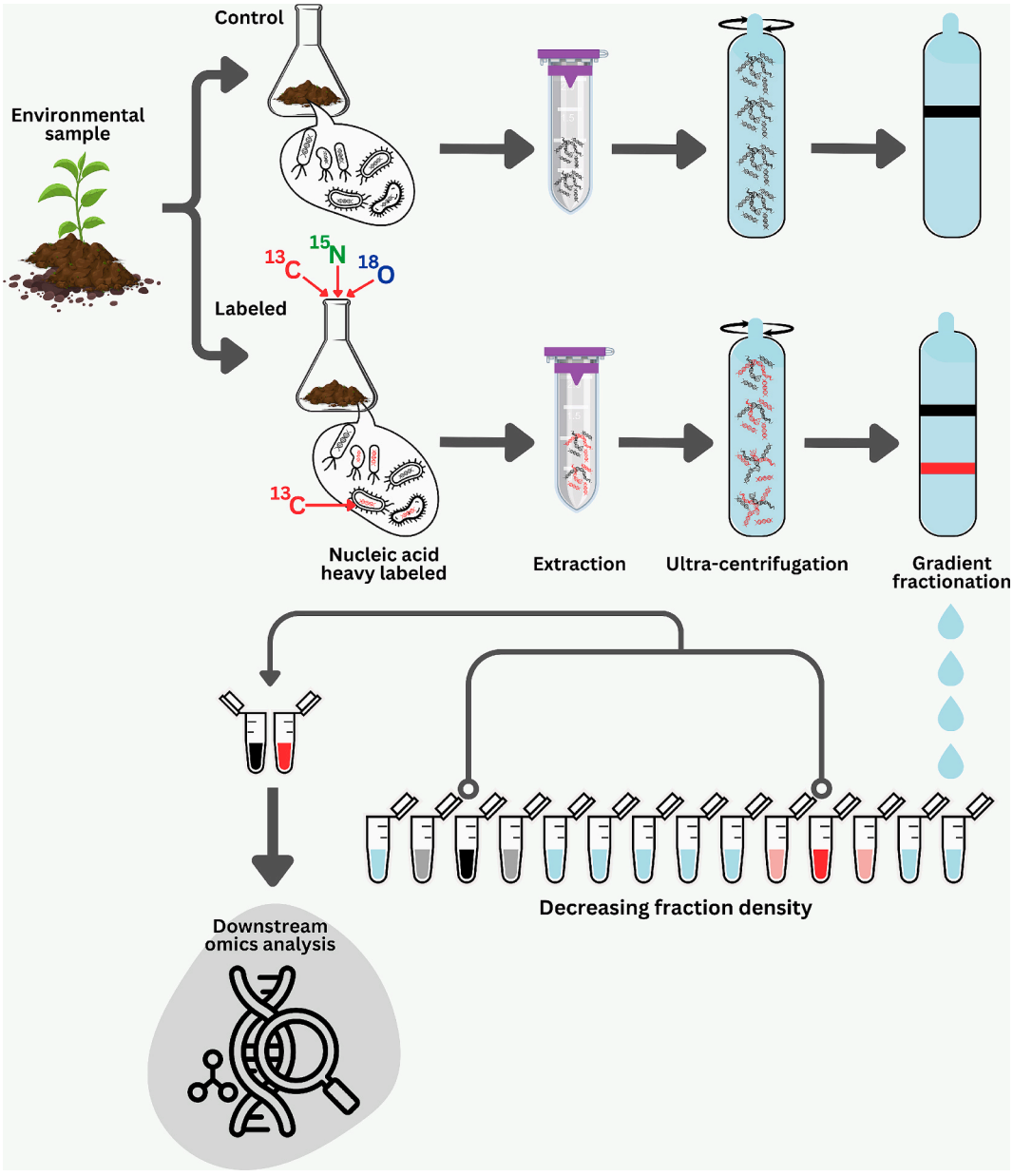


**Fig. 3.** Workflow of nucleic acid Stable Isotope Probing (SIP). A diagram illustrating the SIP process, including the use of isotopically labelled substrates (e.g., $^{13}$C-glucose), density gradient centrifugation, and downstream analysis (e.g., DNA/RNA sequencing or 16S/shotgun sequencing) to link metabolic activity to microbial identity.
Adapted from (Ghori, 2019).

Applications of DNA-SIP span a wide range of ecosystems, including soil, sediment, wastewater, and host-associated microbiomes. It has been used to trace assimilation of labelled substrates such as cellulose, methane, and acetate, directly linking specific microbial taxa to key biogeochemical processes. For example, Eichorst and Kuske (Eichorst and Kuske, 2012) applied $^{13}$C-labeled cellulose DNA-SIP to uncover uncultivated lignocellulose degraders in forest soils, while during the Deepwater Horizon oil spill, DNA-SIP provided direct evidence for hydrocarbon degradation by specific microbial populations, highlighting their role in in situ bioremediation (Gutierrez, 2013). When paired with metagenomics—whether short- or long-read sequencing—DNA-SIP supports genome-resolved profiling of active taxa, thereby bridging substrate use with genetic potential.

### 3.2.2. RNA-SIP

RNA-SIP tracks organisms that rapidly incorporate isotope labels into RNA, which has a much faster turnover than DNA. Because of the inherent instability of RNA, careful handling during extraction and processing is essential. Isotope incorporation is typically detected via ultracentrifugation followed by transcriptomic analysis (Radajewski et al., 2003). The short half-life of RNA allows for significantly shorter incubation times—often as little as 2–8 h—making it ideal for capturing early, transient, or stress-responsive metabolic activity (Manefield, 2002; Herrmann, 2017; Gülay, 2019). This fine temporal resolution makes it particularly useful for investigating dynamic environments or processes that fluctuate on short timescales.

Several studies have demonstrated the strengths of RNA-SIP in linking function to identity. In groundwater-fed biofilters, for example, $^{13}$C-RNA-SIP revealed that *Nitrospira* spp. were actively oxidizing ammonia (Gülay, 2019). Advanced applications have uncovered the chemoorganoautotrophic behaviour of anaerobic methane-oxidizing archaea (ANME-1) and *Bathyarchaeota* subgroup 8 (Bathy-8), which assimilated both organic and inorganic carbon (Yin, 2024). In the human gut, Tannock et al. (Tannock, 2014) employed $^{13}$C-RNA-SIP to identify fast-responding *Bacteroides* species involved in arabinogalactan degradation, demonstrating how the method can resolve rapid responses to dietary inputs.

Taken together, DNA- and RNA-SIP provide complementary insights into microbial activity. DNA-SIP excels at identifying slow-growing taxa and processes that require sustained assimilation over long timescales, whereas RNA-SIP reveals short-term transcriptional responses and the early dynamics of resource use. Used in tandem, they allow researchers to capture both the long-term assimilators that dominate nutrient cycling and the fast responders that initiate metabolic shifts, offering a more complete view of microbial function across temporal scales.

### 3.2.3. Protein-SIP

Protein-based Stable Isotope Probing (Protein-SIP) extends traditional SIP approaches by tracing isotope incorporation into the proteome, thereby linking substrate assimilation directly to expressed enzymes and metabolic pathways (Jehmlich, 2010; Seifert, 2012; Kleiner, 2019). In a typical workflow, microbial samples are incubated with labelled substrates (e.g. $^{13}$C, $^{15}$N, or $^{2}$H). Proteins are then extracted, digested (typically with trypsin), and analysed via high-resolution LC-MS/MS (Fig. 4). Mass shifts in peptide isotopologues reveal the degree of isotope incorporation, which can be quantified with specialized software to identify active enzymes, metabolic fluxes, and functional taxa (Kleiner, 2019; Jehmlich, 2008; Bastida, 2010; Li, 2019).

Early applications demonstrated its value for resolving pollutant degradation. Jehmlich et al. (Jehmlich, 2008) used $^{13}$C-toluene to track degradation in *Aromatoleum aromaticum* strain EbN1 under anoxic conditions, while Taubert et al. (Taubert, 2012) applied Protein-SIP with metagenome-based proteomics to study benzene degradation in a sulphate-reducing consortium to resolve temporal carbon flux among Clostridiales, Deltaproteobacteria, and Bacteroidetes/Chlorobi. Similarly, Herbst et al. (Herbst, 2013) identified Burkholderiales,

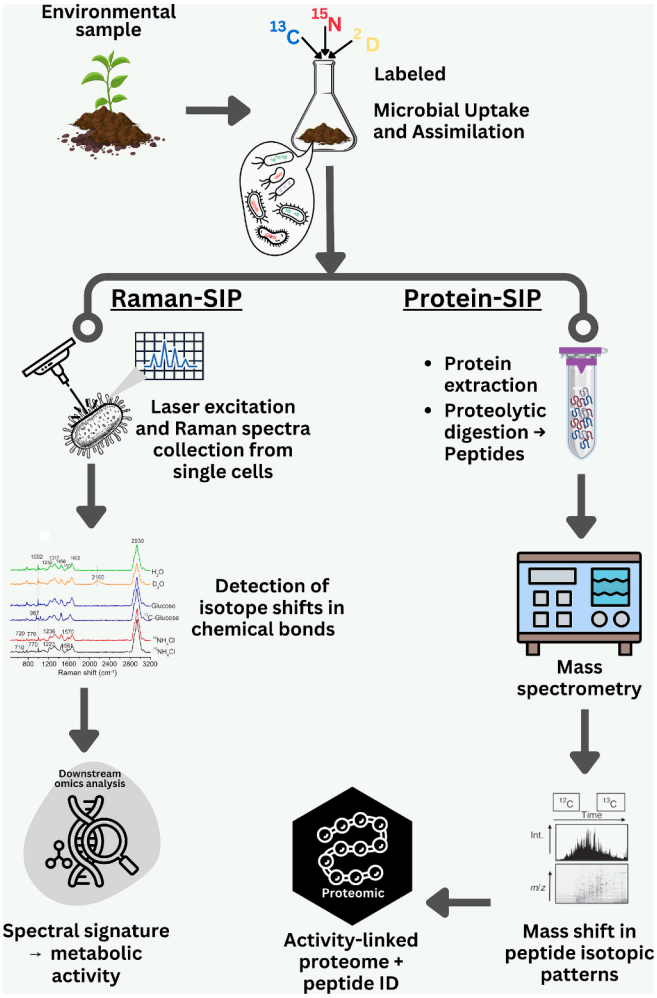


**Fig. 4.** Comparison of Raman-based stable isotope probing (Raman-SIP) and protein-based stable isotope probing (Protein-SIP) workflows. Both methods begin with microbial assimilation of stable isotope-labeled substrates (e.g., $^{13}$C or $^{15}$N or $^{2}$D). In Raman-SIP (left panel), isotope incorporation induces detectable spectral shifts in specific molecular bond regions (e.g., C–H to C–D) within single cells using Raman microspectroscopy, providing a rapid, non-destructive readout of metabolic activity. In Protein-SIP (right panel), isotope-labelled proteins are extracted, digested into peptides, and analysed by liquid chromatography-tandem mass spectrometry (LC-MS/MS). Isotope incorporation is detected as characteristic mass shifts in peptide spectra, enabling the identification of active taxa and functional pathways through proteomic database matching. Single-cell Raman spectra and the mass shift in peptide isotopic patterns are
adapted from (Jehmlich, 2010; Cui, 2017).

