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# MINI REVIEW

# Capturing the genetic makeup of the active microbiome *in situ*

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**More than any other technology, nucleic acid sequencing has enabled microbial ecology studies to be complemented with the data volumes necessary to capture the extent of microbial diversity and dynamics in a wide range of environments. In order to truly understand and predict environmental processes, however, the distinction between active, inactive and dead microbial cells is critical. Also, experimental designs need to be sensitive toward varying population complexity and activity, and temporal as well as spatial scales of process rates. There are a number of approaches, including single-cell techniques, which were designed to study *in situ* microbial activity and that have been successively coupled to nucleic acid sequencing. The exciting new discoveries regarding *in situ* microbial activity provide evidence that future microbial ecology studies will indispensably rely on techniques that specifically capture members of the microbiome active in the environment. Herein, we review those currently used activity-based approaches that can be directly linked to shotgun nucleic acid sequencing, evaluate their relevance to ecology studies, and discuss future directions.**
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## Introduction

Microbial communities perform essential roles in natural and engineered ecosystems. They contribute to the dynamics and complexity of global biogeochemical cycles and are the main drivers for important biotechnological processes. One of the main challenges in evaluating the microbial impact in a given system is that organism abundance, diversity and (re-)action potential can vary across systems and across different temporal and spatial scales and may be correlated with a combination of biological, chemical and physical stimuli. The identification of important microbial factors in a given habitat using 16S ribosomal RNA (rRNA) gene surveys (Tringe and Hugenholtz, 2008), shotgun metagenomics (Handelsman, 2004) and single-cell genomics (Lasken, 2007) has provided important insights into the potential functions of particular microbes. However, using these techniques in their traditional sense does not offer the possibility to fully capture the plasticity of microbial communities and its effect on local and global processes. Instead, it is becoming essential to complement sequencing with methods that distinguish cells that are growing and metabolically active from those that are dormant or

dead (or even extracellular DNA), and to understand under what circumstances microbial dormancy could be followed by stochastic awakening (Buerger *et al.*, 2012). Meaningful ecological units, such as microbial guilds (Simberloff and Dayan, 1991), could be identified by monitoring the activity of individual microbes in relevant time series experiments coupled with spatially comprehensive study designs. Several of the discussed tools offer single-cell resolution and thus provide spatial resolution in addition to information on phylogeny and metabolism (Table 1, Figure 1). This review is aimed at highlighting the different ecological and physiological insights into active community members of a given microbiome that can be gained with currently used approaches (in chronological order of published studies) in conjunction with shotgun nucleic acid sequencing. We note that methods that yield activity-labeled samples *in situ* cannot provide answers to all ecological challenges, however, when applicable, these tools present valuable assets in microbial ecology research. We further discuss prospects of exciting emerging technologies in this field.

## An array of methods available for studying microbial *in situ* activity linked to shotgun sequencing

Methods detecting microbial *in situ* activity target a variety of physiological processes to determine the

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**Table 1** Comparison of basic characteristics associated with selected techniques for studying active microbial populations in situ that can be combined with shotgun sequencing

| Cell process targeted | Technique | Advantages | Disadvantages | Biases/assumptions | Reference |
|---|---|---|---|---|---|
| DNA synthesis | BrdU labeling (B, S) | Can be used as single-cell tool if combined with fluorescent antibody staining | Low labeling efficiency | Rate of uptake varies by cell; may be toxic to some cells | Borneman, 1999; Yin et al., 2000* |
| | DNA-SIP (B) | Link of metabolic function to identity | Long incubation time; cross-feeding problem; contamination prone; high GC DNA problematic; dependent on commercially available labeled compounds[a] | Cell division may be stimulated by substrate addition and not reflect in situ growth rates | Radajewski et al., 2000* |
| | Peak-to-trough ratios in metagenomes (S+B), iRep | No sample incubation necessary; samples can be frozen immediately | Reliant on database; affected by community complexity. | | Korem et al., 2015;* Brown et al., 2016* |
| Transcription | RNA-SIP (B) | High phylogenetic resolution; link of metabolic function to taxonomic identity | Dependent on commercially available labeled compounds; cross-feeding potential[b] | Cell division may be stimulated by substrate addition and not reflect in situ growth rates | Radajewski et al., 2003* |
| | Metatranscriptomics (S+B) | Samples can be frozen immediately; inexpensive; fast | mRNA abundance not necessarily indicative of protein levels and enzymatic activities; high transcriptome coverage necessary; modulation may occur during sampling | RNA abundance does not directly correlate with cell activity | Leininger et al., 2006 |
| Amino-acid biosynthesis | BONCAT (+FISH/FACS) (S +B) | No known effect on growth and translation activity | High amino acid conc. in sample diminishes/suppresses signal | Cell growth may be stimulated by added amino acids; dependence on uptake mechanism | Hatzenpichler et al., 2014; Hatzenpichler et al., 2016* |
| Lipid, carbohydrate, amino-acid biosynthesis | $D_2O$+Raman microspectrometry (S) | $D_2O$ concentration required not known to be toxic to any cells; no stimulation effect expected in hydrated samples; can be combined with FISH; stimulation experiments with unlabeled substrates possible | Low-throughput so far | Different physiologies lead to different incorporation rates of deuterium (for example, autotrophs vs heterotrophs) | Berry et al., 2015* |
| Bacterial reduction potential | Redox sensor green+FACS (S+B) | Rapid response to redox activity within cell; stable fluorescence signal; quick cell penetration; no detrimental effect on core cell functions known; stimulation experiments with unlabeled substrates possible | Incubated cells cannot be frozen; not all microbial species may transport redox sensor green equally well into their cells | | Kalyuzhnaya et al., 2008* |

