Tracking microbial interactions with NanoSIMS

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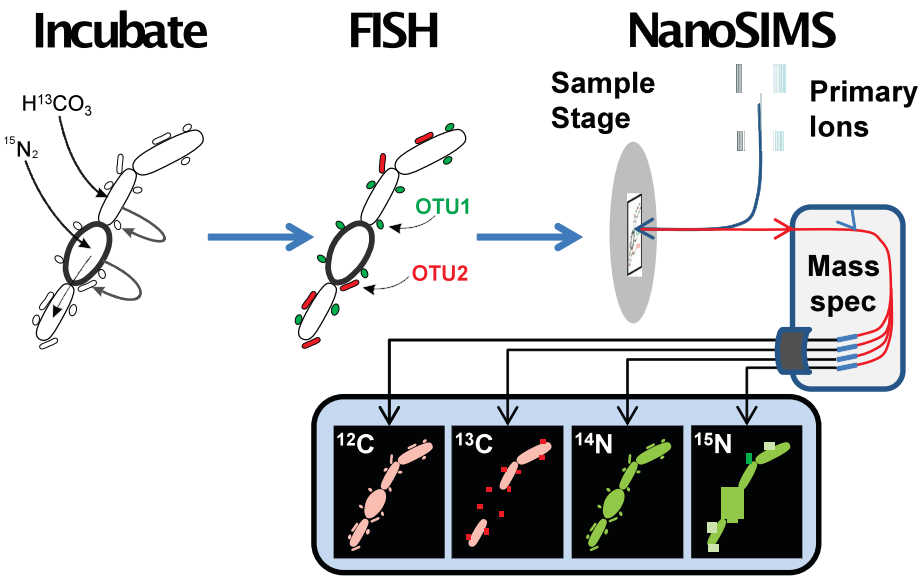
Graphical abstract



ABSTRACT

The combination of stable isotope probing (SIP), NanoSIMS imaging and microbe identification via fluorescence in situ hybridization (FISH) is often used to link identity to function at the cellular level in microbial communities. Many opportunities remain for nanoSIP to identify metabolic interactions and nutrient fluxes within syntrophic associations and obligate symbioses where exchanges can be extremely rapid. However, additional data, such as genomic potential, gene expression or other imaging modalities are often critical to deciphering the mechanisms underlying specific interactions, and researchers must keep sample preparation artefacts in mind. Here we focus on recent applications of nanoSIP, particularly where used to track exchanges of isotopically labelled molecules between organisms. We highlight metabolic interactions within syntrophic consortia, carbon/nitrogen fluxes between phototrophs and their heterotrophic partners, and symbiont-host nutrient sharing.

Introduction

Understanding the functional roles and interactions of individual microorganisms within complex communities is a major goal of environmental microbiology. Significant strides have been made to identify the *in situ* function of individual microbes [1-8]. Yet interactions among cells in environmental communities remain difficult to disentangle. Many factors obscure microbe-microbe interactions, including functional redundancy, transient metabolites, proteins with unknown function, and disconnects between genomic potential and actual function. Researchers are increasingly overcoming these challenges with multi-prong approaches—"community system biology"—where next generation sequencing methods ('-omics') are used in combination with direct process measurements (e.g. stable isotope probing 'SIP') to map functions, energy flows, and biotic relationships in previously intractable complex communities.

Here, we review recent studies of microbial interactions where high-resolution imaging secondary ion mass spectrometry (SIMS) was combined with stable isotope probing (‘nanoSIP’ [9]) to allow tracing of stable isotope assimilation into specific microbial cells. SIMS is a type of imaging mass spectrometry where a primary ion beam is used to generate secondary ions from the surface of a solid sample. The Cameca NanoSIMS enables 'nanoSIP' because of its particularly high spatial resolution (50 nm), high sensitivity, and high mass specificity [9,10]. NanoSIP usually requires targeted microbes to be phylogenetically identified, therefore we also emphasize the *in situ* hybridization methods (e.g., FISH, CARD-FISH, SIMS-ISH, EL-FISH, HISH) which help link identity to isotopic enrichment [11-14]. Many studies have shown these *in situ* hybridization methods are crucial to characterizing

45 microbial activity in the context of spatial relationships [6,15] that range from microbe-host
46 and microbe-microbe interactions, to cell-cell nutrient or metabolite exchanges, and
47 interactions between cells and their organic/inorganic matrix.