Actinomycetales, and Rhizobiales as key polycyclic aromatic hydrocarbon (PAH) degraders in groundwater. These studies highlight how Protein-SIP uniquely captures the enzymatic machinery responsible for substrate turnover in complex microbiomes.

Compared to DNA- or RNA-SIP, Protein-SIP offers two major advantages: (i) It enables quantitative analysis of isotope incorporation (e.g., relative isotope abundance, RIA; labelling ratio, Lr) without cell replication, allowing the study of slow-growing or non-dividing populations, and (ii) it directly connects isotope incorporation to enzymes, providing functional resolution of active metabolic pathway (Jehmlich, 2008; Taubert, 2012; Jehmlich, 2016; Pan, 2011; Sachsenberg, 2015). It is highly sensitive—detecting as low as 1 % incorporation in pure cultures and ~ 10 % in environmental samples—and especially useful in dynamic contexts such as nutrient pulses or syntrophic interactions (Jehmlich, 2016; Vogt;, 2016). However, it has limitations. Phylogenetic

resolution is lower, often restricted to the family or order level (Taubert, 2012; Herbst, 2013). Its utility is limited in scenarios involving co-metabolized micropollutants or low-concentration substrates, which may not be incorporated into biomass (Jehmlich, 2016; Fischer and Majewsky, 2014). It requires high biomass, advanced instrumentation (e.g., Orbitrap or Q-TOF mass spectrometers), robust peptide databases, and specialized analysis software (Jehmlich, 2010). For ecosystems like oceans, where direct substrate addition is impractical, Protein-SIP must be done in lab microcosms. Here, combining it with RNA-SIP can provide complementary insights into both genetic potential and protein-level activity, particularly for uncultivated microbes (Lee et al., 2020).

### 3.2.4. *Raman-SIP*

Raman-SIP is a cutting-edge technique that combines stable isotope labelling (e.g., $^{13}C$, $D_2O$) with Raman microspectroscopy (Fig. 4), a vibrational imaging method that detects bond-specific shifts in molecular structures (Caro, 2024). When isotopically labelled substrates are assimilated, characteristic Raman band shifts occur—for example, C–H bonds in lipids and proteins shift to C–D when deuterium is incorporated—reflecting active anabolic processes (Wang, 2020; Berry, 2015).

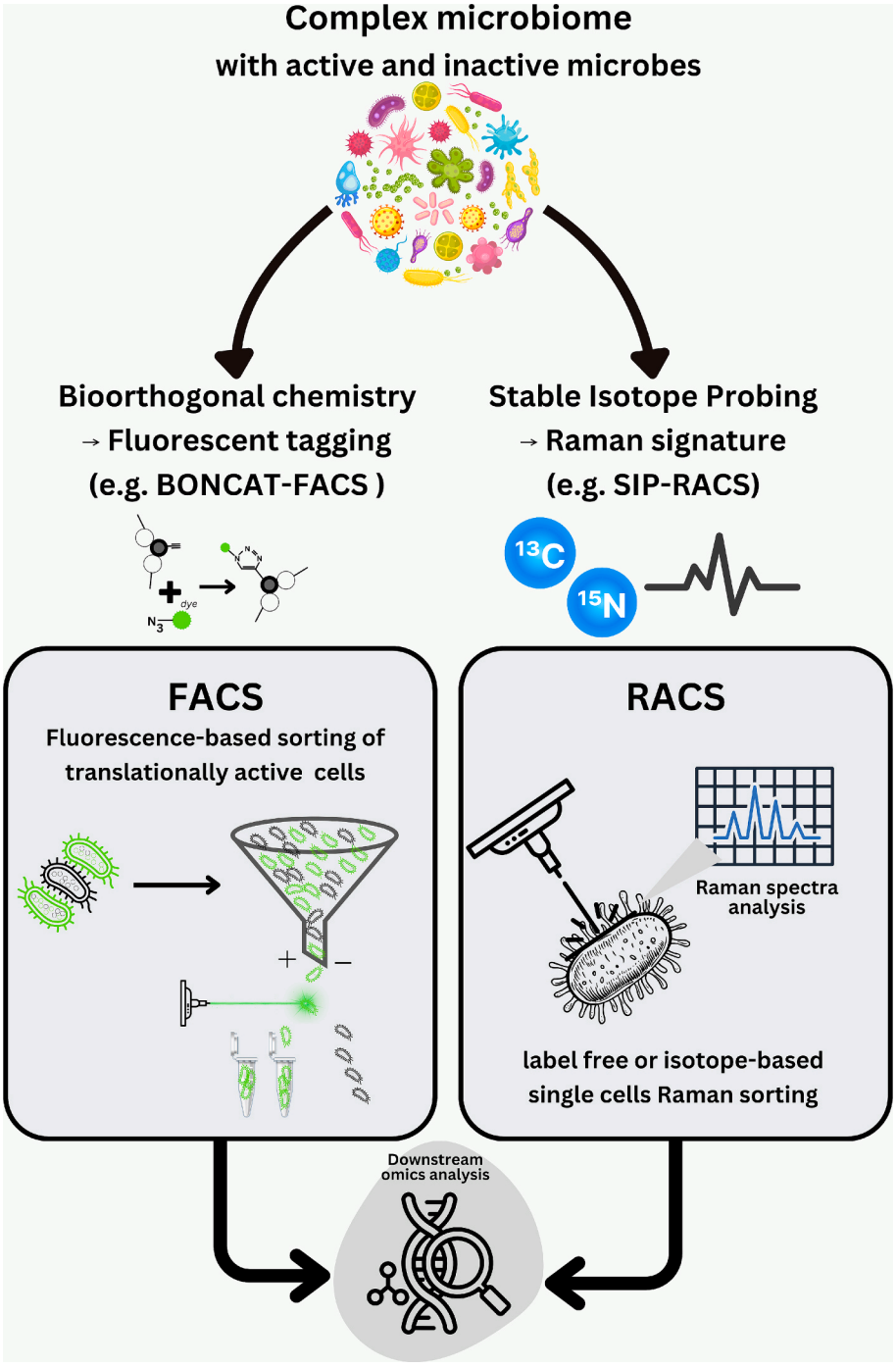


**Fig. 5. Single-cell phenotyping of active microbes in complex microbiomes using FACS and RACS combined with activity-targeting techniques.** Complex microbial communities are first labelled using either bioorthogonal chemistry (e.g., BONCAT) for fluorescent tagging or stable isotope probing (SIP) for isotopic enrichment. After labeling, BONCAT/click-labeled cells can be sorted by FACS, whereas Raman-SIP (or label-free Raman) positive cells can be sorted by RACS, allowing isolation and downstream analysis of translationally or metabolically active single cells from environmental samples.

These spectral changes can be measured non-destructively at the single-cell level, enabling in situ detection of metabolically active microbes without nucleic acid extraction (Lee, 2019; Manefield, 2002; Berry, 2015).

Raman-SIP has been successfully applied in various ecosystems. Jing et al. (Jing, 2018) used $^{13}$C-Raman-SIP to identify uncultivated carbon-fixing bacteria (e.g. *Synechococcus*; *Pelagibacter*) in marine systems. Other studies applied the technique to analyse carbon fixation in marine autotrophs (Li, 2012), track bisphenol A-degradation in activated sludge (Manzi, 2025) and identify *Pseudomonas* populations responsible for naphthalene degradation in groundwater microcosms (Huang, 2007). These applications underscore Raman-SIP's value in uncovering active microbial populations in complex environments, providing spatial, temporal, and functional resolution that complements nucleic-acid and protein-based SIP approaches. Importantly, Raman-SIP differs fundamentally from Protein-SIP in that it offers spectroscopic readouts of isotope incorporation at the single-cell level, rather than proteomic identification of labelled enzymes. When coupled with downstream cell-sorting methods such as RACS (see Section 3.3), Raman-SIP further enables functional sorting and genomic analysis of uncultivated microbes without the need for chemical fluorescent tags.

### 3.3. Cell isolation and sorting approaches

Single-cell phenotyping techniques have revolutionized microbial ecology by enabling researchers to quantify cell-specific functional traits—such as metabolic activity, substrate uptake, or pigment production—and directly link them to microbial identity or genomic features (He, 2019). These approaches provide critical resolution for uncovering microbial heterogeneity, revealing distinct eco-physiological behaviours even among closely related taxa (Lee, 2021; Yan, 2021).

Importantly, sorting methods operate after the labelling step. Whereas BONCAT, SIP, and Raman-SIP provide the labels that mark metabolically active cells, cell isolation platforms such as fluorescence-activated cell sorting (FACS) and Raman-activated cell sorting (RACS) are downstream technologies that physically separate labelled cells from the background community. Both are therefore complementary rather than alternative to the labelling methods described in Sections 3.1–3.2.

Two of the most widely used technologies for microbial single-cell phenotyping are fluorescence-activated cell sorting (FACS) and Raman-activated cell sorting (RACS). Although they rely on different detection principles—fluorescence versus vibrational Raman spectra—both enable targeted recovery of phenotypically distinct cells for downstream analyses such as genomics, transcriptomics, proteomics, or cultivation (Fig. 5).