Techniques are further characterized as allowing for single-cell approaches (S) and/or bulk community analysis (B). References given denote those of either first use of the respective method or the first combined use with (any) sequence data (marked with an asterisk).
Abbreviations: BONCAT, bioorthogonal non-canonical amino-acid tagging; BrdU, bromodeoxyuridine; FACS, fluorescence-activated cell sorting; FISH, fluorescence in situ hybridization; iRep, index of replication; SIP, stable isotope probing.
[a]DNA-SIP may be used with $^{18}O$-$H_2O$, which—to our knowledge—has not been used with metagenomics or single-cell sequencing.
[b]RNA-SIP may be used with $^{18}O$-$H_2O$, which—to our knowledge—has not been used with metagenomics or single-cell sequencing.
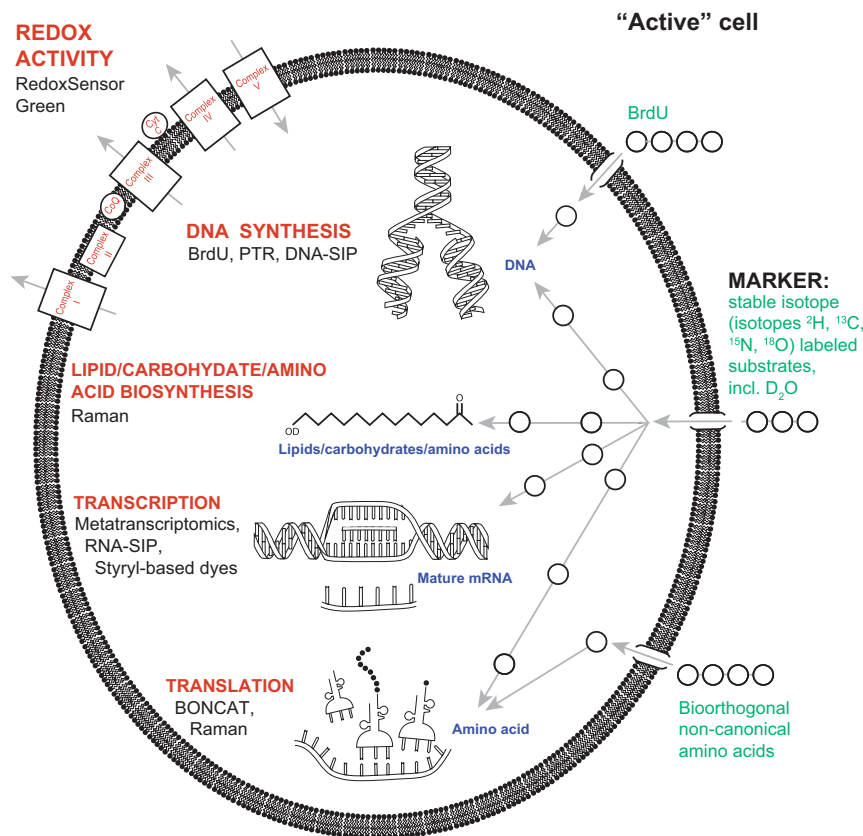
**"Active" cell**

**REDOX ACTIVITY**
RedoxSensor Green

**DNA SYNTHESIS**
BrdU, PTR, DNA-SIP

**LIPID/CARBOHYDATE/AMINO ACID BIOSYNTHESIS**
Raman

**TRANSCRIPTION**
Metatranscriptomics, RNA-SIP, Styryl-based dyes

**TRANSLATION**
BONCAT, Raman

BrdU

**MARKER:**
stable isotope (isotopes $^2H$, $^{13}C$, $^{15}N$, $^{18}O$) labeled substrates, incl. $D_2O$

DNA

Lipids/carbohydrates/amino acids

Mature mRNA

Amino acid

Bioorthogonal non-canonical amino acids

**Figure 1** Methods that yield activity-labeled samples and are targeting cell processes in an 'active' microbial cell that can be coupled with shotgun sequencing. Colors denote resources (green), cell components (blue) and cell processes (orange). Raman: Raman microspectroscopy. For DNA- and RNA-SIP, total nucleic acids are extracted from the samples, and labeled and unlabeled DNA/RNA is separated by density gradient centrifugation. The 'heavier' labeled nucleic acid fractions can be used for construction of metagenomic libraries (Neufeld *et al.*, 2007; Whiteley *et al.*, 2007), whereas PLFAs are analyzed on a mass spectrometer and cannot be combined with nucleic acid sequencing (Jehmlich *et al.*, 2010). Many $^{13}C$-, $^{18}O$-, $^{15}N$-labeled fine chemicals are available (for example, phenol, methanol, ammonia, methane, carbonate, etc.), but the wide-ranging application of SIP is limited by the commercial availability of complex labeled compounds that require expensive custom synthesis. Furthermore, sensitivity of the SIP technique is a function of substrate concentration and the duration of substrate incorporation. Successful SIP is dependent on optimization of substrate concentration to guarantee a significant signal-to-noise ratio and incubation length and avoid enrichment bias (Neufeld *et al.*, 2006) (Table 1). 'Cross-feeding', that is, the flow of the isotope label from primary metabolizers to secondary consumers has also been documented (Hutchens *et al.*, 2004; Dumont *et al.*, 2006). RSG is a fluorogenic redox indicator dye available from Molecular Probes, Invitrogen (Carlsbad, CA, USA). RSG yields green fluorescence (488 nm excitation) when modified by bacterial reductases, many of which are parts of electron transport systems. SIP-Raman microspectroscopy has been performed using $^{13}C$-, $^{15}N$-labeled compounds, as well as with $D_2O$. The addition of $D_2O$ (up to a certain concentration and for limited time) is expected to have negligible effects on the microbial community composition and activity patterns, for example, compared with nutrient substrates (Lester *et al.*, 1960; Berry *et al.*, 2015; Kopf *et al.*, 2015) that are traditionally used for SIP experiments. Incorporation of $D_2O$-derived deuterium into the biomass of autotrophic and heterotrophic bacteria and archaea can be unambiguously detected via C-D signature peaks in single-cell Raman spectra (Ashkin, 1970; Berry *et al.*, 2015). However, for comparative studies between active taxa it should be kept in mind that microbes with different physiologies will incorporate different amounts of deuterium at similar growth rates.

