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RNA-stable isotope probing: from carbon flow within key microbiota to targeted transcriptomes

Tillmann Lueders[a], Marc G. Dumont[b], Lauren Bradford[a], Mike Manefield[c]

[a] Helmholtz Zentrum München – German Research Center for Environmental Health, Institute for Groundwater Ecology, Neuherberg, Germany

[b] Centre for Biological Sciences (CfBS), University of Southampton, Southampton, United Kingdom

[c] Centre for Marine Bioinnovation, School of Biotechnology and Biomolecular Sciences, University of New South Wales, Sydney, Australia

Correspondence: Tillmann Lueders, Helmholtz Zentrum München (GmbH), Institute of Groundwater Ecology, Ingolstädter Landstr. 1, 85764 Neuherberg, Germany.

E-Mail: tillmann.lueders@helmholtz-muenchen.de

Abstract

Stable isotope probing of RNA has enthused researchers right from its first introduction in 2002. The concept of a labelling-based detection of process-targeted microbes independent of cellular replication or growth has allowed for a much more direct handle on functionally relevant microbiota than by labelling of other biomarkers. This has led to a widespread application of the technology, and breakthroughs in our understanding of carbon flow in natural microbiomes, autotrophic and heterotrophic physiologies, microbial food webs, host-microbe interactions and environmental biotechnology. Recent studies detecting labelled mRNA demonstrate that RNA-SIP is not limited to the analysis of rRNA, but is currently developing towards an approach for accessing targeted transcriptomes. In combination with next-generation sequencing and other methodological advances, RNA-SIP will continue to deliver invaluable insights into the functioning of microbial communities.

Introduction

Microorganisms that utilize a specific growth substrate can be identified in a sample by stable isotope probing (SIP). A substrate artificially labelled with a rare stable isotope (e.g. ^{13}C, ^{15}N, ^{18}O) is provided as smart tracer, which becomes assimilated into the biomolecules of target organisms. Methods are available to analyse the incorporation of label into various biomarkers, including phospholipid fatty acids (PLFAs), DNA, RNA, proteins and entire cells. Each of these is discussed within this themed issue. Here we focus on the most recent advances in the development and application of RNA-SIP (Fig. 1).

For both DNA- and RNA-SIP, labelled nucleic acids are physically separated by isopycnic gradient centrifugation. Gradients of cesium chloride (CsCl) are used for DNA [1], whereas cesium trifluoroacetate (CsTFA) amended with a small percentage of formamide is used for RNA [2]. Fractionation of SIP gradients was first introduced for RNA, an important advance compared to ethidium bromide-based band detection. Fractionation allows access to the full range of buoyant densities resolved in gradients, including only partially labelled nucleic acids. In combination with quantitative analyses of gradient fractions, the distribution of specific RNA-populations across gradient fractions can be compared [3].

Most of the RNA-SIP studies to date have targeted rRNA, generating taxonomic information on the microbes involved in label assimilation. Undoubtedly, this is where the approach has had its greatest appeal in tracing lineage-specific carbon flow within complex microbial communities. However, Huang et al. [4] noted that labelled mRNA transcripts can also be detected in gradient fractions. Dumont and colleagues [5] compared the results of labelling methanotrophs with $^{13}CH_4$ in lake sediment by targeting 16S rRNA and *pmoA* markers by both DNA- and RNA-SIP and showed that the labelling of *pmoA* transcripts was more rapid than that of *pmoA* genes. This study laid the foundation for the combination of SIP with next-generation sequencing-based metatranscriptomics [6]. This emerging approach to target specific microbial transcriptomes has the potential to alleviate some of

the most fundamental limitations of non-targeted 'omics [7] in complex systems. Considerable advances can be expected from the implementation of this novel strategy in different research fields.

RNA-SIP: strengths and limitations

RNA and DNA are preferred biomarkers for taxonomic identification of labelled microbes (Table 1). RNA labelling, unlike DNA, is independent of cellular replication, making RNA-SIP more sensitive [2,5]. A community's metagenome may remain static over time scales where its transcriptome dynamically responds to environmental change. Moreover, the targeted microbes might be slow growing. For example, in a study on autotrophic ammonia-oxidizing archaea (AOA) in an agricultural soil, labelled AOA were only detectable by RNA-SIP, suggesting that they did not replicate sufficiently for DNA labelling [8]. Similarly, ^{13}C-acetate assimilation by putative mixotrophic atmospheric methane oxidizers in a forest soil was apparent after 3 weeks in labelled mRNA but not in DNA [9]. On the other hand, work with RNA requires caution, as it is less stable than DNA. This is especially true for mRNA. Thus optimized laboratory routines are required to mitigate these issues. The prolonged ultracentrifugation times at room temperature required for RNA-SIP are fortunately not of concern because of the RNase-inhibitory function of CsTFA, a chaotropic salt [5].