#### 3.3.1. RACS

Raman-activated cell sorting (RACS) is a downstream method typically applied after isotopic labelling (e.g., Raman-SIP) or in label-free mode, allowing researchers to physically recover single cells identified as metabolically active based on their intrinsic biochemical or isotopic signatures (Lee, 2019; Lee et al., 2020). It integrates Raman microspectroscopy with microfluidic-based sorting to isolate individual cells according to their intrinsic biochemical or isotopic signatures. As cells flow through a microchannel, a focused laser excites molecular vibrations, generating a Raman spectrum—a unique chemical fingerprint of each cell. These spectra can detect natural biomolecular differences (e.g., carotenoids, lipids) or isotope incorporation, as in Raman-SIP experiments.

Identified isotopically enriched cells can be further isolated for downstream analysis using several techniques (Zhang, et al., 2015; Jing, 2022; Zhang, et al., 2024; Huang et al., 2009). Optical tweezers employ focused lasers to trap and move cells precisely within a medium, suitable for detailed studies but with limited throughput (Huang et al., 2009; Xie et al., 2005; Bustamante, 2021). Laser-induced ejection systems dislodge target cells from microscope slides using short laser pulses, useful in structured environments like biofilms while preserving spatial context (Zhang, et al., 2024; Wang, 2013; Liang, 2022). Microfluidic Raman-activated cell sorting (RACS) integrates Raman detection with flow cytometry-like sorting (Lee, 2019; Zhang, 2015). It can process hundreds to thousands of cells per hour—and advanced systems achieving rates of up to 3000 cells per minute—making it ideal for analysing free-living microbial populations (Zhang, 2015; Wang, 2017; Lindley, 2022; Xiaozhi, 2021).

Each of these methods presents trade-offs. Optical tweezers provide exceptional precision but at the cost of throughput and technical simplicity. Laser ejection allows selective targeting within complex samples but requires careful optimization to avoid cell damage. Microfluidic RACS, while technically more complex, offers a balance of throughput and specificity suitable for ecological surveys and cell-enrichment workflows.

RACS has been used to isolate carotenoid-rich bacteria from the Red Sea (Song, 2017), distinguish anabolically active cells involved in $CO_2$ fixation (Jing, 2018), and resolve spectral differences in co-cultures of bacteria and yeast (Jing, 2022; Song, 2017). Its key advantage lies in the ability to sort viable, label-free cells, avoiding perturbations associated with fluorescent stains or genetic tagging—especially valuable for uncultured or environmentally sensitive microbes (Lee et al., 2020). Once a target Raman signature is identified, actuators (e.g., acoustic, hydrodynamic, dielectrophoretic) divert the cell into a separate collection stream (Lee et al., 2020; Zhang, 2015; Lyu, 2020).

Although traditional RACS systems process only tens to hundreds of cells per hour, recent advances have boosted throughput to 200–500 cells/hour with high accuracy (e.g., $98.3 \pm 1.7$ % for deuterium-labelled cells) (Lee, 2019; Lee et al., 2020). Despite this progress, several challenges remain. RACS requires high-powered lasers, sensitive optics, and real-time computational analysis to handle inherently weak Raman signals (Xiaozhi, 2021; McIlvenna, 2016). Signal noise is a key limitation, particularly in complex or debris-laden samples. Background scattering from microfluidic materials (e.g., polydimethylsiloxane (PDMS), glass) can obscure cellular signals, necessitating clean suspensions and optimized substrates. Novel designs, such as 3D-printed detection chambers, have improved signal-to-noise ratios by reducing optical interference (McIlvenna, 2016).

Overall, RACS stands out as a powerful platform for non-invasive, label-free sorting of functional phenotypes—especially in ecological or environmental studies where conventional labelling is impractical. Its ability to preserve cell viability and sort based on metabolic or isotopic cues makes it uniquely suited for high-resolution functional microbiome analysis (Xiaozhi, 2021; McIlvenna, 2016; Li, 2012).

#### 3.3.2. FACS

Fluorescence-activated cell sorting (FACS) is a high-throughput sorting platform that is most commonly used downstream of non-isotopic labelling approaches, particularly BONCAT, where translationally active cells are tagged with non-canonical amino acids (Hatzenpichler, 2016; Blainey, 2013; Rinke, 2014). It isolates cells based on their fluorescent properties, which can be intrinsic (e.g., chlorophyll autofluorescence in cyanobacteria) or extrinsic (e.g., BONCAT or dye labelling). FACS supports extremely high throughput—up to $5 \times 10^4$ cells per second—making it particularly advantageous for large-scale microbiome profiling (Stavrakis, 2019; Rane, 2017; Stepanauskas and Sieracki, 2007).

In a typical FACS workflow (Fig. 5), microbial cells are suspended in a buffered liquid stream and passed through a flow cell. Lasers excite fluorophores associated with each cell, and detectors measure emitted fluorescence. Based on fluorescence intensity and scatter properties, droplets containing target cells are electrostatically charged and sorted into collection tubes (Rinke, 2014; Li, 2021). This process enables the rapid recovery of fluorescently labelled cells for downstream molecular analyses, including DNA or RNA sequencing. Rinke et al. (Rinke, 2014)

applied this method and showed efficient isolation of genomic DNA from individual microbial cells in less than 12 h of bench time over a 4-day period, yielding DNA quantities sufficient for downstream PCR and sequencing.

FACS is most commonly integrated with BONCAT in microbiome studies to isolate metabolically active cells for genomic or transcriptomic investigation. For example, BONCAT-FACS has been used to uncover novel consortia between methanotrophic archaea and Verrucomicrobia in deep-sea methane seep sediments (Hatzenpichler, 2016), identify early responding Firmicutes following hydration in arid biocrusts (Trexler et al., 2023), and profile active fermentative and methanogenic microbes in coal seams (McKay, 2022). In engineered environments like wastewater treatment systems, it has revealed functional microbial groups operating under distinct process conditions (Du and Behrens, 2021), while in salt marsh sediments, BONCAT-FACS enabled spatial mapping of anabolic activity linked to redox gradients and mineral associations (Marlow, et al., 2021).

Despite its versatility, the success of genome amplification from FACS-sorted cells varies depending on sample type. Marine and freshwater samples often yield high recovery rates (up to 40 %), but soil samples frequently pose challenges due to inhibitory substances and resistant cell structures, resulting in lower genome amplification efficiency (<10 %) (Rinke, 2014). Nonetheless, when successful, FACS combined with single-cell genomics enables high-resolution exploration of the metabolic potential of uncultivated and low-abundance microbes in complex ecosystems (Trexler et al., 2023).

### 3.4. Comparative overview of chemical biology tools

The techniques described above differ not only in their labelling strategies but also in their resolution, incubation requirements, downstream outputs, and cost. Table 1 provides a side-by-side comparison that highlights how these approaches complement each other and where their trade-offs lie.

Non-isotopic approaches such as BONCAT offer rapid readouts of translational activity with single-cell resolution, particularly when paired with FACS, which enables high-throughput sorting of fluorescently labelled active cells. This combination is especially effective for identifying newly active subpopulations, but it requires efficient probe uptake and can be influenced by toxicity or matrix effects.

In contrast, stable isotope probing (SIP) methods use isotopically labelled substrates to directly link metabolic function to identity. DNA-SIP is the most established, capturing long-term assimilation processes but requiring high biomass and long incubation periods. RNA-SIP complements this by capturing short-term transcriptional activity, though RNA instability can pose challenges. Protein-SIP takes the approach further by resolving enzyme-level dynamics, offering insights into flux and pathway engagement, but at the cost of high biomass and lower phylogenetic resolution. Raman-SIP, meanwhile, provides a non-destructive, single-cell view of isotope incorporation, bridging the gap between population-level SIP and cell-specific analysis, though throughput remains limited.

Cell-sorting platforms stand apart in function but are often used downstream of these labelling methods. FACS is most commonly applied after BONCAT, enabling the recovery of active subpopulations for genomics or transcriptomics. RACS plays a similar role for Raman-labelled or label-free cells, isolating metabolically distinct individuals based on spectral fingerprints. While FACS offers much higher throughput, RACS has the advantage of working without chemical probes, preserving viability and capturing functional heterogeneity even in uncultured taxa.

Table 1 makes clear that no single technique is universally superior. BONCAT excels in speed and specificity, DNA- and RNA-SIP in linking function to taxonomy over different timescales, Protein-SIP in connecting substrate use to enzymes, and Raman-SIP in non-destructive, single-cell resolution. FACS and RACS then serve as powerful extensions, turning labelled (or spectrally distinct) cells into material for deeper genomic or cultivation-based analyses. By matching method choice to research goals and, where possible, combining complementary strategies, researchers can achieve far more comprehensive views of microbial activity and its ecological drivers.

### 3.5. Choosing the right method for specific research applications

The choice of chemical biology tool depends less on the method itself than on the specific biological question and practical constraints of the study. BONCAT excels when the aim is to detect newly active cells on short timescales and is most effective when coupled with FACS for downstream analysis. SIP approaches (DNA-, RNA-, Protein-, Raman-SIP) are better suited for tracing substrate flow and linking metabolic activity to taxa, with RNA-SIP capturing rapid responses, DNA-SIP resolving longer-term assimilation, and Protein-SIP adding enzyme-level resolution. Raman-SIP is distinct in offering non-destructive, single-cell metabolic readouts, especially when combined with RACS for functional sorting.

From a practical standpoint, FACS is the method of choice when high-throughput sorting of labelled subpopulations is required, while RACS provides label-free isolation at lower throughput but with higher phenotypic specificity.

In many cases, a multi-method approach yields the most comprehensive insight. For example, applying BONCAT and SIP in parallel can reveal both protein synthesis and substrate assimilation in comparable microbial populations, providing complementary perspectives on activity. Similarly, Raman-SIP combined with RACS and metagenomics has been demonstrated as a proof-of-concept for functional sorting and genomic analysis of uncultured microbes, offering a label-free alternative to chemical tagging.

Recent developments are expanding the scalability of these methods. High-throughput SIP (HT-SIP) platforms now incorporate automation, robotics, and multi-omics pipelines, enabling fine-scale tracking of substrate utilization across time points and conditions (Nuccio, 2022). By aligning method choice with experimental goals and combining complementary techniques, researchers can overcome the limitations of individual tools and gain deeper insights into microbial activity, interactions, and ecosystem dynamics.