48 **NanoSIP in microbial ecology**

49 In the past decade, microbial ecology has benefitted greatly from the use of stable isotope
50 tracing (SIP) techniques (DNA-SIP, RNA-SIP, Protein-SIP, PLFA-SIP, metabolite-SIP) to link
51 identity and function within interacting microbial populations, via detection of specifically
52 labeled cellular components [16-19]. In these approaches, isotope enrichment of specific
53 DNA sequences, proteins or lipids extracted from bulk samples is measured, yet it is not
54 possible to resolve the contributions of individual cells or cell compartments to a particular
55 process [20]. Many critical questions in microbe-microbe and host-microbe interactions
56 require micro-scale spatial information that is lost during bulk sample processing. This is
57 where the NanoSIP approach plays an important role, as the high resolution of NanoSIMS
58 elemental and isotopic images can document cellular metabolism and transfers between
59 individual, closely associated cells. For many researchers, direct measurements of how
60 microbial partners function, and cell-specific rates of metabolic activities are necessary to
61 link symbiont activity, host ecological processes and ecosystem-level biogeochemistry. As
62 access to NanoSIMS instruments becomes more and more available, particularly via user
63 facilities in the USA and Europe, researchers will increasingly be able to balance the
64 availability, cost, and appropriateness of the NanoSIP approach to their individual scientific
65 questions.

66 The nanoSIP combination has been broadly used in cell biology [10,21-27], microbiology
67 [2,28-30] and phytoecology [31-34] (Fig. 1). NanoSIP is particularly valuable for microbial
68 ecology because it provides spatially resolved information about uptake and transfer of
69 isotopically-labelled compounds in environmental samples where the component taxa
70 cannot be cultured individually. Moreover, by imaging the intracellular localization of stable
71 isotope-labelled molecules, nanoSIP can provide direct evidence of cellular metabolism,
72 distribution of labelled molecules and even quantitative tracking of molecules as they are
73 assimilated into cell biomass or exchanged with other cells [20,21,35]. The most commonly
74 used metabolic tracers in labelling experiments are ^{13}C and ^{15}N [9,35]; additional studies
75 have used ^{34}S and ^{2}H [6,36].

76

77 **Phylogenetic labelling for nanoSIP**

In nanoSIP studies, cells or subcellular structures (ribosomes or proteins) of target organisms are labelled with oligonucleotide probes or antibodies to provide phylogenetic identification. This procedure takes place immediately after a SIP experiment and typically includes chemical fixation and hybridization steps prior to NanoSIMS analysis [9,35]. Label detection can be done by either (a) sequential imaging by fluorescence microscopy followed by NanoSIMS (FISH-NanoSIMS) e.g. [7,8] or (b) direct label imaging during NanoSIMS analysis (EL-FISH-NanoSIMS, HISH-SIMS) [5,11,12]. In FISH-NanoSIMS applications, the cells are commonly labelled with fluorescent dyes using conventional FISH or CARD-FISH protocols. When target cells are hindered by particles or embedded in an organic matrix or exoploymeric substances (EPS), marking with a laser microdissection microscope or using a coordinate system can ensure that the same fields of interest are imaged by fluorescence microscopy and NanoSIMS. Alternatively, an orthogonal type of microscopy (e.g. scanning electron microscopy (SEM), atomic force microscopy (AFM), or helium ion microscopy) can be used to check the morphological appearance of fluorescently labelled target cells prior to NanoSIMS analysis. For direct label imaging, a rare element such as a halogen (attached to oligonucleotide probes or tyramides) or metal (gold-labelled oligonucleotide probes or antibodies) is introduced into the target cells, these elements are then concurrently mapped during the NanoSIMS analysis (Fig. 2) [5,11-13].