activity state of a cell. The earliest developed microbial method that yields activity-labeled samples *in situ* and can be combined with shotgun sequencing takes advantage of the incorporation of bromodeoxyuridine (BrdU) into newly synthesized DNA (and RNA) of a viable cell and subsequent density gradient centrifugation or immunocapture of BrdU-labeled DNA (Urbach *et al.*, 1999). BrdU labeling has been successfully used in a variety of environments to identify DNA-synthesizing populations. Studies have investigated phosphate stimulation in soil (Borneman, 1999), the active population of lake water (Urbach *et al.*, 1999), bacterial functional redundancy with respect to various

carbon substrates in soil (Yin *et al.*, 2000), microbial association with arbuscular mycorrhizal hyphae (Artursson and Jansson, 2003), microbial response to inoculation of various plants with arbuscular mycorrhizal (Artursson *et al.*, 2005), nitrogen addition in Alaskan boreal forest (Handelsman, 2004; Allison *et al.*, 2008), vertical redox gradients in Baltic Sea sediment (Lasken, 2007; Edlund *et al.*, 2008), fungal response to different carbon sources in forest soils (Hanson *et al.*, 2008; Buerger *et al.*, 2012; McGuire *et al.*, 2012) and microbial response to carbon sources of varying recalcitrance in soil (Urbach *et al.*, 1999; Goldfarb *et al.*, 2011). In addition, studies have targeted bacterial and archaeal

(Borneman, 1999; Yin *et al.*, 2000; Artursson and Jansson, 2003; Artursson *et al.*, 2005; Goldfarb *et al.*, 2011) as well as fungal (Urbach *et al.*, 1999; Allison and Treseder, 2008; Allison *et al.*, 2008; Hanson *et al.*, 2008) DNA synthesis activity in response to various environmental stimuli in soils and sediments (Yin *et al.*, 2000; Edlund *et al.*, 2008). Although BrdU-labeling experiments can help identify replicating microorganisms and determine average activity in response to various changing environmental conditions, organisms vary in their ability to incorporate the relatively large BrdU molecule into their DNA, and the rate at which incorporation occurs can deviate up to 10-fold between cells (Urbach *et al.*, 1999; Artursson and Jansson, 2003; Hellman *et al.*, 2011). Such variability may lead to incomplete and skewed representation of the active microbial population in a given environment. In addition, owing to low labeling efficiency, BrdU labeling linked to sequencing requires a relatively large number of sample replicates to obtain the amount of DNA needed for 16S rRNA gene and metagenome sequencing, which renders this technique relatively costly and labor intensive (Tables 1 and 2). The aforementioned studies have yielded important insights into the functioning of microbial communities, yet combining BrdU labeling with either fluorescence-activated cell sorting (FACS) and subsequent high-throughput sequencing or solely with high-throughput sequencing has found limited application to date (Mou *et al.*, 2008; David *et al.*, 2014; Hamasaki *et al.*, 2016). Published studies have predominantly included PCR-amplified rRNA gene surveys and only a few metagenome studies are available (Figure 2, Table 3). However, as DNA requirements for shotgun sequencing decrease, sequencing of BrdU-labeled environmental DNA may become more prevalent.

Stable isotope probing (**SIP**) labeling technology can be used to selectively enrich the DNA or RNA of active microbial populations (Radajewski *et al.*, 2003). For SIP analysis, stable isotope (for example, $^2$H, $^{13}$C, $^{15}$N or $^{18}$O) labeled substrates are added to the environment and as these substrates are consumed by active organisms, labeled atoms are incorporated in their DNA or RNA. Radajewski *et al.* (2000) first demonstrated this approach by adding various $^{13}$C-enriched carbon sources ($^{13}$CH$_3$OH, $^{13}$CH$_4$) to soil and subsequently identifying active methylotrophs. The authors found that methylotrophy was confined to *Alphaproteobacteria* lineages and identified various new methanol dehydrogenase alpha subunit (*mxaF*) sequences. DNA-SIP is currently the most applied method, having been used by many research groups to study *in situ* microbial activity (Figure 2). RNA-SIP provided insights that led to the isolation and subsequent genome sequencing of a *Thauera* sp., which was identified as key phenol degrading organisms in an industrial wastewater treatment plant (Manefield *et al.*, 2002). Recently, Dumont *et al.* (2013) obtained a $^{13}$CH$_4$ labeled-RNA metatranscriptome from methanotrophs in lake sediment. The labeled metatranscriptome was predominantly enriched in sequences from *Methylococcaceae* and transcripts of methane monooxygenase (*pmoCAB*) genes. The authors suggested that SIP and metatranscriptomics can be broadly applicable, but this approach has only been used one other time to our knowledge (Fortunato and Huber, 2015). Although limitations of DNA- and RNA-SIP include availability of labeled compounds, cross-feeding, sensitivity and potential for enrichment bias (Figure 1, Table 1), continuous efforts to optimize SIP, applying it to wider ranges of environments, and combining it with other techniques, such as Raman microspectroscopy and fluorescence *in situ* hybridization (FISH; Huang *et al.*, 2007) make DNA-SIP the best current method for sequence-based characterization of key populations synthesizing DNA in the presence of isotope-labeled compounds.

Metatranscriptome studies enable the analysis of expressed genes in environmental communities, portraying the near real-time condition of microorganisms because of the relatively short half-life of mRNA (Moran *et al.*, 2012). Studies of microbial communities in soil and marine environments were

**Table 2** Comparison of practical considerations of activity-based techniques in combination with sequencing

| Technique | Incubation time | Labor intensity | Cost of disposables |
|---|---|---|---|
| BrdU labeling | Hours to days | H | $$-$$$ |
| DNA-SIP | Hours to days | M | $$ |
| Peak-to-trough ratios in metagenomes, iRep | NA | L-M | $ |
| RNA-SIP | Minutes | M | $$ |
| Metatranscriptomics | NA | L | $ |
| BONCAT (+FACS) | Hours to days | H | $$$* |
| D$_2$O+Raman spectrometry | Hours to days | H (currently because of lack of automation) | $$$* |
| Redox sensor green+FACS | 10 min | H | $$$* |