## 4. Application of chemical biology tools across microbiomes

Activity-based labelling and single-cell phenotyping techniques have greatly expanded our ability to interrogate microbial function in natural environments. Unlike bulk approaches or predictive metagenomics, these tools directly capture physiological states and substrate assimilation in situ. In this section, we present case studies organized by ecosystem type, but with an emphasis on process-based comparisons—showing how different methods such as BONCAT, SIP, and Raman-based single-cell tools have been applied to the same substrates or microbial activities. By contrasting how each method reveals complementary aspects of microbial function, we highlight their respective advantages, limitations, and opportunities for integrated application in future microbiome research.

### 4.1. Soil and rhizosphere microbiomes

Soil and rhizosphere microbiomes are among the most taxonomically diverse and spatially heterogeneous microbial communities. Their high complexity makes them an important testbed for chemical biology tools, which can distinguish between dormant, active, and substrate-assimilating populations. Recent studies show how different methods—SIP, BONCAT, and Raman-based techniques—provide complementary perspectives when applied to the same ecological processes, such as nitrogen cycling, carbon assimilation from plant inputs, and resolving microbial dormancy and activity states.

**Table 1**
Comparative overview of chemical biology tools for microbial activity detection.

| Method | Labelling Type | Target Molecule | Resolution | Typical Incubation Time | Sample Input Requirement | Downstream Output | Instrumentation | Cost Category (per sample)* | Notable Strengths | Key Limitations | Typical throughput |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **Non-isotopic approaches** | | | | | | | | | | | |
| BONCAT (with click chemistry) | AHA / HPG (bioorthogonal amino acids) | Nascent Proteins | Single cell or population | Minutes–hours | ~$10^5$–$10^6$ cells for FACS analysis; ~1–10 μg protein for proteomics | Fluorescent cells, DNA, protein | Fluorescence microscope, FACS | $$ | High specificity; FACS-compatible; short-term detection | Uptake varies across taxa; potential probe toxicity; requires translation | Medium |
| **Cell sorting (fluorescence-based)** | | | | | | | | | | | |
| FACS (BONCAT downstream)[1] | Fluorescent tags (BONCAT, dyes, autofluorescence) | Labelled whole cells | Single cell | Minutes–hours | ~$10^6$–$10^7$ cells per sample; higher input ($10^7$–$10^8$) for rare populations | Sorted fractions, genomes | Flow cytometer | $$ | High throughput; genome recovery; widely available | Label-dependent; variable genome amplification; Lower recovery in complex matrices | High |
| **Isotopic labelling (SIP)** | | | | | | | | | | | |
| DNA-SIP | $^{13}C$, $^{15}N$ | DNA | Community / Taxon | >12 days | 1–5 μg DNA input; starting from ~ $10^8$–$10^9$ cells (high biomass) | Labeled DNA; sequencing | Ultracentrifuge, qPCR, sequencer | $$$–$$$$ | Captures long-term assimilation; metagenome recovery | Based on replicating taxa; expensive; high substrate needs | Low |
| RNA-SIP | $^{13}C$, $^{15}N$ | RNA | Community / Taxon | 2–8 h | <1 μg total RNA; starting from ~ $10^9$–$10^{10}$ cells | Labeled RNA; transcriptomics | Ultracentrifuge, RNA-seq | $$$–$$$$ | sensitive to short-term activity; high temporal resolution | RNA instability; yield-sensitive; costly | Medium |
| Protein-SIP | $^{13}C$, $^{15}N$ | Proteins / Peptides | Community / Taxon | Hours – days | ~10–100 μg total protein; starting from ~ $10^9$–$10^{11}$ cells | Peptide MS profiles | LC-MS/MS | $$$$ | Direct enzyme-level insight; quantitative flux info | High input needs; complex data; lower phylogenetic resolution; expensive and require MS expertise | Low |
| Raman-SIP | $^2H_2O$, $^{13}C$-glucose | Whole-cell biomass | Single cell | Hours – days | ~$10^5$–$10^6$ cells; depends on Raman sensitivity and labeling | Raman spectra, isotopic enrichment | Raman microscope | $$–$$$ | Non-destructive; live-cell compatible; single cell view | Low throughput; expensive equipment (requires advanced optics) | Low |
| **Cell sorting (Raman-based)** | | | | | | | | | | | |
| RACS (with Raman-SIP or label free)[2] | Raman spectral shift (label-free or isotopic) | Whole cell biochemical profile | Single cell | NA for label-free; 2–12 h for isotopic labelling | Typically $10^4$–$10^6$ cells; sorting is slower than FACS, so input is usually lower. | Sorted cells; genomes | Raman + microfluidic sorter | $$$ | Label-free sorting; integrates with SIP for phenotype-genotype linkage | Low throughput than FACS; highly specialized instruments (expensive setup) | Low |

Legend: *Cost Categories are divided into $: Low (<$100/sample) – Basic reagents & minimal instrument use. Suitable for simple workflows.; $$: Medium ($100–500/sample) – Standard reagents & instrument time (e.g., FACS hourly charge, basic sequencing).; $$$: High ($500–1500/sample) – Isotope labelling, advanced instrumentation (e.g., Raman, ultracentrifugation), and complex workflows. $$$$: Very High (>$1500/sample) – Multiple expensive steps (deep sequencing, proteomics, advanced sample prep), specialized instruments. BONCAT: Bioorthogonal non-canonical amino acid tagging; FACS: Fluorescent activated cell sorting; RACS: Raman activated cell sorting; SIP: Stable isotope probing. [1] BONCAT is often followed by FACS for sorting and downstream analysis.; [2] Raman-SIP is often followed by RACS for single-cell isolation.

#### 4.1.1. Carbon assimilation from plant inputs

Stable isotope probing (SIP), BONCAT, and complementary single-cell techniques have provided critical insights into how microbial taxa assimilate plant-derived carbon in soils and rhizospheres. Plant inputs such as root exudates, litter, and crop residues are major drivers of microbial activity, yet the mechanisms of carbon transfer and the identity of active taxa vary depending on substrate type and environmental conditions. Comparative applications of these methods highlight both bacterial and fungal roles in carbon cycling, as well as how agricultural management practices shape carbon flow.

*4.1.1.1. Root exudates and rhizosphere carbon flow.* Root exudates represent a major input of labile carbon into soils, shaping rhizosphere microbial communities and fuelling plant–microbe interactions. To trace this process, Achouak and Haichar (Achouak, 2019) provided a standardized DNA/RNA-SIP protocol for incorporating $^{13}$C-labeled plant carbon into microbial nucleic acids, which has since become widely adopted in rhizosphere studies.

Applying this approach, Haichar et al. (Haichar, 2008) demonstrated through DNA-SIP that root exudates are selectively assimilated by distinct bacterial taxa, with Sphingobacteriales and *Myxococcus* consistently identified as key exudate utilizers across different plant species. This highlighted the strong role of exudates in structuring rhizosphere bacterial communities. Building on this, Hernández et al. (Hernández, 2015) used RNA-SIP in rice rhizospheres and roots to capture immediate transcriptional responses, showing that Proteobacteria (Alpha-, Beta-, Delta-) and Verrucomicrobia (*Spartobacteria*, *Opitutae*) incorporated exudate-derived $^{13}$C. Importantly, label incorporation was markedly higher on roots (~20 % of reads) than in surrounding rhizosphere soil (~4%), underscoring the spatial concentration of carbon flow near root surfaces.

Adding a functional layer, Li et al. (Li, 2019) employed proteomic SIP in maize, wheat, and Arabidopsis rhizospheres, recovering metagenome-assembled genomes (MAGs) from labelled proteins. They found high representation of XoxF-type methanol dehydrogenases and proteins involved in phytohormone modulation, quorum sensing, and nutrient transport, directly linking carbon assimilation with ecological traits underpinning root colonization and host–microbe interactions.

Beyond SIP, emerging activity-based single-cell methods extend these insights. In a pioneering study, BONCAT-Live was applied in *Populus* rhizospheres to label, isolate, and cultivate microbes that actively translated proteins in response to root exudates such as malate and salicin, capturing functional responders beyond static SIP snapshots (Mulay et al., 2025). Similarly, Chip-SIP, which combines phylogenetic microarrays with dual isotopic tracers ($^{13}$C-root exudates, $^{15}$N-litter), resolved substrate preferences among microbial groups, showing that Actinobacteria preferentially incorporated litter-derived substrates while fungi assimilated both exudate- and litter-derived carbon (Nuccio et al., 2021). Though still developing, RACS leverages single-cell Raman spectra for label-free sorting and has been highlighted as a promising avenue for rhizosphere studies (Song, 2017).

SIP approaches provide the backbone for identifying which taxa incorporate root-derived carbon and at what timescales, while BONCAT captures the translational activation of subpopulations responding directly to exudates. Raman-based sorting adds the possibility of resolving heterogeneity within these responders. Instead of offering redundant perspectives, these methods together outline a multi-layered picture: SIP highlights long-term incorporators, BONCAT reveals immediate responders, and Raman distinguishes active cells within seemingly uniform populations.

*4.1.1.2. Crop residue decomposition under fertilization regimes.* Crop residues represent a major pool of organic matter returned to soils, and their decomposition is central to soil fertility and nutrient cycling. Stable isotope probing has been particularly powerful for linking residue-derived carbon to active microbial taxa and for dissecting how management practices shape these dynamics.

Using DNA-SIP with $^{13}$C-labeled rice residues, Kong et al. (Kong, 2020) identified bacterial taxa such as *Lysobacter* (Proteobacteria) and *Streptomyces* (Actinobacteria), along with fungal genera including *Chaetomium*, *Penicillium*, *Aspergillus*, and other Ascomycota, as active assimilators of residue carbon. Fertilization regime strongly influenced decomposition dynamics: bacterial incorporation patterns remained consistent across treatments, but fungal genera responded more rapidly under manure-amended soils (NPKM) than under mineral fertilization alone (NPK). This acceleration was attributed to fungal enzymatic breakdown of recalcitrant polymers such as cellulose and lignin, which generated soluble products subsequently assimilated by bacteria. Network analyses revealed more complex fungal–bacterial interactions under NPKM, underscoring how management practices shape residue turnover.