Protein-SIP, the youngest amongst the different strategies to detect biomarker labelling, allows for the most direct indication of specific metabolic activities by a given microbial population [10]. However, it requires a considerable depth of à-priori (meta)genomic information in order to phylogenetically place labelled peptide sequences. This is often difficult to accomplish for more complex communities or as-yet uncultivated lineages. The most recent advances in mRNA-SIP demonstrate that it is a powerful alternative to detect process-related gene expression [4,5,8,9,11,12].

Based on mass spectrometry as opposed to buoyant density separation, protein-SIP and PLFA-SIP have detection limits at ~1% isotope incorporation or below for ^{13}C [13]. Until recently, the detection

limits of DNA- or rRNA-SIP were estimated to be ~20% ^{13}C enrichment, controlled mainly by the limits of gradient fractionation. However, the application of high-throughput sequencing of gradient fractions does not only allow for a very sensitive detection of highly labelled populations at extremely low-abundance [14]. If combined with models assuming normal distribution of distinct rRNA species across buoyant densities, the estimation of population-specific labelling of only a few atom % ^{13}C can be inferred via buoyant density shifts [15]. The comprehensive sequencing-based quantitative interpretation of DNA-SIP gradients has recently been introduced [16], which substantiates this enhanced sensitivity for both ^{13}C- and ^{18}O-labelling at an unprecedented level of lineage-specific resolution. The integration of novel analytical strategies and interpretation routines will continue to increase the sensitivity of rRNA-SIP.

Applications of RNA-SIP

Carbon flow in complex natural microbiota

Since its introduction for the identification of aerobic phenol degraders in an industrial bioreactor [2], RNA-SIP has been applied to elucidate microbial key players involved in a wide range of catabolic and respiratory processes, and in chemolithoautotrophy. A number of RNA-SIP studies have addressed aerobic methano- and methylotrophs in different habitats. Recent work has investigated aerobic methanotroph diversity in the sediment of an oligotrophic German lake [5,6], revealing that mostly type I methanotrophs, closely related to phylotypes also found in other lakes, were active in methane turnover. The labelled metatranscriptome provided a wealth of detail on expressed metabolic pathways active in methane and nitrogen cycling [6]. Other studies have pulsed methanotroph communities in soil with ^{13}C-acetate instead of ^{13}C-methane [9,12], showing that distinct uncultured lineages of type II methanotrophs assimilated carbon from acetate, proving them to be facultative methanotrophs.

RNA-SIP has also frequently targeted ammonia oxidizers, for which potential mixotrophy is also relevant. Pratscher et al. showed using rRNA/mRNA-SIP that ammonia-oxidizing archaea in an agricultural soil relied primarily on autotrophic CO_2 fixation involving the 3-hydroxypropionate pathway [8]. RNA-SIP has also been employed to unravel trophic interactions of nitrifiers. In ammonia-oxidizing activated sludge, protozoan grazing of bacteria was revealed by tracing carbon flow from ^{13}C-bicarbonate into rRNA of ciliate grazers (*Epistylis* spp.) [17]. More recently, a *Micavibrio*-like bacterial micropredator was demonstrated to prey on nitrite-oxidizing *Nitrospira* spp. in a similar system [18].

Carbon usage in sulfur-oxidizing *Sulfurimonas* spp. at a pelagic redoxcline has also been traced by rRNA-SIP [19]. *In situ* experiments with ^{13}C-pyruvate found no rRNA labelling of *Sulfurimonas* GD17, despite its known pyruvate metabolism. Pure culture incubations found that the ^{13}C was incorporated only into amino acids, not nucleic acids. Using a differential labelling strategy with CO_2 and pyruvate, the authors were able to show that these presumed chemolithoautotrophic denitrifiers could assimilate pyruvate as supplementary carbon source *in situ.* Thus, whenever possible, different SIP approaches should be combined to elucidate peculiar physiologies. In a follow-up study, the authors used ^{13}C-labelled cells of *Sufurimonas* spp. to reveal that specific marine ciliate and flagellate populations grazed on the labelled cells and thus controlled the daily bacterial production of lithoautotrophs at the investigated redoxcline [20].