Labor intensity is a rough estimate of time required to handle 10 retrieved samples/data sets, where 1 sample may either be 1 cell or a population: L ~ <1 week; M ~ >1–2 weeks; H ~ 2–4 weeks. Cost of disposables is divided into three brackets for processing and analysis of 10 individual samples (excluding replicates): $ ~ <$100; $$ ~ $100–500; $$$ ~ >$500. These costs do not include labor or equipment time. Cost differences that may occur depending on whether samples are defined as single cells, which require MDA, or as sub-populations were determined to be negligible for 10 samples. Costs affected by this sample differentiation are denoted with an asterisk.
Abbreviations: BONCAT, bioorthogonal non-canonical amino-acid tagging; BrdU, bromodeoxyuridine; FACS, fluorescence-activated cell sorting; iRep, index of replication; MDA, multiple displacement amplification; NA, not applicable; SIP, stable isotope probing.
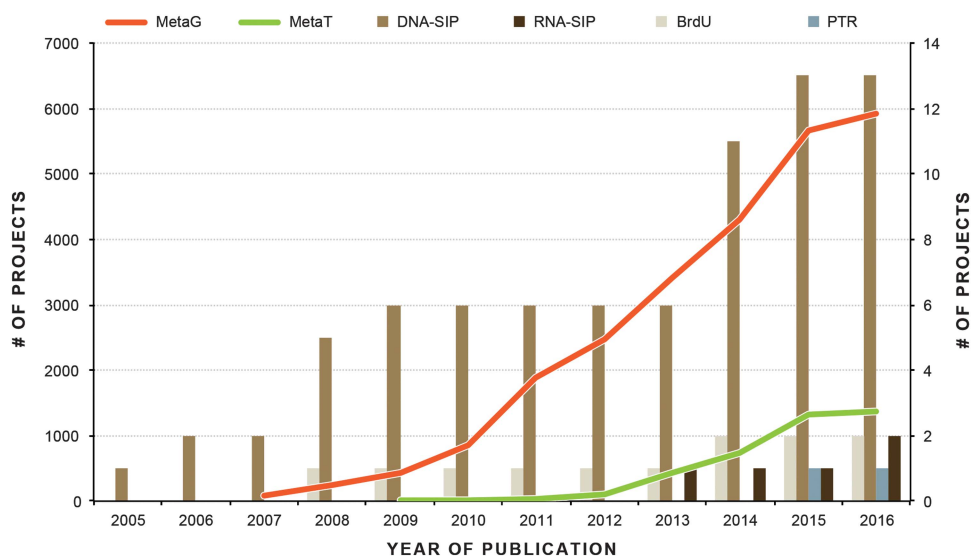
**Figure 2** Project statistics by method over the last decade. Counts displayed exclusively include projects using high-throughput sequencing. Metagenome (MetaG) and metatranscriptome (MetaT) projects are depicted by lines (primary y axis), whereas BrdU, DNA-SIP, RNA-SIP and PTR projects are displayed as bars (secondary y axis). Project abundances are cumulative. Number of MetaG and MetaT projects include public sequencing projects as recorded in the Genomes OnLine Database (GOLD) (Pagani et al., 2011) retrieved 15 January 2016. Number of all other activity-based projects include published records that feature high-throughput (next-generation) shotgun sequence data.

the first to use high-throughput sequencing technology for mRNA analysis (Leininger et al., 2006; Frias-Lopez et al., 2008; Urich et al., 2008). Since then, several studies in various environments have shown that only a subset of the total microbial community contributes to the sequenced RNA pool, and can therefore be considered transcriptionally active. For example, Lesniewski et al. (2012) were able show that transcriptional microbial activity of hydrothermal plume communities was largely attributed to water column microorganisms rather than members from the seafloor. mRNA is regularly used as an indicator of microbial activity and is logistically advantageous because samples can be frozen instantaneously, thereby enabling delayed processing after freezing, which is not an option for other methods that yield activity-labeled samples in situ (Table 1). This is relevant when in situ conditions cannot easily be maintained during sampling or downstream sample processing. A critical issue is how deeply a community transcriptome is 'covered' by the sequence data. If too shallow, libraries will be dominated by transcripts from metabolic pathways shared by most cells and lacking in those representing specialized biogeochemical pathways (Hewson et al., 2009; Gifford et al., 2010; Poretsky et al., 2010). As a consequence, unique expression patterns within a community may be missed, and comparative analyses between communities can become insensitive. Also, depletion of rRNA before metatranscriptome sequencing is not always trivial. Nevertheless, the near-real-time response of mRNA levels to environmental changes experienced by a cell renders assessing inventories of mRNA pools highly informative for identifying ecologically

relevant processes while also determining the identities of their microbial drivers. Declining costs, greater sequencing depth and more comprehensive genomic databases render metatranscriptomics an increasingly powerful and popular tool.

With the advent of fluorescence microscopy activity stains have found an application in microbial ecology. These include stains that rely on cell membrane integrity, enzyme activity or the presence of biomolecules (lipids/nucleic acid). For instance, redox sensor green (RSG) and denaturing gradient gel electrophoresis (DGGE) profiling were used to detect and enumerate microbes actively metabolizing the $C_1$ substrates methane, methanol, methylamine and formaldehyde in Lake Washington sediment (Kalyuzhnaya et al., 2008). According to Kalyuzhnaya et al., RSG does not suppress cellular metabolism as was previously found for tetrazolium salts, such as CTC (5-cyano-2,3-ditolyl tetrazolium chloride) (Ullrich et al., 1996). Using FACS, RSG could likely be scaled to separate bacterial and archaeal cells with an electron membrane potential in the presence of environmental stimuli, therefore studying population activity levels.