Beyond DNA-SIP, protein-SIP has been used in microbial ecology to trace substrate-derived carbon into expressed proteins, offering functional insights into active metabolic pathways in mixed communities. For example, Protein-SIP has linked $^{13}$C incorporation to proteins in anaerobic consortia (Jehmlich, 2008) and, more recently, ultra-sensitive methods have been developed to quantify isotope assimilation using metaproteomics in complex microbiomes (Kleiner, 2019). Although these studies have not yet focused on crop residues, they underscore protein-SIP's potential to reveal enzymatic functions—such as carbohydrate-active enzymes—underlying residue decomposition when applied in agricultural soil systems. RNA-SIP, while less commonly applied to crop residues, offers the potential to resolve short-term transcriptional responses to residue inputs, complementing DNA- and protein-SIP by capturing more rapid metabolic dynamics.

Although BONCAT has not yet been directly applied to crop residue decomposition, its use in related soil contexts illustrates its potential. For example, BONCAT-FACS studies in biocrust wet-up events showed that Firmicutes rapidly became translationally active upon rewetting, while other groups such as Actinobacteria and Proteobacteria remained dormant in the early stages (Couradeau, 2019; Trexler et al., 2023). Extrapolating to crop residues, BONCAT could provide valuable insight into the temporal activation sequence of fungal versus bacterial taxa—helping to distinguish primary degraders initiating residue breakdown from secondary consumers of soluble metabolites released later.

Raman-based approaches extend resolution to the single-cell level. Raman-SIP has demonstrated assimilation of isotopically labelled substrates by individual soil bacteria, while RACS provides the potential to sort and identify active residue assimilators in a label-free manner (Cui, et al., 2018; Song, 2017). Although still technically demanding and not yet widely applied in residue contexts, these methods could bridge population- and single-cell scales of residue carbon flow.

SIP has clarified the population-level assimilation of residue carbon and revealed how fertilization regimes shape fungal–bacterial interactions. Protein-SIP and RNA-SIP point to the specific enzymes and rapid transcriptional responses driving decomposition, while BONCAT provides a means to resolve which taxa activate first following residue addition. Raman-based techniques potentially extend this further by allowing cell-level discrimination between primary degraders and secondary consumers. Collectively, these tools emphasize not only who decomposes residues, but when and in what physiological state—insight critical for linking soil management practices to decomposition dynamics.

*4.1.1.3. Plant litter decomposition.* Plant litter, enriched in cellulose, lignin, and other complex polymers, represents a more recalcitrant form of carbon input compared to exudates or crop residues. Its decomposition requires cooperation between fungi and bacteria, and SIP approaches have been central to uncovering the succession and division of labour among these microbial guilds.

Using $^{13}$C-labeled substrates, Pepe-Ranney et al. (Pepe-Ranney, 2016) applied high-resolution DNA-SIP to trace xylose and cellulose assimilation in soils. They observed a clear successional pattern: Firmicutes dominated xylose assimilation within the first day, followed by Bacteroidetes at day 3 and Actinobacteria by day 7. In contrast, $^{13}$C-cellulose assimilation occurred later (from day 14 onward), primarily involving Verrucomicrobia, Chloroflexi, and Planctomycetes. Similarly, Lee et al. (Lee et al., 2011) used DNA-SIP with $^{13}$C-labeled rice callus to identify active decomposers and found that Bacilli (Firmicutes) were early incorporators, whereas Actinobacteria maintained activity across all stages. Additional phyla assimilating residue-derived carbon included Proteobacteria, Acidobacteria, Chloroflexi, and Cyanobacteria. Together, these studies demonstrate that fungi and bacteria contribute sequentially: fungi and certain copiotrophic bacteria initiate polymer degradation, while slower-growing taxa assimilate the resulting soluble products.

Protein-SIP has been demonstrated in other complex microbial systems (Kleiner, 2019; Jehmlich, 2008), and although not yet applied to litter specifically, these studies underscore its potential to identify carbohydrate-active enzymes (CAZymes) involved in polymer breakdown. RNA-SIP, though rarely applied to litter, offers complementary temporal resolution by capturing short-term transcriptional responses of both fungal and bacterial decomposers during early stages of litter addition.

While BONCAT has not yet been applied directly to litter decomposition, its unique strength in this context would be separating hyphal fungi actively producing extracellular enzymes from bacterial cross-feeders metabolizing soluble products. In solid litter environments where SIP captures community-level assimilation, BONCAT could resolve which guilds are immediately protein-synthesizing in response to polymeric substrates, providing a functional map of decomposer hierarchies.

Raman-based single-cell approaches have demonstrated the feasibility of detecting isotope assimilation at the individual-cell level in soil microbiomes (Wang, 2016). Although not yet directly applied to litter-carbon decomposition, these methods offer the potential to resolve heterogeneity in assimilation within decomposer guilds. Coupled with Raman-activated cell sorting (RACS) (Song, 2017), this could enable isolation and genomic characterization of specific fungal or bacterial cells actively metabolizing litter-derived carbon, adding a single-cell perspective to community- and enzyme-level insights.

SIP studies have uncovered the successional turnover from fast-growing bacteria to slower decomposers, while proteomic and RNA-based variants highlight the functional pathways underpinning these shifts. BONCAT, although not yet widely applied, could differentiate between fungal enzyme producers and bacterial cross-feeders in real time. Raman-based single-cell methods add a layer of heterogeneity, showing that even within dominant guilds, assimilation can vary cell to cell. This layered perspective reframes litter decay not as a uniform process, but as a sequence of temporally staggered, physiologically diverse interactions between fungi and bacteria.

#### 4.1.2. Nitrogen cycling

Microbial transformations of nitrogen underpin soil fertility, agricultural productivity, and ecosystem function. Processes such as nitrification, denitrification, and organic nitrogen turnover are carried out by diverse microbial guilds, but their active members in situ have historically been difficult to pinpoint. Because many of these transformations occur at fine temporal and spatial scales, chemical biology tools are invaluable for resolving the identity and activity of microbial taxa involved.

Stable-isotope probing (SIP) has been instrumental in assigning nitrogen-transforming functions to microbial groups in soil. For instance, Buckley et al. (Buckley et al., 2007) used $^{15}N_2$-DNA-SIP in temperate soils to show that free-living diazotrophs—including Clostridia (Firmicutes) and diverse Proteobacteria—are actively fixing nitrogen, resolving function at the taxonomic level. In a successional soil crust context, SIP similarly revealed non-cyanobacterial diazotroph activity before phototrophs establish, suggesting temporal dynamics in functional roles (Pepe-Ranney et al., 2016). More recently, Wang et al. (Wang et al., 2020) applied $^{15}N_2$-DNA-SIP in flooded rice soils and attributed nitrogen fixation to both cyanobacterial groups in the orders Nostocales and Stigonematales (e.g., *Anabaena*, *Nostoc*, *Fischerella*) and proteobacterial lineages like Rhizobiales and Burkholderiales. Meanwhile, investigations into nitrification using $^{13}C$-$CO_2$-SIP demonstrated concurrent activity of both archaea (Thaumarchaeota e.g., *Nitrosopumilus*-like AOA) and bacterial (AOB) ammonia oxidizers in agricultural soil (Pratscher et al., 2011). The most recent advance comes from Hu et al. (Hu et al., 2025), who used SIP-based methods to reveal that mixotrophic cyanobacteria—previously overlooked—play a critical role in $N_2$ fixation, especially in transitional microbiome contexts. Together, these SIP studies connect specific nitrogen cycle pathways with the microbial taxa responsible, offering ecosystem-level insight into nutrient turnover.

Protein-SIP has been demonstrated in soils as a way to trace $^{15}N$ assimilation into expressed proteins. For example, Starke et al. (Starke et al., 2016) used $^{15}N$-protein-SIP to show that bacteria dominate short-term assimilation of plant-derived nitrogen in agricultural soils. Methodological advances now allow ultra-sensitive quantification of isotope incorporation into proteins from complex microbiomes (Kleiner et al., 2023). While no Protein-SIP studies yet directly label canonical nitrification or denitrification enzymes (AmoA, NirS/NirK, Nor, NosZ), the approach holds promise for linking isotope assimilation to enzyme systems once applied in this context. RNA-SIP complements this by capturing short-term transcriptional responses: for instance, Pratscher et al. (Pratscher et al., 2011) demonstrated that ammonia-oxidizing archaea and bacteria actively incorporated $^{13}CO_2$ during nitrification, resolving chemolithoautotrophic activity at the transcript level.

Although BONCAT has not yet been widely applied to nitrogen cycling, its potential is evident. BONCAT-FACS could be used to identify translationally active ammonia oxidizers or denitrifiers immediately after nitrogen inputs (fertilizer or root exudates), distinguishing organisms actively producing proteins in situ from dormant populations. This would complement SIP by revealing the timing of activity and differentiating primary nitrogen transformers from cross-feeding taxa.

Raman-based single-cell methods offer a powerful extension for nitrogen cycling studies. Cui et al. (Cui, 2017) combined surface-enhanced Raman spectroscopy with $^{15}N$ tracers to monitor nitrogen assimilation in individual soil bacteria, while Cui et al. (Cui, et al., 2018) applied resonance Raman-SIP with $^{15}N_2$ to identify diazotrophs directly in soils, exposing functional heterogeneity within nitrogen-fixing guilds. When coupled with Raman-activated cell sorting (RACS), these approaches allow isolation and genomic characterization of active nitrogen fixers and nitrifiers from complex soil consortia, adding a cell-specific layer to population- and enzyme-level insights.

Population-level SIP studies provide clear evidence of which taxa drive nitrification, denitrification, and nitrogen fixation under field conditions. Protein-SIP offers a route to link isotope incorporation with the enzymes that mediate these processes, while BONCAT highlights taxa that activate immediately after nitrogen inputs. Raman-SIP/RACS then extend resolution down to single-cell differences, capturing functional heterogeneity even within canonical groups such as *Trichodesmium* or *Nitrospinae*. In combination, these approaches move nitrogen cycle studies from bulk flux measurements to a more nuanced view of guild-specific timing, physiology, and micro-scale variability.

#### 4.1.3. Resolving microbial dormancy and activity states

A large fraction of soil and rhizosphere microbes exist in dormant or low-activity states, yet may transition rapidly to activity following environmental change. Distinguishing dormant from active cells is therefore essential for understanding microbial contributions to nutrient cycling and ecosystem resilience. Chemical biology tools offer

complementary strategies for resolving these physiological states.