In anoxic marine sediments, recent RNA-SIP work has focused on the identification of acetate-oxidizing microbes in manganese reducing incubations [21]. In distinct sediments from Sweden, Norway and Korea, the labelling of *Colwiella* spp., *Arcobacter* spp. and the *Oceanospirillaceae* were surprisingly consistent. A similar experiment indicated that members of the *Desulfuromonadales* were the key acetate consumers under strictly iron or manganese-reducing conditions [22].

Host-microbe interactions

RNA-SIP has also been extensively used in disentangling interactions between unicellular and multicellular organisms, especially plant-microbe interactions. In a climate-change oriented study, Drigo et al. pulsed $^{13}CO_2$ into mycorrhizal and non-mycorrhizal C-3 plants [23], revealing that elevated atmospheric CO_2 concentrations induced changes in rhizospheric C flow, especially in mycorrhizal plants. A stimulation of mycorrhizal fungi resulted in feedbacks on the entire soil food web. These effects were also shown to develop over multiple seasons [24]. Using $^{13}CO_2$, a greater proportion (~20%) of the root-colonizing bacteria of rice plants was shown to draw directly on fresh plant assimilates vs. bacteria in the rhizosphere (~4%) [25]. RNA-SIP with $^{13}CO_2$ has also shown that genetically modified potato cultivars exert distinct selective forces on rhizosphere communities than unmodified cultivars [26], an important advance in studying potential impacts of GM plants. The principle applicability of mRNA-SIP has also been demonstrated for plant-microbe systems [11]. Here, the labelling of distinct coding and non-coding bacterial mRNAs was found comparing the rhizosphere and rhizoplane of *Arabidopsis thaliana*, providing valuable insights on how microbes adapt to the host environment.

RNA-SIP is also highly useful for the functional dissection of the gut microbiome. Its application for the tracing of microbial starch metabolism in the human colon in an *in vitro* gut model was already demonstrated in 2009 [27], but related *in vivo* studies are yet to come. For animal systems, Godwin et al. compared the assimilation of $^{13}CO_2$ pulses in communities from the kangaroo foregut and the bovine rumen [28]. Evidence for dominant acetogenesis was found in the kangaroo foregut, with *Blautia coccoides* identified as the key acetogen. This explained why kangaroos have much lower methane emissions in comparison to cows, in which the rumen is dominated by hydrogenotrophic methanogens. In the same year Tannock et al [29] used RNA-SIP to identify bacteria in the rat cecum degrading the dietary fructane inulin. *Bacteroides uniformis*, *Blautia glucerasea*, *Clostridium indolis*, and *Bifidobacterium animalis* dominated the assimilation of ^{13}C from inulin fed to rats. In an elegant laboratory verification, representative isolates of the RNA-SIP identified bacteria were then tested

for growth on inulin. Here, *B. uniformis* was the only strain that could actually ferment inulin, whilst the others were only consuming hydrolysis products.

Environmental biotechnology

RNA-SIP was first developed in the context of phenol biodegradation in an activated sludge community treating coking effluent [2]. The identification of microbes responsible for pollutant degradation remains a key application today, along with processes in wastewater treatment and anaerobic digestion. The primary report on mRNA-SIP addressed aerobic naphthalene degradation in contaminated groundwater [4]. By combining rRNA-SIP, mRNA-SIP and Raman-FISH, the authors showed that an *Acidovorax sp.* which eluded laboratory cultivation was responsible for degradation of naphthalene under the low µM concentrations relevant *in situ*. Jechalke et al [30] recently published a comprehensive dissection of a benzene degrading biofilm from an aerated groundwater treatment pond. rRNA-SIP identified *Zoogloea* and *Dechloromonas* spp. as the dominant assimilators of ^{13}C from benzene. Compound-specific isotope fractionation analysis implicated a dihydroxylation reaction for aromatic ring cleavage, consistent with ^{13}C incorporation by specific dioxygenases detected in protein-SIP. A further recent combination of rRNA-, DNA- and protein-SIP investigated anaerobic hydrocarbon degradation in marine sediments [31]. ^{13}C-labelled butane and dodecane were pulsed into seep samples from the Mediterranean and the Gulf of California under sulfate-reducing conditions. Members of the *Desulfobacteraceae* were found to be the dominating degraders for all treatments. But while butane degraders were closely related, long-chain alkane degraders appeared more distinct between sites. Labelling of several key enzymes involved in anaerobic alkane oxidation was also found via protein SIP in the same study. rRNA-SIP has also been applied to elucidate the role of different bacterial populations active in methanogenic toluene-degradation [32]. A network of interactions between *Desulfosporosinus* spp. as primary degraders and distinct *Syntrophaceae*, *Desulfovibrionales* and *Chloroflexi* as syntrophic partners was suggested in a respective enrichment culture.