One of the most recently developed methods that yield activity-labeled samples in situ is bioorthogonal non-canonical amino-acid tagging (BONCAT). This technique is based on the in vivo incorporation of artificial amino acids that carry modifiable chemical tags into newly synthesized proteins (Dieterich et al., 2006). These chemical tags can then be fluorescently labeled via click chemistry (Hatzenpichler et al., 2014). BONCAT has been demonstrated to be effective in labeling the proteomes of a wide range of taxonomically and

**Table 3** Availability of nucleic acid sequence data retrieved using *in situ* microbial activity approaches

| Technique | Environment | Sequence data | References for shotgun sequencing approaches |
|---|---|---|---|
| BrdU labeling | | 16S rRNA, MetaG | Mou *et al.*, 2008; David *et al.*, 2014 |
| DNA-SIP | | 16S rRNA, MetaG | Kalyuzhnaya *et al.*, 2008c; Dumont *et al.*, 2006; Chen *et al.*, 2008; Kalyuzhnaya et al., 2008b; Neufeld *et al.*, 2008; Sul *et al.*, 2009; Gutierrez *et al.*, 2013; Zhang *et al.*, 2013; Chemerys *et al.*, 2014; Kim *et al.*, 2014; Pinnell *et al.*, 2014; Verastegui *et al.*, 2014; Werner *et al.*, 2014; Eyice *et al.*, 2015; Abu Laban *et al.*, 2015; Schwartz *et al.*, 2016 |
| RNA-SIP | | 16S rRNA, MetaT | Borneman, 1999; Mou et al., 2008; Haroon *et al.*, 2013; Fortunato and Huber, 2015 |
| Metatranscriptomics | | 16S rRNA, MetaT | Leininger *et al.*, 2006; Dumont *et al.*, 2006; Frias-Lopez *et al.*, 2008; Chen *et al.*, 2008; Urich *et al.*, 2008; Kalyuzhnaya *et al.*, 2008b; Neufeld *et al.*, 2008; Shi *et al.*, 2009; Sul *et al.*, 2009; He *et al.*, 2010; Lin *et al.*, 2010; Helbling *et al.*, 2011; Feike *et al.*, 2011; Damon *et al.*, 2011; Bomar *et al.*, 2011; Burow *et al.*, 2012; Ottman *et al.*, 2012; Vila-Costa *et al.*, 2012; Zhang *et al.*, 2013; Chaparro *et al.*, 2013; Gutierrez *et al.*, 2013; Embree *et al.*, 2013; Carvalhais *et al.*, 2013; Sanders *et al.*, 2013; Baker *et al.*, 2013; Ursell and Knight, 2013; Lim *et al.*, 2013; Lehembre *et al.*, 2013; Orsi *et al.*, 2013; Chemerys *et al.*, 2014; Kim *et al.*, 2014; Pinnell *et al.*, 2014; Verastegui *et al.*, 2014; Werner *et al.*, 2014; Franzosa *et al.*, 2014; Jorth *et al.*, 2014; Schwab *et al.*, 2014; Lamendella *et al.*, 2014; Satinsky *et al.*, 2014; Klindworth *et al.*, 2014; Hua *et al.*, 2014; Simón-Soro *et al.*, 2014; Hilton *et al.*, 2014; Eyice *et al.*, 2015; Abu Laban *et al.*, 2015; Alexander *et al.*, 2015a; Chapelle *et al.*, 2015; Bikel *et al.*, 2015; Bello-Ortí *et al.*, 2015; Ishii *et al.*, 2015; Alexander *et al.*, 2015b; Eren *et al.*, 2015; Jones *et al.*, 2015; Steen *et al.*, 2016; Jiang *et al.*, 2016; Marzano and Domier, 2016; Kodzius and Gojobori, 2016; Mondot and Lepage, 2016; De Filippis *et al.*, 2016; Radajewski *et al.*, 2000; Kalyuzhnaya *et al.*, 2008a, c; Konopka *et al.*, 2010 |
| Redox sensor green | | 16S rRNA | |
| BONCAT | | 16S rRNA | Hatzenpichler *et al.*, 2014; Korem *et al.*, 2015 |
| Raman+D₂O | | 16S rRNA | Berry *et al.*, 2015; Brown *et al.*, 2016 |
| Peak-to-trough ratios in metagenomes, iRep | | MetaG | Radajewski *et al.*, 2003; Leininger *et al.*, 2006; Hatzenpichler *et al.*, 2014; Korem *et al.*, 2015; Brown *et al.*, 2016; Hatzenpichler *et al.*, 2016 |

Sequence data denote those from published studies in listed references. ⌿: sediment or soil; ◣: marine or fresh water; ⌸: (enrichment) culture or bioreactor; ◷: association studies between bacteria and/or archaea and eukaryotes. References do not include molecular marker studies. Abbreviations: BONCAT, bioorthogonal non-canonical amino-acid tagging; BrdU, bromodeoxyuridine; iRep, index of replication; rRNA, ribosomal RNA; SIP, stable isotope probing. References for metatranscriptomics studies are too comprehensive to list, and thus cover a broad selection of studies.

physiologically distinct archaea and bacteria (Hatzenpichler *et al.*, 2014). For example, dominant cells in an aerobic methanotrophic enrichment culture from deep-sea sediments were identified as *Methylococcaceae*-related gammaproteobacterium based on FISH analyses. The translational activity of these *Methylococcaceae* cells was found to be dependent on the addition of methane to the enrichment culture. Genome information from this *Methylococcaceae* strain confirmed the methanotrophic nature of this bacterium. Rather than