Using nanoSIP to measure nutrient transfers and microbial interactions

Microbe-host interactions

The nanoSIP approach is increasingly used to provide insight on interactions between microorganisms or fungi and their animal or plant hosts. Work in this area includes some of the earliest nanoSIP studies, including a seminal study on shipworms, where N-fixing symbionts were readily identified based on their $^{15}N_2$ assimilation [1]. More recently, symbionts in reef building corals have been examined [37-39], in studies focused on demonstrating and characterizing predicted functions, such as symbiont C fixation. NanoSIP was also recently used to investigate C fixation by microbial symbionts in gutless *Olavius algarvensis* worms living in seagrass sediments. It confirmed predictions from prior metaproteomic studies, demonstrating the worm symbionts could fix CO derived from seagrass rhizome decomposition [40].

As systems become more complex, the need for additional metadata and experimental treatments increases. For example, in a study where lower termites were fed ^{13}C-cellulose, NanoSIMS images revealed ^{13}C incorporation by gut protists and their symbiotic bacteria

[41], but without additional data, the nature of this symbiosis could not be determined. In a more recent study, Tai and coworkers combined genomics and nanoSIP to show that Bacteroidales ectosymbionts fix nitrogen and supply it to their cellulose-degrading protist host within the hindgut of a wood-eating cockroach [42]. Berry and coworkers [6] took the investigation of gut microbes a step further, using isotopically labelled protein incubations (threonine), FISH, NanoSIMS imaging, community sequencing, and sterile mouse experiments to identify specific microbes that forage for host proteins in the mouse gut. This study was the first to couple single cell, isotope, and meta-'omics approaches *in vivo* in a mammalian system, and is an exemplar for future studies of interactions between uncultivated taxa in human and environmental microbiome research.

In plants, there are also many opportunities to study plant-microbe interactions using NanoSIMS. For example, root-associated mycorrhizal fungi are particularly important in root nutrient acquisition and exchange [31,33,34,43], and in Nuccio et al. [31], NanoSIMS imaging was used to illustrate mycorrhizal fungal transport of nitrogen derived from decaying organic matter, and transfer to the host plant.

Phototroph-Heterotroph Interactions

Many recent NanoSIMS studies of microbial interactions have focused on partnerships between phototrophs and heterotrophs. This emphasis reflects a growing recognition of the varied roles heterotrophs play in supporting phototrophic productivity (e.g. in the rhizosphere [31,43], phycosphere [30,44], and possibly even the endosphere [45]). But the nature of phototroph-heterotroph relationships can be complex (e.g. evolving from beneficial to opportunistic [46]) and recent work shows primary producers can also have complicated ecological roles, for example acting as organic consumers [47]. Combinations of nanoSIP and whole genome or ‘omics approaches become critical as researchers attempt to unravel these relationships.

Many nanoSIP studies have explored interactions involving cyanobacteria [15,48-54]. In mats and biofilms, cyanobacteria produce an extensive organic extracellular matrix, providing the surrounding heterotrophic community with a rich source of nutrients. Using a combination of stable isotope tracing and proteomics, Stuart et al. demonstrated that mat phototrophs may assimilate their own EPS under a range of metabolic conditions [47,55]. Paired proteomics analyses point to multiple enzymes (involved in degradation of amino acids, proteins, nucleotides, and carbohydrates) that indicate the cyanobacteria use their own EPS as an energy/C source. In this study, nanoSIP results indicate that cyanobacteria can effectively compete with heterotrophic community members for organic matter in both

light and dark conditions, and that both nutrient requirements and community interactions contribute to cycling of extracellular organic matter.