185 rRNA-SIP has also been applied for the identification of microbes in activated sludge assimilating ^{13}C
186 from nonyl phenol, a common low-level pollutant and xenoestrogen in the urban water cycle [15].
187 The most intensively labelled degrader phylotype (*Afipia* sp.) was relatively low in rRNA abundance.
188 Conversely, less enriched phylotypes (*Propionibacterium* and *Frateuria* spp.) were more abundant,
189 and therefore made a greater contribution to nonyl phenol biodegradation. In another example of
190 RNA-SIP applied to wastewater treatment, Nielsen et al. [33] identified glucose-fermenting bacteria
191 in a full-scale enhanced biological phosphorus removal (EBPR) system. Mainly Gram-positive
192 *Propionibacteriaceae* and *Streptococcaceae* were identified as primary glucose fermenters. These
193 were subsequently quantified by FISH across a range of distinct wastewater treatment plants, giving
194 an elegant example of how SIP can guide monitoring approaches back in the field.
195 Finally, Ito et al. have pioneered the application of RNA-SIP to carbon flow in anaerobic digestion
196 processes [34]. SIP with ^{13}C-labelled glucose and propionate as well as MAR-FISH
197 (microautoradiography and fluorescence *in situ* hybridization) with ^{14}C acetate suggested that an
198 uncultured *Synergistes* lineage was active as syntrophic acetate oxidizers, outcompeting acetoclastic
199 methanogens. In their follow-up study, the authors demonstrated how rRNA-SIP and population-
200 specific substrate flux analyses can be combined to identify rate-limiting steps in anaerobic digestion
201 [35]. Taken together, the many recent applications of RNA-SIP reviewed here substantiate the
202 approach as a prime research strategy to unravel specific activities, ecophysiologies and interactions
203 in complex natural microbiota.
204

205 ## Future directions

206 Undoubtedly, the application of next-generation sequencing to density-resolved RNA fractions
207 represents the largest recent methodological advance in RNA-SIP. Although the methodologies are at
208 hand and the next-generation sequencing of amplicons from RNA gradients is now routine
209 [14,15,25,28,29,31,36,37], only one study to date has retrieved a labelling-assisted targeted
210 transcriptome [6]. Although this was mostly a proof-of-concept experiment with aerobic

methanotrophs, it clearly shows that the metatranscriptome of target populations in environmental samples can be selectively recovered. The combination of SIP with such 'omics techniques is of significant potential, since it provides functional context to sequence data that is not only inferred, but proven by label incorporation. The physical separation of labelled biomarkers - such as in DNA and RNA-SIP - focuses sequencing analysis and allows for a greater analysis depth of target populations.

All other SIP studies reporting on labelled mRNA to date have used either fingerprinting, RT-qPCR, or cloning and sequencing of transcripts to substantiate labelling [4,5,8,9,11,12]. This is surprising since total RNA sequencing strategies are long established [38]. However, the rather small total quantities of labelled RNA (~10s of ng) obtainable from gradient fractions represents a major technical limitation to this end. Both pre-gradient rRNA depletion and post-fractionation RNA amplification (Fig. 1) have the potential to skew transcript ratios [39] and thus to interfere with the detection of labelling. Yet, sequencing technologies and strategies to work with extremely small transcript quantities are rapidly evolving [40]. Thus we expect to see substantial advances in SIP-mediated targeted transcriptomics in the next years.

For 'classical' rRNA-SIP, the application of high-throughput sequencing has added valuable taxon-level precision to quantitative gradient interpretation. Combined with turnover rates and net substrate fluxes, the labelling intensity can be used to infer the contribution of distinct populations to a transformation process, and to quantify population-level substrate utilization [15]. In DNA-SIP, the combination of ^{13}C- and ^{18}O-labelling has recently been suggested as a quantitative measure to infer general bacterial growth rates [16], as discussed in another review of this thematic issue [41]. However, it is also clear that extensive efforts are required to identify sequencing OTUs whose distribution between density fractions is significantly altered by label incorporation, and to sensitively quantify those density shifts [15,16,42]. In essence, all strategies chosen to substantiate lineage-specific label incorporation must fulfil the criterion of comprehensively comparing template

abundance in high vs. low density fractions of gradients from labelled treatments and unlabelled controls [43].