studying the bulk proteome, BONCAT is able to specifically target proteins that have been expressed in response to an experimental condition. The method can also be used in conjunction with rRNA-targeted FISH and thereby allow the simultaneous identification of a microbial cell and its translational activity. In a very recent effort to describe anaerobic methane oxidizers from methane seep sediments, BONCAT was combined with FACS to sort translationally active methane-oxidizing consortia. Whole-genome amplification and 16S rRNA
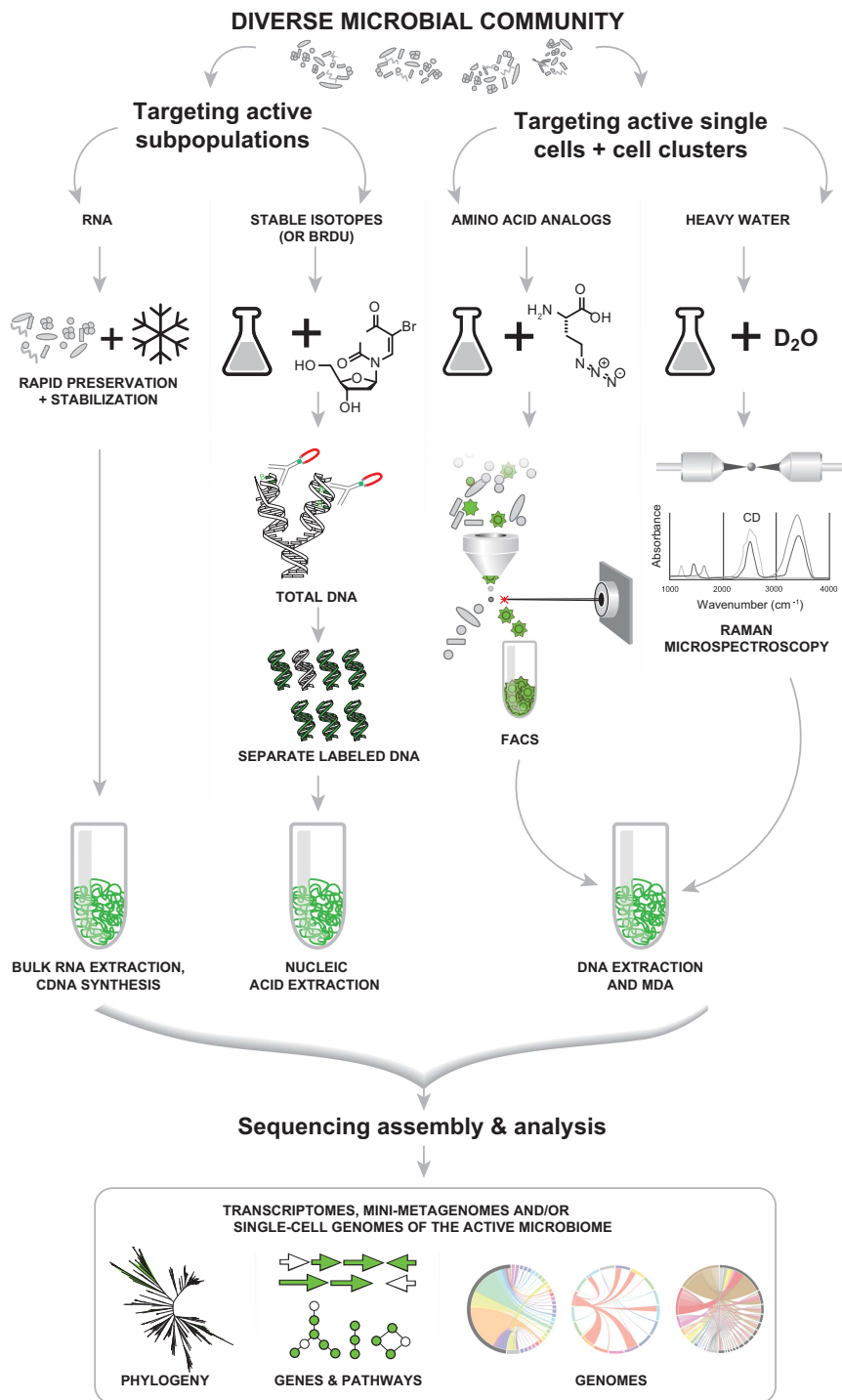
**Figure 3** High-throughput workflows of current and emerging *in situ* microbial activity approaches linked to sequencing. Metatranscriptomics and stable isotope labeling are the most commonly used techniques coupled with next-generation shotgun sequencing technology. Emerging methods that are currently still subject to development and/or optimization involve the incubation of cells and cell clusters with, for example, fluorescent compounds or $D_2O$ before selective sorting of active cells using FACS or Raman OT.

gene sequencing of the aggregates revealed a novel archaeal–bacterial association (Hatzenpichler *et al.*, 2016). This technique presents great prospects for future high-throughput sequencing coupled to BON-CAT and is expected to help answer questions regarding cellular translational activities in response to environmental cues at micrometer resolution, as

well as in a high-throughput manner across larger scales (Figure 3). Limitations of BONCAT include the diversity of amino-acid uptake mechanisms in the environment, the potential for artificial growth stimulation following addition of amino acids and suppression of signal when environmental concentrations of amino acids are high. Aside from these potential

limitations, we expect BONCAT to be combined with shotgun metagenomics in the near future.

Raman microspectroscopy is an established vibrational spectroscopic technique with emerging prospects in microbial ecology. This non-destructive technique enables chemical fingerprinting of individual eukaryotic cells, organelles, or bacterial and archaeal cells. Chemical information derived from a Raman spectrum provides comprehensive and intrinsic information on the chemical composition of the analyzed cell without the need of external labeling, which, for example, enables microbiologists to identify storage compounds, cytochromes and pigments on a single-cell level within a few seconds (Gruber-Vodicka *et al.*, 2011; Majed *et al.*, 2012; Milucka *et al.*, 2012). Interestingly, the incorporation of the stable isotopes $^{13}$C, deuterium and $^{15}$N into microbial biomass can also be detected via characteristic band shifts in the Raman spectra of the labeled microbes (Wang *et al.*, 2016), but it should be noted that the $^{15}$N-induced shifts are difficult to interpret in complex samples. As Raman microspectroscopy can be combined with FISH (Huang *et al.*, 2007) and optical tweezers (OTs), it offers a culture-independent approach to study the physiology of uncultivated microorganisms in their natural ecosystem. For example, Raman-FISH was applied to identify and measure *in situ* $^{13}$C-naphthalene degraders within a microbial community from a complex groundwater system and proved that an uncultured species (*Acidovorax* sp.) had the key role in naphthalene biodegradation, rather than the cultivated naphthalene biodegrading *Pseudomonas* sp. obtained from the same groundwater (Huang *et al.*, 2008). OT-Raman (Raman microspectroscopy coupled with OTs) has been applied to trap and measure single cells of blood (Xie *et al.*, 2002) or *Escherichia coli* (Xie and Li, 2003) and can also be used to sort isotope-labeled microbial cells for subsequent whole genome amplification and sequence-based 16S rRNA gene identification (Huang *et al.*, 2009; Berry *et al.*, 2015). Alternatively, Raman-activated cell ejection using laser-induced forward transfer can be exploited as a low-throughput method for physically removing individual cells from a sample based on their Raman spectrum (Wang *et al.*, 2013; Song *et al.*, 2016).