Other work in cyanobacteria mats has combined nanoSIP with metatranscriptomics, specific inhibitors, and CARD-FISH to illustrate carbon uptake patterns associated with different diel cycles and acetate consumers, and metabolite flows between cyanobacteria, *Chloroflexi* and sulfate reducers [48-50]. In these studies, while nanoSIP-FISH pointed to likely players and quantitatively important nutrient exchanges, the corresponding metatranscriptome and isolate genome data was critical to reconstruct the catabolic pathways involved (fermentation of glycogen by the dominant cyanobacteria and conversion of glycogen to polyhydroxyalkonates by *Chloroflexi*). Similarly, the combination of nanoSIP and genome sequencing was critical to understand the symbiotic relationship between an N-fixing unicellular cyanobacterium (whose genome has lost many common metabolic functions) and photosynthetic picoeukaryotic cells from the Haptophyta division [15]. In combination with a particularly gentle sample preparation approach (to preserve loosely partnered cells), isotope tracing with $^{15}N_2$ and $H^{13}CO_3$ showed these cyanobacteria provide fixed N to their eukaryotic partners in exchange for fixed C. More recent studies of this symbiosis have revealed the additional importance of Fe availability to the N-fixing partner [53].

Single cell isotope tracing becomes slightly more complicated, but even more valuable, in studies of multitrophic interactions. For example, a substantial amount of algal cell organic matter can be transferred to surrounding bacteria following viral lysis [30]. Sheik et al used a combination of nanoSIP with amplicon sequencing to track bacterial community succession following lysis of ^{13}C- and ^{15}N-enriched algal cells, which provided both ecological context and helped guide CARD-FISH targeting. Other studies have used multiple isotope tracers simultaneously (e.g. ^{13}C-inorganic carbon and $^{15}N_2$ gas), to track assimilation within a food web. The colony-forming cyanobacterium *Aphanizomenon*, common in the Baltic Sea, actively fixes N_2, but then releases nearly half as NH_4^+, which is taken up by surrounding prokaryotic and eukaryotic plankton, diatoms and copepods, forming the basis of the Baltic Sea food web [56].

Anaerobic Oxidation of *Methane*

Over the past 15 years, FISH-SIMS and more recently FISH-NanoSIMS have been used to explore the physiology of anaerobic oxidation of methane (AOM) consortia in marine sediments, via cultivation-independent incubations with ^{13}C-labelled methane and ^{15}N-ammonia, ^{15}N-nitrate and $^{15}N_2$ [4,57-61]. Syntrophic associations between methanotrophic archaea (anaerobic methanotrophs, ANME) and sulfate reducing bacteria (SRB), drive AOM in marine methane seep sediments. This system is where combined FISH and SIMS was

first applied [57], and continues to be an archetype for the approach [62]. Still, it is important to recognize that FISH-NanoSIMS alone could not resolve the nature of the syntrophic interaction between ANME and SRB, but was most powerful in combination with other microscopy techniques, physiology experiments and 'omics investigations.

The nature of the ANME-SRB syntrophy was initially elusive, with experimental evidence showing that hydrogen, methanol, formate or acetate did not served as electron donors for sulfate reduction [63]. FISH-SIMS helped reveal that ANME cells could assimilate methane-derived carbon, showed higher N assimilation by ANME vs. SRB cells when ^{15}N-ammonia was provided, and proved that ANME cells were able to fix N_2 and transfer it to SRB [4,58,59]. A syntrophic model for AOM, through the transfer of partly-reduced sulfur compounds, was proposed based on FISH-immunolabelling-NanoSIMS results [64]. NanoSIMS analyses showed that ANME cells, identified by mapping of Au deposited by specific antibody labelling, accumulated high amounts of sulfur, correlated with the assimilation of methane-derived carbon. Identified as polysulfides by Raman microspectroscopy, these sulfur compounds are thought to act as diffusible electron shuttles between ANME and SRB cells [64].

Recently, additional NanoSIMS analyses of AOM consortia showed that biosynthetic activities of single ANME and SRB cells are unrelated to the proximity of syntrophic partners [7]. Moreover, ANME cells maintained similar biosynthetic activities when sulfate was replaced with artificial electron acceptors [62], apparently due to direct interspecies electron transfer (DIET) from ANME to SRB cells. Firm evidence in support of DIET was provided via the discovery of multi-haem cytochromes genes in the genomes of ANME, the detection of cytochromes in the extracellular space of AOM consortia, and descriptions of nanowire-like structures in thermophilic AOM cultures [7,65].