Stable isotope probing links substrate assimilation to metabolic activity and can thus provide indirect evidence of dormancy when large fractions of the detected community fail to incorporate label. For example, in hyperarid Negev Desert biological soil crusts, DNA-SIP using $H_2^{18}O$ (water) revealed that only a small percentage (approximately 4–8 %) of bacterial taxa became metabolically active upon wetting, while the majority remained inactive even after hydration (Gabay et al., 2023). Similarly, $^{15}N_2$ RNA-SIP in grassland soils recovered labelled RNA from only a restricted set of diazotrophs such as Clostridia and Proteobacteria, indicating that many potential nitrogen fixers were dormant (Buckley et al., 2007). Even in substrate-amended soils, high-resolution $^{13}$C-SIP has demonstrated that only 10–20 % of OTUs incorporated label during the first week of cellulose and xylose metabolism, with the majority showing no assimilation signal (Pepe-Ranney, 2016). Collectively, these SIP studies suggest that dormancy is widespread in soils, and only a minority of taxa are metabolically active at any given time.

BONCAT-FACS provides a direct way to distinguish active from dormant populations by labelling newly synthesized proteins. Couradeau et al. (Couradeau, 2019) used BONCAT-FACS in soil and biological soil crusts to show that between 25–70 % of cells were translationally active—far higher than earlier estimates of ~ 5 % activity based on rRNA staining. Trexler et al. (Trexler et al., 2023) extended this approach, coupling BONCAT with sequencing to reveal taxonomic differences in activation: Firmicutes activated rapidly after wet-up, while Actinobacteria and Proteobacteria remained dormant. These findings challenge the long-standing view of near-universal soil dormancy, highlighting BONCAT as a transformative tool for directly quantifying active fractions of soil communities.

Raman microspectroscopy provides a complementary approach by detecting stable-isotope incorporation in single cells without cultivation. Studies using $D_2O$ as a general metabolic marker have shown that translationally active cells display distinct C–D Raman bands, while dormant cells do not. Applied to soils, Raman-SIP can thus distinguish heterogeneity in metabolic states within the same taxonomic group, revealing that dormancy is not uniform even among closely related strains. Combined with Raman-activated cell sorting (RACS), this enables isolation and downstream genomic analysis of active vs dormant subpopulations (Wang, 2016; Berry, 2015).

SIP indicates that large fractions of soil communities fail to assimilate labelled substrates, implying dormancy. BONCAT challenges earlier assumptions by directly revealing higher-than-expected fractions of translationally active cells, while Raman-SIP provides single-cell confirmation that activity is unevenly distributed within taxa. Together, these methods converge on a new view of soil microbiomes: dormancy is not universal, but dynamically modulated, with a substantial minority of cells remaining active and poised to respond to environmental change.

### 4.2. Marine and aquatic systems

Marine and freshwater microbiomes mediate key global biogeochemical cycles, including carbon fixation, nitrogen transformations, and the degradation of pollutants such as hydrocarbons and plastics. Because these systems are highly dynamic and often oligotrophic, chemical biology tools are essential for resolving which taxa are actively involved in resource utilization at any given time.

#### 4.2.1. Carbon assimilation and DOM turnover

Dissolved organic matter (DOM) is the largest pool of reduced carbon in the oceans, and its microbial transformation is central to the marine carbon cycle. Different chemical biology tools have illuminated complementary aspects of this process.

Stable isotope probing has been widely used to link DOM assimilation to specific microbial groups. In surface waters of the South China Sea, Liu et al. (Liu, 2020) applied $^{13}$C-DNA-SIP with amino acids and glucose, identifying uptake by SAR11 clade Alphaproteobacteria and Gammaproteobacteria, while in parallel, Thaumarchaeota assimilated $^{13}$C-bicarbonate, reflecting mixotrophic strategies (Seyler, 2018). Similarly, Campana et al. (Campana, 2021) used DNA-SIP to examine uptake of $^{13}$C-labeled high-molecular-weight DOM in coastal seawater, showing strong enrichment of Flavobacteria and other Bacteroidetes specialized for polysaccharide degradation. Together, these SIP studies demonstrate that DOM turnover is partitioned across generalist heterotrophs (e.g., SAR11) and specialized degraders (e.g., Flavobacteria), with Thaumarchaeota contributing via autotrophy and mixotrophy. Beyond surface waters, RNA-SIP combined with metatranscriptomics has revealed functional partitioning in extreme environments. At deep-sea hydrothermal vents, Fortunato and Huber (Fortunato and Huber, 2016) showed that Epsilonproteobacteria and methanogenic archaea dominated distinct thermal niches, employing different carbon fixation pathways.

BONCAT provides a dynamic view of which cells become translationally active in response to fresh DOM pulses. Hatzenpichler et al. (Hatzenpichler, 2016) pioneered BONCAT-FACS for environmental samples and demonstrated its utility in seawater communities, capturing rapid translational responses of subsets of bacteria during substrate additions. This approach highlights that even when SIP shows broad potential for DOM utilization, only a fraction of cells translate proteins immediately after input, providing temporal resolution into activation dynamics.

At the single-cell level, Raman microspectroscopy can track carbon assimilation without cultivation. Using $^{13}$C-bicarbonate as a tracer, Li et al. (Li, 2012) showed—via resonance Raman shifts of carotenoid bands—that only a subset of carotenoid-containing photoautotrophic cells in mixed seawater communities fixed $CO_2$ during short incubations, demonstrating single-cell heterogeneity in carbon assimilation. Similarly, Raman-activated cell ejection (RACE) has been used to sort individual seawater cells incorporating $^{13}$C, including *Synechococcus* and *Pelagibacter*, enabling direct genomic linkage of DOM assimilation at the single-cell level (Jing, 2018). In addition to labelled substrates, Raman-SIP has also detected carbon fixation based on intrinsic spectral signatures of pigments, offering a label-free strategy for monitoring autotrophic activity in situ (Song, 2017).

SIP identifies the broad spectrum of taxa assimilating different DOM fractions, BONCAT potentially reveals which subsets activate immediately following pulses, and Raman-based approaches demonstrate that even within phototrophic assemblages, only a fraction of cells incorporate label during short incubations. These perspectives reinforce that DOM turnover is not a community-wide phenomenon but a mosaic of temporally staggered, physiologically heterogeneous responses across microbial guilds.

#### 4.2.2. Nitrogen cycling

Marine nitrification and denitrification are crucial for nitrogen availability and greenhouse gas regulation in aquatic ecosystems. Stable isotope probing has provided key insights into these processes. SIP studies using $^{15}$N-labeled ammonium have identified Thaumarchaeota such as *Nitrosopumilus* as the dominant ammonia-oxidizing archaea in surface waters (Tolar et al., 2016; Wan et al., 2023), while $^{15}$N tracer approaches have also revealed Nitrospinae as the principal nitrite oxidizers in open-ocean systems (Liu et al., 2018; Kitzinger et al., 2020). BONCAT has been successfully applied in aquatic samples to quantify translationally active subsets of bacterioplankton (Leizeaga, 2017; Lindivat, 2020; Samo et al., 2014), and although not yet targeted specifically at nitrifiers or denitrifiers, its demonstrated utility suggests strong potential for application in oxygen minimum zones where denitrification is concentrated (Hatzenpichler, 2016).

At the single-cell level, Raman-SIP offers a powerful approach to link microbial identity and function. Although this technique has not yet been applied directly to open-ocean diazotrophs such as *Trichodesmium*,

proof-of-concept studies in other systems demonstrate its potential. Raman-based approaches with $^{15}N$ labeling—using surface-enhanced and resonance Raman spectroscopy—to detect nitrogen fixation in soil diazotrophs and to trace nitrogen assimilation at both bulk and single-cell levels (Cui, et al., 2018; Cui, 2017). More broadly, Raman-SIP has been recognized as a versatile tool for investigating microbial activity in complex communities (Wang, 2016). These advances suggest that Raman-SIP could be adapted to aquatic environments to explore whether $N_2$ fixation is heterogeneously expressed within diazotrophic populations, as has already been demonstrated for *Trichodesmium* using NanoSIMS (Finzi-Hart et al., 2009).

SIP has firmly established the roles of Thaumarchaeota, Nitrospinae, and denitrifiers in marine nitrogen cycling, while BONCAT has shown that only subsets of bacterioplankton are translationally active during nitrogen transformations. Raman-SIP, although not yet applied to open-ocean diazotrophs, has demonstrated in other systems that nitrogen fixation and assimilation can be tracked at single-cell resolution, highlighting functional heterogeneity within microbial populations. Together, these approaches could shift our understanding of marine nitrogen cycling from uniform guild activity to a partitioned and heterogeneous process, underscoring the importance of future single-cell applications in aquatic environments.

#### 4.2.3. Pollutant degradation

Marine microbes play crucial roles in the breakdown of anthropogenic pollutants, including hydrocarbons from oil spills and plastic-derived compounds. Chemical biology tools have been applied to identify the active degraders and to resolve temporal and single-cell dynamics of pollutant utilization.

Stable isotope probing has been central to identifying hydrocarbon degraders in seawater. In oil-amended seawater microcosms, DNA-SIP with $^{13}C$-labeled alkanes identified *Oceanospirillales*, *Colwellia*, and *Cycloclasticus* as key degraders of linear and aromatic hydrocarbons, providing direct linkage of substrate assimilation to known hydrocarbonoclastic bacteria (Valentine et al., 2010; Redmond and Valentine, 2012; Sieradzki et al., 2021). Similarly, Gutierrez et al. (Gutierrez, 2013) used $^{13}C$-hexadecane DNA-SIP to demonstrate enrichment of *Alcanivorax*; *Marinobacter*, *Pseudoalteromonas*, and *Alteromonas* during oil degradation in marine systems. Complementing these SIP-based findings, Dombrowski et al. (Dombrowski et al., 2016) reconstructed draft genomes from marine bacteria enriched under SIP experiments and revealed extensive genetic potential for hydrocarbon degradation—identifying pathways such as alkane degradation and PAH catabolism across *Marinobacter* and uncultured alpha- and gammaproteobacterial lineages derived directly from the Deepwater Horizon spill plume. Extending this approach to plastics, Odobel et al. (Odobel, 2025) applied $^{13}C$-labeled polyhydroxybutyrate (PHB) in seawater incubations and revealed *Marinobacter*, *Pseudoalteromonas*, and *Alteromonas* as active plastic-degrading microbes, confirming that SIP can also resolve the assimilation of biodegradable polymers in marine environments. Together, these studies demonstrate how SIP provides the experimental evidence of who actually assimilates pollutants, while genome-resolved omics unveils the underlying metabolic pathways and broader functional landscape within hydrocarbon and plastic degraders.