In a recent study, active microbial cells were identified under near-natural conditions in complex systems via combined use of heavy water ($D_2O$) and Raman microspectroscopy (Berry *et al.*, 2015). $D_2O$-Raman spectroscopy can be combined with FISH for parallel identification of the metabolically active microbes. Furthermore, unlabeled substrates can be added and changes in activities of microbial groups of interest can be quantified (Berry *et al.*, 2015; Eichorst *et al.*, 2015). For example, it was revealed that in the cecal microbiota of mice *Akkermansia muciniphila* and *Bacteroides acidifaciens*, two host-compound foragers, exhibited distinctive response patterns to amendments of mucin and sugars (Berry *et al.*, 2015). By applying $D_2O$-labeling and OT-Raman, cecal microbial cells stimulated by mucin or glucosamine were obtained for multiple displacement amplification and subsequent identification through 16S rRNA gene sequencing (Berry *et al.*, 2015). Although offering direct access to genomic information from microbes with a defined activity, sorting via OTs is slow. In this context, it is interesting to note that recently a fast Raman-activated cell sorting system (RACS), was designed and used to collect carotenoid-producing yeast cells (Zhang *et al.*, 2015a). Other recent advances include microfluidics-based cell manipulation techniques, such as flow-based OT-RACS and Dielectrophoresis (DEP-) RACS, and advanced Raman techniques, for example, Surface-enhanced Raman spectroscopy (SERS) flow cytometry, Resonance Raman spectroscopy (RRS) cell counting and Coherent anti-Stokes Raman spectroscopy (CARS) flow cytometry, which are discussed elsewhere (Zhang *et al.*, 2015b). Microfluidic device-based Raman sorting can be readily integrated with downstream processes, such as cell incubation, chemical analysis and PCR (Wang *et al.*, 2005). Unlike FACS, Raman sorting can use more criteria, including the incorporation of stable isotopes, to identify and sort individual cells (Table 1).

Finally, two very recently developed non-invasive bioinformatic approaches for linking metagenomics and bacterial activity use sequence read depth across microbial genomes. DNA replication typically begins at a single origin of replication in bacterial and archaeal chromosomes (Mott and Berger, 2007). Thus, during DNA replication, regions that have already been passed by the replication fork will have two copies while the yet unreplicated regions will have a single copy. Korem *et al.* (2015) show that metagenome read coverage patterns for different microbial genomes contain a single trough and a single peak, the latter coinciding with the bacterial origin of replication. The peak-to-trough ratio (PTR) was also demonstrated to provide a quantitative measure of a species growth rate *in vitro* and *in vivo*, under different growth conditions and in complex bacterial communities. For instance, it was shown that the proliferative behavior of virulent and non-virulent strains of *C. rodentium* could be accurately predicted by their PTRs. The application of PTRs is promising, not least, because it considers genomic variance between strains, copy number variation of different genomic regions and variable coverage resulting from sequencing depth. However, it requires information on origin of replication sites of the investigated bacteria and archaea and, on a case-by-case basis, manual correction for relative abundance levels of taxa (Table 1). In an effort to expand this method to metagenome data with draft genomes, Brown *et al.* (2016) developed the index of replication (iRep), which is based on mapping metagenome sequencing reads to the collection of assembled sequences that represent a draft genome. Excluding extremely high and low coverage regions, the overall

slope of coverage across a genome is used to calculate iRep (Brown *et al.*, 2016). Applied to metagenomic data, iRep showed, for instance, that Candidate Phyla Radiation bacteria are generally characterized by slower growth rates compared with various human microbiome organisms. iRep requires a ⩾75% complete genome in order to produce accurate measurements and was shown to be then correlated to PTRs and experimentally determined growth rates (Brown *et al.*, 2016).

## Choosing an appropriate method for assessing microbial activity *in situ*

Our view of microbial activity is strongly shaped by the method and parameters chosen. Microbial contribution to processes can neither always be definitively assessed in every environment nor at a scale that allows interpretation at the systems level. But when experiments are designed carefully to address specific ecological questions at relevant and yet tangible temporal or spatial scales and under consideration of the limitations of the chosen tool (as discussed in the following), methods that yield activity-labeled samples *in situ*, are a great resource. The choice of an appropriate tool for targeting the *in situ* active microbial population will depend on various factors. The physicochemical characteristics of the environment of interest, for example, water saturation, pH, amino acid and sulfide concentration, determine whether a water- or bioorthogonal amino-acid-based labeling method is practical. Although microbial activity is generally correlated with the availability of water in a system, the addition of water may lead to artificially increased microbial response, for example, in systems that are heterogeneously wetted, such as soil aggregates. On the other hand, adding isotope-labeled substrates other than water as typically done in conventional SIP studies changes the nutrient composition of the system and often leads to biases in microbial activity patterns (Table 1). The presence of a reductase activity, DNA synthesis, transcription and translation may occur in cell maintenance mode, as well as during growth and division, which is problematic for the correlation of these processes to quantitative rates of specific functions (Blazewicz *et al.*, 2013). Estimates for maintenance requirements between cultures and microbes living, for example, in low activity subsurface environments vary widely (Hoehler and Jørgensen, 2013). Similarly, ranges of metabolic rates for the same reaction should be modeled for their specific environmental parameters to optimize incubation durations (Hoehler and Jørgensen, 2013). As defining meaningful scales for microbiology research is a recurring challenge (Paerl and Steppe, 2003), the scale of the microbial impact radius determines the choice of method. For example, in a soil environment techniques based on single-cell resolution rarely provide adequate insights for systems-level mass balance and flux calculations. Each method should be tested for the particular type of microbial community studied before results are interpreted, because discrimination against various cell types or taxa may occur (Berney *et al.*, 2007; Teske and Sørensen, 2007; Narasingarao *et al.*, 2011; Franklin *et al.*, 2013; Lloyd *et al.*, 2013; Eloe-Fadrosh *et al.*, 2016).