Challenges and future directions

While nanoSIP is now widely recognized as a powerful tool in microbial ecology, users must be aware of its limitations, some of which are inherent to the complexity of biological systems. Technical challenges, such as alterations of the elemental and isotopic composition of cells during hybridization protocols, are a concern for those interested in measuring truly quantitative metabolic fluxes. On the other hand, 'biological' or 'metabolic' challenges (e.g. complex microbial communities, intricate or rapid nutrient exchanges) may be overcome by multi-prong approaches that include 'omics, complementary microscopy techniques and/or physiology experiments.

Cross Feeding

Cross feeding occurs with the transfer of labelled metabolites from primary processors of a substrate to a second group of microorganisms, and can make interpretation of nanoSIP results ambiguous. The primary ways to control for this effect are 1) to use the shortest possible incubations, 2) quantify the relative level of incorporation of the label, and 3) use genomic data to constrain metabolic potential. Remarkably, in many environmental systems, using short incubations is sufficient to avoid cross feeding. For example, nitrogen fixers tend to become enriched well before other non-fixers in $^{15}N_2$ experiments [e.g. [66,67]. However, we cannot take this outcome for granted for symbionts. At the extreme, terminally differentiated cells within a single organism may be the site of initial isotope uptake but are not the site of isotope assimilation into biomass, such as in the nitrogen-fixing heterocysts of *Anabeana* [67] or field populations of *Aphanizomenon* sp. [66]. NanoSIP may also fail to identify slow-growing organisms as primary substrate users. In structurally less-integrated microbial systems, microorganisms using secondary metabolites released by fast-growing primary users may assimilate an isotope label at the same time as slow-growing primary users [e.g. [68]. Ultimately, investigators must address the potential for cross-feeding within each system individually, and bring to bear accessible metadata to interpret nanoSIP results.

Artefacts of Phylogenetic labelling

In many nanoSIP approaches, protocols used for fixation and phylogenetic labelling have numerous cell treatment steps. Recently, concerns have been raised that these treatments may alter the cellular isotopic composition following SIP experiments by loss of low molecular-mass compounds (permeabilization), or addition of nonlabeled elements (during chemical fixation or tyramide deposition). NanoSIMS studies with ^{13}C- and ^{15}N-labelled type strains showed a stepwise dilution of both carbon and nitrogen isotopic composition after chemical fixation, FISH and CARD-FISH protocols [14,54]. Additional experiments suggest that alteration of the isotopic composition may depend not only on the isotope in question and fixation method, but also the taxa in question (J. Pett-Ridge and S. Behrens unpublished results). For example, in gram-negative *Vibrio cholerae*, CARD-FISH reduced cell ^{13}C enrichment by 60-80%, and $\Delta^{15}N$ values between 30-60%, relative to chemical fixation alone. For gram-positive *Bacillus subtilis*, CARD-FISH treatment resulted in 55-80% lower ^{13}C enrichment, and 70-75% lower $\Delta^{15}N$ values (Fig. 3). The high variance in dilution effect attributable to fixation and/or FISH treatments is a particularly important consideration for researchers interested in the absolute enrichment of a particular cell population.

Despite the caveats associated with isotopic dilution, FISH-based cell identification is still widely used to target individual populations within samples of unknown microbial

composition, and preserves cell integrity for subsequent imaging analyses. This is especially true for phylogenetically cohesive populations where a confined rRNA oligonucleotide probe may be used. In samples where good community composition data exists, it may even be possible to use multiple reporter elements or fluorescent dyes (e.g. Multiplex FISH or CLASI-FISH) for simultaneous identification of multiple phylotypes [69,70] followed by NanoSIMS analysis.