BONCAT has been applied to assess which subsets of marine microbial communities become translationally active in response to pollutant exposure. In seawater incubations with plastic leachates, BONCAT combined with CARD-FISH and 16S rRNA gene sequencing revealed that only a fraction of the bacterial community became protein-synthesizing, including taxa in *Alteromonadales*, *Oceanospirillales*, *Nitrosococcales*, *Rhodobacterales*, and *Sphingomonadales* (Romera-Castillo et al., 2024). BONCAT's ability to discriminate active from inactive cells makes it especially valuable in complex pollutant-degrading consortia where traditional SIP may detect broader substrate incorporation occurring over longer timescales. Beyond pollutants, BONCAT has also been used to detect translationally active antibiotic-resistant bacteria in aquatic environments, enabling rapid single-cell resolution monitoring of antimicrobial resistance (Wang, 2022).

Raman-based approaches extend resolution to the single-cell scale. Using $^{13}C$-labeled substrates, Raman microspectroscopy has successfully identified hydrocarbon-assimilating bacteria in oil-enriched seawater, revealing heterogeneity even within clonal populations of known degraders (Huang, 2007). More recent developments coupling Raman with activated cell sorting (RACS) open the possibility of isolating active pollutant degraders for downstream genomic characterization (Huang et al., 2009), enabling the discovery of novel degradation pathways otherwise masked in bulk community analyses. Although applications to plastic degradation remain scarce, proof-of-principle Raman-SIP studies underscore its potential to map single-cell variability in pollutant assimilation.

SIP anchors pollutant degradation to specific hydrocarbonoclastic and plastic-degrading taxa, BONCAT resolves which subsets of the community rapidly become translationally active, and Raman-based approaches reveal that even within canonical degraders, activity is heterogeneously expressed at the single-cell level. Together, these complementary methods provide a multi-layered perspective on pollutant degradation, connecting substrate assimilation with functional potential, temporal dynamics of microbial activation, and fine-scale heterogeneity within degrader populations. This integrative framework offers a more complete narrative of how marine microbes respond to anthropogenic inputs and highlights pathways for discovering novel pollutant-degrading functions.

### 4.3. Engineered and industrial system

Microbial communities in engineered environments such as bioreactors, wastewater treatment plants, and anaerobic digesters underpin critical biotechnological applications, from energy production to pollutant remediation. Because these systems are designed for high rates of substrate conversion, they provide tractable models for linking microbial activity to measurable outputs (e.g., methane yield, pollutant removal). Chemical biology tools have been increasingly applied to these microbiomes, enabling dissection of functional guilds, metabolic handoffs, and responses to operational parameters.

#### 4.3.1. Substrate degradation and functional guilds

Stable isotope probing (SIP) has been the most widely applied method for resolving degradative guilds in engineered systems. DNA-SIP with $^{13}C$-labeled long-chain fatty acids in anaerobic digesters, combined with genome-resolved metagenomics, showed that about 70 % of the $^{13}C$-enriched genome bins belonged to *Syntrophomonas*, underscoring its central role in syntrophic long-chain fatty acid (LCFA) degradation (Ziels, 2018). Fluorescence-assisted methods such as fluorescently labeled polysaccharides with FACS further expand this toolbox, enabling the direct isolation of active degraders and revealing novel lineages (*Candidatus Cellulosimonas argentiregionis*) relevant to biomass conversion (Doud, 2020). Together, these approaches demonstrate how isotope-based and fluorescence-assisted strategies can directly link metabolic activity to specific lineages and expand the catalog of organisms driving bioconversion in engineered environments.

Protein-SIP can be used to extend this resolution by tracing different stable isotope atoms incorporation into carbohydrate-active enzymes (CAZymes) and stress-response proteins, directly linking assimilation to enzymatic function (Jehmlich, 2016). BONCAT complements SIP by detecting translationally active populations in real time. In lab-scale biogas reactors, Hellwig et al. (Hellwig, 2024) used BONCAT with click chemistry to enrich for newly synthesized proteins, identifying Clostridia and Bacteroidetes as active fermenters during feeding cycles. Similarly, BONCAT-FACS has been applied to sludge to separate translationally active cells involved in volatile fatty acid (VFA) metabolism from dormant background taxa (Amekan, 2021). Raman microspectroscopy adds further resolution, with $D_2O$-based Raman-SIP

distinguishing active *Methanosarcina* and *Methanothermobacter* from dormant conspecifics in digester sludge (Fernando et al., 2025).

SIP identifies the core degraders of cellulose and lignocellulose, Protein-SIP potentially connects assimilation to expressed enzymes, BONCAT tracks immediate translational responses during feeding cycles, and Raman-SIP distinguishes active from dormant conspecifics. Together these methods move substrate degradation studies beyond static taxonomic inventories toward dynamic maps of who acts, when, and how.

#### 4.3.2. Syntrophy and methanogenesis

Syntrophic interactions are essential for converting VFAs and long-chain fatty acids to methane in anaerobic digesters. DNA-SIP with $^{13}$C-butyrate has repeatedly highlighted *Syntrophomonas* as a keystone syntroph, while $^{13}$C-propionate SIP identified *Smithella* and *Syntrophobacter* as primary oxidizers linked to *Methanothrix* via interspecies electron transfer (Ziels et al., 2019; Wang, 2019). Feeding strategy experiments demonstrated that adjusting reactor inputs could selectively enrich different *Syntrophomonas* species, optimizing long-chain fatty acid conversion to biomethane (Ziels, 2018).

Protein-SIP has reinforced these findings by directly linking isotope assimilation to syntrophic enzymes. Under acetate accumulation, $^{13}$C-labeled peptides mapped to *Methanosarcina*, *Methanoculleus*, and Clostridia lineages encoding the FTHFS enzyme, providing functional confirmation of SAO–hydrogenotrophic methanogenesis as a resilience pathway (Mosbæk, 2016). Recent BONCAT studies suggest it may be possible to capture short-term activation of syntrophic partners during feeding cycles, although direct evidence for sequential activation of syntrophic acetate-oxidizing bacteria (SAOB) and methanogens remains limited. Raman-based approaches have provided complementary evidence by showing that only subsets of *Methanosaeta* and *Methanosarcina* are active methane producers at a given time, underscoring heterogeneity within key archaeal taxa (Fernando et al., 2025).

Together, SIP defines the keystone syntrophs and their methanogenic partners, Protein-SIP confirms the enzymes enabling these partnerships, and emerging BONCAT and Raman approaches point to temporal phasing and population heterogeneity. These insights reframe syntrophy not as a uniform community response, but as a dynamic and physiologically uneven process that underpins digester stability.

#### 4.3.3. Stress responses and operational resilience

Operational upsets in engineered anaerobic systems—particularly ammonia accumulation and volatile fatty acid (VFA) overload—reconfigure carbon flow between acetoclastic methanogens and syntrophic acetate oxidizers (SAO) coupled to hydrogenotrophic methanogens. Stable isotope probing has been central to unraveling these shifts. In acetate-fed incubations under high Total Ammonia Nitrogen (TAN), $^{13}$C-DNA-SIP revealed that acetoclastic *Methanosarcina* remained partially active despite inhibition, while heavy-DNA fractions also contained Clostridia-related bacteria, supporting coexistence of acetoclastic methanogenesis and SAO under ammonia stress (Hao et al., 2015). Complementary protein-SIP experiments traceˆ13C into peptides from *Methanosarcina*; *Methanoculleus*, and Clostridia encoding formyltetrahydrofolate synthetase (FTHFS), confirming SAO–hydrogenotrophic methanogenesis as a key resilience pathway during VFA stress (Mosbæk, 2016). At the single-cell level, FISH–Raman combined with $D_2O$ labelling has further extended resolution of stress responses. These analyses revealed that only subsets of *Methanosarcina* and *Methanothermobacter* remained metabolically active under different substrate regimes, highlighting significant within-guild heterogeneity in stress tolerance (Fernando et al., 2025).

Together, these methods reveal resilience not as a uniform community trait but as an emergent property shaped by the persistence of acetoclastic lineages, the activation of SAO–hydrogenotrophic pathways, and heterogeneity in stress tolerance among individual methanogens.

### 4.4. Host-Associated microbiomes and human health

Host-associated microbiomes in humans and animals play pivotal roles in nutrient processing, immune function, and disease progression. Because these environments are tightly linked to host physiology, they provide powerful testbeds for activity-resolved methods. Comparative applications of SIP, BONCAT, and Raman-based approaches reveal not only which taxa are active, but also the timing and heterogeneity of their activity during health and disease.

#### 4.4.1. Gut microbiome metabolism and nutrient processing

Stable isotope probing has been widely applied to trace dietary carbohydrates in gut consortia. In human and animal gut microbiomes, RNA-SIP with various $^{13}$C-labeled substrates (e.g., glucose,starch, amino acids, galacto-oligosaccharides, inulin) has revealed key fermenters and degraders such as *Ruminococcus bromii*, *Allobaculum*, *Prevotella*, and *Bifidobacterium*, including evidence for cross-feeding interactions (Egert, 2007; Herrmann, 2017; Young, 2015; Ralls, 2016; Kovatcheva-Datchary, 2009; Maathuis, 2012). These studies provide a detailed map of functional specialization among gut microbes, even in disease contexts such as inflammation.

BONCAT adds a temporal and physiological layer by identifying translationally active taxa. In murine models of colitis, BONCAT-FACS detected early shifts in bacterial physiology, including membrane damage and a rise in *Akkermansia* activity before symptom onset, highlighting BONCAT's potential for predicting inflammatory disease (Taguer, 2021). In faecal microbiomes exposed to xenobiotics, BONCAT revealed that the translationally active fraction shifts independently of community composition, showing that BONCAT can capture real-time responses that sequencing alone cannot resolve (Taguer, 2021).