As the numbers of bulk metagenome and meta-transcriptome studies have greatly increased over the last few years (Figure 2), we expect other *in situ* activity targeted approaches coupled to shotgun sequencing to follow this trend, as in part already observed by the recent increase in DNA-SIP projects. Although DNA- and RNA-SIP methods are still hampered by throughput, high-throughput pipelines will likely become available for broader community use. FACS-based enrichment of active microbial cells may contribute sequence data in the near future because of its accessibility to users and compatibility with a variety of staining or tagging assays (Figure 3). Similarly, SIP in combination with Raman microspectroscopy and sorting is a promising approach, which may soon become available as high-throughput RACS (Zhang *et al.*, 2015b) (Figure 3). A future venture will be determined by the amenability of methods that yield activity-labeled samples *in situ* to unicellular eukaryotic microbes, including protists and fungi. Although many nucleic acid sequence-based studies encompass well-characterized microbial communities providing a level of understanding of their role in a particular habitat, allowing community modeling (Ruff *et al.*, 2015), we often lack an understanding of eukaryotic microbes. This group of organisms is extremely heterogeneous and there is evidence for its significance in various systems, for example, in airways of cystic fibrosis (Eickmeier *et al.*, 2014), soil (Taylor *et al.*, 2014) and contaminated aquifers (Holmes *et al.*, 2013). On the other hand, inactive eukaryotic microbes may significantly add to the pool of relic DNA. Hence, methods that yield activity-labeled samples *in situ* should include these organisms. Availability and cost-effectiveness of the necessary equipment, as well as the scalability of data throughput will likely guide the future of methods that yield activity-labeled samples *in situ* linked to genomics.

## Integrating genomic and functional information from the active microbiome

Capturing the genetic makeup of the active microbiome *in situ* holds great promise to substantially advance our understanding of microbial community functioning. It enables a shift from cataloging the mere presence of taxa and encoded (potential) functions to understanding their ecological roles, including contributions to ecosystem processes. Some of the intriguing and timely research foci in

microbial ecology revolve around expanding our knowledge of microbial guilds, groups of species that exploit the same resource, and of reactions that are thermodynamically feasible enough to sustain microbial growth that are lacking associated micro-organisms to date. In these cases, nucleic acid sequencing combined with specific labeled substrate assays and coupled to continuous geochemical profiling would, for instance, allow targeting novel organisms that perform redox reactions of interest and enable comprehensive flux modeling of systems (LaRowe and Amend, 2016). Another timely ecological topic is the significance of so-called 'strongly interacting species' (SISs) and temporarily dormant cells. SISs are species that have strong positive or negative impacts on the species they directly interact with (Gibson *et al.*, 2016). SISs are not necessarily keystone species as the removal of SISs from a community does not coercively result in mass extinction (Paine, 1995), but they may include organisms of relatively low abundance (Gibson *et al.*, 2016). SISs can have an important role in shaping the steady states of microbial ecosystems regardless of their relative abundance (Gibson *et al.*, 2016). Hence, identifying SISs and studying the processes in their 'interaction hub' in a comparative analysis can help reveal metabolic strategies of ecologically relevant microorganisms and interaction strength heterogeneity, for example, across geochemical gradients. SISs may be periodically dormant and hence require time series sampling and/or stimulation experiments (Epstein, 2013). Dormancy represents a bet-hedging strategy, in which an organism can enter a state of low metabolic activity when environmental conditions are disadvantageous and resuscitate when conditions become favorable (Lennon and Jones, 2011). Dormant cells thus generate seed banks, which are likely to impact diversity dynamics and maintenance in a given ecosystem (Jorgensen, 2011). To better understand the contribution of key population size vs organism state to variable environmental conditions, data on total community (for example, targeted using total DNA), population growth (for example, targeted using BrdU, metagenomics) and specific (for example, targeted using SIP, metatranscriptomics, BON-CAT and $D_2O$-Raman microspectroscopy with substrate amendments), as well as general activity (for example, targeted using BONCAT, metatranscriptomics, RSG, $D_2O$-Raman microspectroscopy, iRep, PTR) should each be collected in time series experiments. Similarly important is the characterization of the ecological niche of SISs, which may be difficult because of micro-environmental heterogeneity, for instance in soil (Pester *et al.*, 2010) and biofilms (Augspurger *et al.*, 2010). Owing to intricate structures, including channels and voids, a biofilm can host multiple functional groups of cells in various spatial relationships of each other (Gieseke *et al.*, 2003). These spatial arrangements affect nutrient accessibility and therefore influence levels

of competition and mutualism between microbial factors (Santegoeds and Damgaard, 1999). The opportunity to resolve the spatial distribution of active microbial cells will disclose the relevance of proximity for microbial interactions with each other, higher organisms or substrate surfaces. Relevant scaling of experimental designs can be achieved using geochemical profiling and flux analysis before and paralleling the use of community analysis. Retrieval of shotgun sequence information remains essential, for instance, when studying genomic variation and gene regulation within species, resulting in increased ecosystem functioning complexity (Darch *et al.*, 2015). Culture-based approaches, metaproteomics and metabolomics studies will effectively complement these studies. Finally, novel single-molecule sequencing methods, such as nanopore technologies (Oxford Nanopore Technologies (ONT; Schneider, 2012), may soon enable the direct measurement and sequencing of chemically modified bases incorporated into the nucleic acids of transcriptionally active or replicating cells. ONT has recently demonstrated the detection of 5-methylcytosine in bacterial and human DNA (Simpson *et al.*), highlighting its potential to detect other non-canonical bases in the near-term future (Woyke and Rubin, 2014).

Exciting technological advances have shaped the trajectory of microbial community studies, resulting in fascinating insights into our microbial world. We expect this trend to continue as sequence quality improves at reduced costs with a shift from bulk microbial community analyses to more refined studies that target active populations of interest. These improved and novel technologies will assist in exploring the surging scientific questions and applications that have been engaging the scientific community in the field of microbial ecology.

## Conflict of Interest

The authors declare no conflict of interest.

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