Sample preparation

Sample preparation for nanoSIP experiments should attempt to conserve the ultrastructural organization of cells and, if possible, their elemental and isotopic composition and spatial distribution. NanoSIMS analyses can resolve some subcellular structures (e.g. cell wall, nucleoid, ribosomes) and these ultrastructural features may be imaged in advance with high spatial resolution by electron microscopy [32]. However, conventional sample preparation methods for TEM or SEM aiming to maintain high structural integrity of samples may lead to isotope dilution or physical rearrangement of molecules. High pressure freezing followed by freeze-substitution and sectioning is an excellent alternative to conventional protocols even for samples with a high-water content and has been used to map the distribution of ions and light elements in plant and mammalian tissue [71-74]. Similar protocols need to be developed for correlative analyses in microbiology, ideally in combination with phylogenetic labelling.

Secondary metabolites

Identification of the secondary metabolites that are transferred among microorganisms in complex systems adds additional challenges to microbe interaction studies. Since nanoSIP can only track isotopes or elements, the biochemical pathways, excreted metabolites and molecular forms by which a tracer is assimilated have to be inferred by other methods. One approach is to predict and test for secondary metabolites based on genetic potential and bulk analytical methods, and to conduct follow-up nanoSIP experiments by adding the target metabolite [49]. This combination offers direct fingerprinting of catabolic pathways, thus resolving the flow of primary intermediates within microbial communities. Another approach is correlative application of complementary single cell imaging techniques, such as Raman microspectroscopy, ToF-SIMS or scanning transmission X-ray microscopy with NanoSIMS imaging [64,75]. In experiments with known starting substrates, biochemical pathways and potential intermediates can also be inferred from orthogonal methods such as genomics, transcriptomics and proteomics.

Conclusions

The study of microbial interactions with nanoSIP is rapidly advancing and expanding, and we expect this trend to continue. The level of sophistication in these studies has increased over the last decade as questions move from “which microbe uses which substrate?” to “how does this organism interact with another and its host?”. While nanoSIP alone in an unperturbed system can identify potentially interesting interactions, data from orthogonal methods (geochemistry to ‘omics) and/or manipulation experiments are critical to providing deep understanding. The key role of nanoSIP is demonstrating that an OTU is involved in an assimilatory function or process at the single cell or subcellular scale. Because microbial interactions are critical to human health, food security and global climate, we expect to see even more studies using nanoSIP to investigate a diverse range of microbe interactions.

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Figures

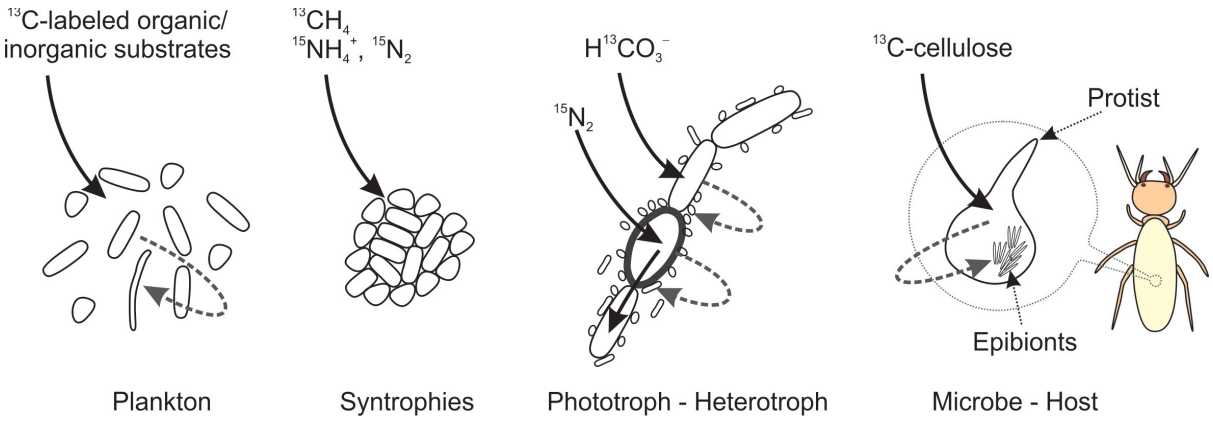

Figure 1. Illustration of microbial communities and associations where NanoSIMS in combination with stable isotope probing (nanoSIP) has been applied to resolve assimilation and exchange of specific metabolic substrates. These include label uptake and transfer from primary to secondary processors in planktonic communities; assimilation of carbon and nitrogen by syntrophic consortia involved in anaerobic oxidation of methane; CO_2 and N_2 fixation by environmental populations of cyanobacteria and the subsequent transfer of organic carbon and ammonia to attached microbial communities; and trophic networks in microbial communities living in the gut of higher organisms, here exemplified by cellulose-degrading protozoa and their epibionts in the gut of termites.