Most RNA-SIP studies to date have relied on ^{13}C-labelling. However, alternative isotope tracers are available and should find wider consideration. ^{15}N-labelling to trace microbes active in nitrogen cycling is well established in DNA-SIP [42,44,45]. Still, to the best of our knowledge, no RNA-SIP study with ^{15}N has been published. In contrast, two studies are available that have performed RNA labelling with $H_2{}^{18}O$. The first study provided insights on a microbial re-activation cascade that occurs when dried soil crusts are rehydrated upon rainfall [36], allowing to better understand microbial feedbacks to increasing aridity and extreme rain events. More recently, it was demonstrated that after a prolonged ~5 weeks of incubation of a soil with $H_2{}^{18}O$, >75% of the rRNA was ^{18}O-labelled [37]. Both studies demonstrate that $H_2{}^{18}O$ is effective as a universal label for active microbes in RNA-SIP.

While the integration of different SIP strategies is becoming increasingly important (e.g. combinations of RNA- & Raman-SIP [4], combined ^{13}C- and ^{14}C-labels [19], RNA-, DNA- and protein-SIP [30,31]), gradient-independent detection methods for labelled RNA are also emerging. The so-called “Chip-SIP” approach relies on the direct isotopic characterization of rRNA hybridised to a phylogenetic microarray by NanoSIMS mass spectrometric imaging [46]. Although the availability of such analytical platforms is still limiting, the approach has been demonstrated to provide valuable quantitative insights into lineage-specific carbon and nitrogen usage in estuarine and marine microbiota [47,48]. Furthermore, a highly sensitive method for the measurement of isotopic enrichment in RNA using ultrahigh-performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS) has recently been published [49]. This protocol can detect an enrichment of 1.5 atom % ^{13}C in as little as 1 ng of nucleic acids and it enables researchers to directly quantify isotope enrichment in RNA from gradient fractions. In combination with the application of targeted RNA cleavage catalysts to RNA-SIP such as LNAzymes [18] or RNaseH [50], we believe that such centrifugation-independent approaches have great unrealised potential to advance RNA-SIP methodologies, and to find more unexpected needles in the many microbial haystacks.

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426 Table

427 **Table 1.** Strengths and limitations of major biomarkers and approaches used in SIP.

Marker	Analysis Method	Strength	Limitation
PLFA	Gas chromatography – isotope ratio mass spectrometry (GC-IRMS)	- High sensitivity, quantitative - Can infer absolute label incorporation	- Very low taxonomic resolution
DNA	Isopycnic centrifugation, various downstream analysis options (qPCR, fingerprinting, marker gene sequencing, 'omics)	- Labelling inferred via rRNA gene community structure - Metabolic potential via functional genes - Potential for targeted (meta-) genome assembly	- Labelling dependent on genome replication and cellular growth - Strong impact of genomic G+C content on buoyant density
rRNA	Isopycnic centrifugation, various downstream analysis options (qPCR, fingerprinting, rRNA amplicon or total rRNA sequencing)	- Labelling inferred via rRNA community structure - Rapid labelling, independent of cell replication - Most active organisms and dynamic changes resolved	- No data on functional genes / gene expression - RNA less stable than DNA, difficult to obtain from some samples
mRNA	Isopycnic centrifugation, various downstream analysis options (qPCR, fingerprinting, mRNA amplicon or total RNA sequencing)	- Labelling of actively transcribed genes, direct ties to community function - Rapid labelling, independent of cell replication - Resolution of rapid changes - Differential gene expression under varying conditions	- Taxonomic precision can be limited - mRNA very unstable; high risk of degradation - Low mRNA quantities (<5% of total RNA) - enrichment or pre-amplification may be necessary
Protein	Protein fractionation, proteolysis, metaproteomics by high-resolution mass spectrometry of peptides	- Direct link between metabolic activity and phylogeny - High sensitivity, quantitative - Rapid labelling, independent of cell replication - Short incubation times	- Labour intensive workflow - Requires à priori metagenomic data for identification of labelled taxa

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Figure legends

Figure 1. General workflow of RNA-SIP and downstream labelling detection approaches. Steps in dashed boxes are optional steps that may be necessary for the detection of labelled transcriptomes.