Raman–DIP (using $D_2O$) enhances this functional resolution further. By resolving metabolic activity at single-cell resolution, it revealed that gut bacteria assimilate different substrates preferentially — with glucose stimulating activity toward fatty acid synthesis in situ versus phenylalanine synthesis under lab conditions — underscoring substrate-specific metabolic heterogeneity within the gut microbiome (Wang, 2020).

Collectively, these approaches provide complementary layers of resolution. SIP traces nutrient flows and cross-feeding hierarchies, BONCAT pinpoints which taxa rapidly activate translation under dietary or stress conditions, and Raman–DIP captures substrate-specific activity at the single-cell level. In combination, these approaches provide a systems-level view of gut metabolism that integrates substrate use, physiological activation, and cell-specific behaviour.

#### 4.4.2. Pathogens, antibiotic resistance, and host interactions

In clinical settings, BONCAT has been used to distinguish active pathogens from inactive bystanders directly in patient-derived samples. For example, in sputum from cystic fibrosis patients, BONCAT-FACS revealed that only 6–56 % of the bacterial cells were translationally active, with *Pseudomonas aeruginosa* frequently dominating the active fraction—demonstrating that activity, rather than abundance, drives infection dynamics in situ (Valentini, 2020). BONCAT has also been adapted to host-infected cells, where selective labeling and proteomic enrichment of *Burkholderia thailandensis* proteins revealed pathogen-specific activity within complex host backgrounds (Franco et al., 2018).

Raman-based single-cell methods extend this resolution to antibiotic resistance. In gut consortia, Raman–DIP (with $D_2O$) combined with Raman-activated cell sorting (RACS) enabled the isolation and genome recovery of metabolically active, antibiotic-resistant bacteria—showing that resistance and metabolic activity are concentrated in distinct subfractions of the community (Wang, 2020). Complementary single-cell phenotyping strategies such as FACS have similarly been used to isolate and characterize resistant bacteria from clinical samples, providing insight into their metabolic adaptations and resistance mechanisms (Grandy et al., 2022; Narayana Iyengar, 2024).

Stable isotope probing (SIP) has been less frequently applied in

clinical infections due to practical constraints, but studies in gut systems demonstrate its feasibility for mapping substrate assimilation and cross-feeding. Extending SIP to pathogen-focused infections could complement BONCAT's translational resolution and Raman's single-cell profiling by directly linking substrate use to functional guilds.

Together, BONCAT pinpoints active pathogens in clinical samples, Raman-based approaches uncover resistant subfractions at the single-cell level, and SIP offers a substrate-level perspective where feasible. Integrating these methods moves diagnostics beyond presence/absence, toward resolving who is active, who is resistant, and who is clinically relevant in host-associated infections.

## 5. Challenges and future directions

While innovative chemical biology tools have significantly advanced our ability to study microbial activity in complex microbiomes, several challenges remain that must be addressed to fully realise their potential. These challenges span technical limitations, integration with other methodologies, and the development of new tools and platforms. Addressing these issues will be critical for advancing microbiome research and unlocking new insights into microbial ecology, host-microbe interactions, and biotechnological applications.

### 5.1. Practical Considerations and technical barriers

One of the primary challenges facing current chemical biology tools is their sensitivity, throughput, and compatibility with diverse sample types. For example, Raman-based techniques, while powerful for non-destructive single-cell analysis, are limited by the complexity of spectral data and the need for advanced data analysis tools. Interpreting Raman spectra requires robust computational methods to distinguish between overlapping peaks and identify specific metabolic signatures (Huang, 2007; Tang, et al., 2024; Bagheri and Komsa, 2023). Additionally, the throughput of Raman-activated cell sorting (RACS) is currently limited by the time required to acquire spectra from individual cells, though recent advancements in microfluidics and automation are beginning to address this issue (Lee, 2019; Lee, 2021; Song, 2017). While bioorthogonal chemistry offers high specificity and non-destructive labelling, several challenges remain. Labelling efficiency can vary significantly between microbial species, particularly in complex matrices such as soil or sludge, where background fluorescence may interfere with detection (Redeker et al., 2018; Hatzenpichler, 2014). Additionally, some synthetic amino acids and probes can be toxic to sensitive microbial cells at high concentrations, potentially altering their metabolic activity (Steward et al., 2020; Landor, 2023). Future research should focus on developing more biocompatible probes and optimizing reaction conditions for diverse microbial systems.

Stable isotope probing (SIP) is a powerful tool for tracking substrate assimilation, but its widespread adoption is hindered by the high cost of isotopically labelled substrates, particularly for rare or expensive compounds (Wang, 2016). Furthermore, SIP techniques such as density gradient centrifugation and Raman microspectroscopy require specialized equipment and expertise, limiting their accessibility for many research groups (Wang, 2016; Tang, et al., 2024). To address these challenges, efforts are underway to develop automated platforms that can reduce the time and resources required for SIP-based studies (Nuccio, 2022).

### 5.2. Integration with Multi-Omics approaches

To gain a more comprehensive understanding of microbiome function, chemical biology tools must be integrated with multi-omics approaches, such as metagenomics, transcriptomics, and metabolomics. For instance, combining stable isotope probing (SIP) with -metatranscriptomics can reveal not only which microbes are active but also which metabolic pathways are being expressed in response to specific environmental conditions (Bradford, 2018; Hu, 2019). Similarly, integrating BONCAT with single-cell genomics can provide insights into the genetic potential of translationally active cells, linking genotype to phenotype in complex communities (Hatzenpichler, 2016). However, integrating these methodologies requires careful experimental design and computational pipelines to harmonise data from different platforms. Future efforts should focus on developing standardised protocols and computational tools to facilitate the seamless integration of chemical biology and multi-omics data.

### 5.3. Development of new probes and Reporters

Expanding the repertoire of bioorthogonal probes and isotopic labels is essential for targeting a wider range of metabolic pathways and biomolecules. Recent advancements in click chemistry have led to the development of new probes, such as alkyne-labelled fatty acids and azido-sugars, which can be used to study lipid and carbohydrate metabolism in microbial cells (Charron, 2009; Shieh et al., 2012). While current probes are therefore effective for tracking protein and carbohydrate synthesis and substrate assimilation, there is a growing need for tools that can monitor secondary metabolite production, and other specialised pathways. The synthesis of fluorogenic probes that become fluorescent only upon reaction with their target molecules offers the potential for reduced background noise and improved sensitivity in complex samples (Shieh, 2015). Continued collaboration between chemists, microbiologists, and engineers will be critical for designing and validating new probes that can address these emerging needs.

### 5.4. High-Throughput and automated platforms

The development of high-throughput and automated platforms is another key area for future innovation. Current methods for single-cell phenotyping, such as RACS and fluorescence-activated cell sorting (FACS), are often labour-intensive and limited in throughput. However, advancements in microfluidics and lab-on-a-chip technologies are paving the way for more efficient and scalable approaches. For example, microfluidic platforms for RACS have been developed to increase sorting efficiency and reduce spectral acquisition times, enabling the analysis of thousands of cells per hour (Lee, 2019; Lee, 2021; Song, 2017). Similarly, automated platforms for activity-based protein profiling (ABPP) are being developed to screen for enzyme activity in complex microbiomes, providing insights into the functional roles of uncultured microbes (Whidbey and Wright, 2019). High-throughput SIP (HT-SIP) platforms—such as the one described by Nuccio et al. (Nuccio, 2022)—used a semi-automated system that significantly reduces manual labour—requiring only one-sixth of the hands-on time compared to traditional SIP methods—and allows for the simultaneous processing of 16 samples. By automating critical steps such as density gradient fractionation and cleanup, HT-SIP enhances reproducibility and throughput, making SIP more accessible for large-scale studies. Additionally, the incorporation of a non-ionic detergent into the gradient buffer has been shown to improve DNA recovery, further optimizing the process. This approach has been successfully applied to study microbial interactions in the hyphosphere of arbuscular mycorrhizal fungi, demonstrating its efficacy in complex environmental samples (Nuccio, 2022).

### 5.5. Future directions and remaining challenges

Each activity-profiling method presents inherent trade-offs. BONCAT is restricted to translationally active cells and may exclude metabolically active populations undergoing dormancy or slow growth. SIP, while robust for substrate assimilation studies, fails to capture non-growing but transcriptionally responsive taxa. Raman-SIP remains sensitive to matrix interference and requires advanced computational deconvolution of overlapping peaks. Additionally, variability in probe uptake, cytotoxicity at higher concentrations, and lack of standardized

normalization complicate quantitative comparisons across taxa and conditions.

Resolving these limitations will require standardized benchmarking frameworks, harmonized metadata reporting, and integration with predictive modelling—such as genome-scale metabolic reconstructions or flux balance analysis—to contextualize single-cell activity within community-scale processes. Further, the development of hybrid methods (e.g., BONCAT-RNA-seq, SIP-metatranscriptomics, or Raman-FISH) promises to enhance resolution across molecular layers. Emphasis should be placed on designing scalable, matrix-agnostic workflows that combine biochemical specificity with high-throughput compatibility, particularly for environmental and host-associated microbiomes.

## 6. Conclusion

The emergence of chemical biology tools—such as bioorthogonal chemistry, stable isotope probing (SIP), and single-cell phenotyping—has transformed our ability to resolve microbial function at single-cell resolution in complex microbiomes. These methods have overcome critical limitations of cultivation and bulk omics by enabling direct linkage of genotype to phenotype in situ.

Integrative approaches, such as BONCAT-FACS or SIP-Raman microspectroscopy, have revealed translational and metabolic activity in rare or uncultured taxa, shedding light on microbial roles in diverse ecosystems and biotechnology. However, challenges remain in sensitivity, throughput, and matrix compatibility, alongside the need for expanded probe chemistries and data integration pipelines.

Future progress hinges on scalable, automated platforms (e.g., RACS, HT-SIP), novel fluorogenic or isotopically labelled probes, and machine learning-assisted analysis. These advances will be pivotal for embedding single-cell activity data into predictive ecosystem models. Ultimately, the convergence of chemical biology, systems microbiology, and multi-omics will not only enhance ecological understanding but also inform rational microbiome engineering for health, sustainability, and industry.

## 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.

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## Data availability

No data was used for the research described in the article.

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