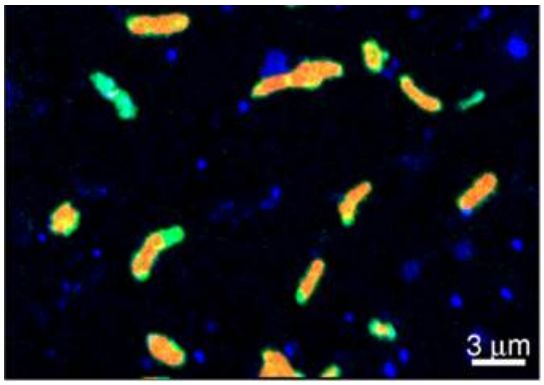

Figure 2. Example of direct label imaging by NanoSIMS for phylogenetic identification of target cells. In this example, key microbes responsible for autotrophic processes at deep sea vents were targeted. The NanoSIMS data is depicted as a composite RGB image showing ^{13}C assimilation from ^{13}C-labelled inorganic carbon (green), phylogenetic identification of the dominant autotrophic microbial group by ^{19}F introduced in the cells via CARD-FISH (red) and ^{32}S as a biomass indicator (blue). Image, courtesy of Stefan Sievert.

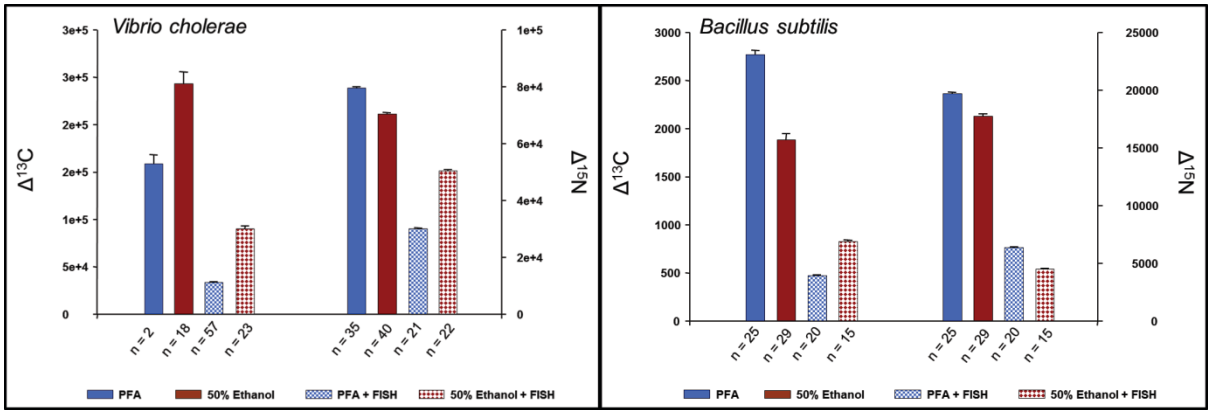

Figure 3. The effects of fixation technique (paraformaldehyde (PFA), ethanol) and CARD-FISH on the isotopic composition (^{13}C and ^{15}N) of *Vibrio cholerae* (92A 1552 El Tor, Inaba, wild type, Rifs) and *Bacillus subtilis* (JH642) cells cultivated on 99.9 atom% $^{13}C/^{15}N$ mixed amino acids (Cambridge Isotope Laboratories) (J. Pett-Ridge and S. Behrens, unpublished data). The number of single cells analysed by NanoSIMS for each treatment is indicated on the *x* axis.

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