Advances in the Analysis of Biogeochemical Interfaces: NanoSIMS to Investigate Soil Microenvironments

Review article for "Advances in Agronomy"

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Abstract

Since a NanoSIMS high-resolution secondary ion mass spectrometry instrument was first used for cosmochemistry investigations over a decade ago, both interest in NanoSIMS and the number of instruments available have significantly increased. However, SIMS comes with a set of challenges that are of both technical and conceptual nature, particularly for complex samples such as soils. Here, we synthesize existing research and provide conceptual and technical guidance to those who wish to investigate soil processes at the sub-micron scale using secondary ion mass spectrometry, specifically with NanoSIMS. Our review offers advice resulting from our own operational experience but also intends to promote synergistic research on yet unresolved methodological issues. We identify and describe the basic setup of a NanoSIMS instrument and important issues that may arise as a soil sample specimen is prepared for NanoSIMS analysis. This is complemented by discussions of experimental design, data analysis and data representation. Next to experimental design, sample preparation is the most crucial prerequisite for successful NanoSIMS analyses. We discuss the requirements and limitations for sample preparation over the size range from individual soil particles to intact soil structures such as macroaggregates or intact soil cores. For robust interpretation of data obtained by NanoSIMS, parallel spatial, textural (scanning electron microscopy, atomic force microscopy) or compositional analyses (scanning transmission X-ray microscopy) are often necessary to provide necessary context. We suggest that NanoSIMS analysis is most valuable when applied in concert with other analytical procedures and can provide powerful inference about small scale processes that can be traced via isotopic labeling or elemental mapping.
1. Introduction

1.1. The importance of nano-scale processes in soils research

Soil is often described as one of the most complex media on earth (Schulze and Freibauer, 2005). This complexity extends from the ecosystem-scale to individual microaggregates, where nanometer-scale interactions between microbiota, organic matter and mineral particles are thought to control the long-term fate of soil carbon, nutrients and pollutants (Lehmann et al., 2007; Schmidt et al., 2011). Processes that have a major impact at the landscape- or global-scale are determined by events occurring at the micro- and nanometer scales. For example, entrapment of soil organic matter (SOM) within microaggregates with a diameter of less than 250 µm and SOM sorption onto even smaller clay and iron oxides is a vital mechanism for long term preservation of organic carbon (OC) in soils (Lehmann et al., 2007; von Lützow et al., 2006). Release of nutrients in the rhizosphere is driven by root exudation at highly active micron-scale biogeochemical interfaces between roots, microbes and minerals (Breland and Bakken, 1991; Hinsinger et al., 2009; Norton and Firestone, 1996). Microbial activity occurs mostly in micro-habitats (Dechesne et al., 2007; Müller and Defago, 2006; Nunan et al., 2007) and involves mineralization of SOM and organic pollutants. Hydrologic processes at the field-scale are also influenced by fine-scale interactions as preferential flow paths may create localized zones of altered water and nutrient flow and thereby impact microbial abundance, community structure and SOM turnover (Chabbi et al., 2009; Morales et al., 2010). Preferential flow zones are themselves heterogeneous at the micro-scale, with a heterogeneous supply of oxygen, water and nutrients driving “hot spots” of microbial growth directly adjacent to areas of lesser microbial activity (Bundt et al., 2001). In all of these cases, activities at nano- to micron-scale soil biogeochemical interfaces determine the expression of higher level ecosystem functions. The majority of soil research, however, is conducted on bulk (> 1 g) samples, which are often significantly altered prior to analysis. Pretreatments and analytical side effects include drying at varying temperatures, sieving/homogenization for process or elemental analysis, thermal alteration (as in pyrolysis GC/MS) or chemical alteration (as in alkaline extraction of “humic”
substances or in cupric oxide oxidation for lignin analyses). With the advent of novel micro-
spectroscopy and spectrometry techniques that allow for the study of micro- to nano-scale
molecular, isotopic, and elemental patterns, it is now possible to make process-oriented
observations (e.g. the stabilization of organic matter, sorption of pollutants and mineral
weathering) at the micron or sub-micron scale.

Elemental and isotopic imaging conducted via secondary ion mass spectrometry (SIMS) is a
particularly promising technique for small-scale soil process research. SIMS uses a high-
energy ion beam to sputter material from a sample surface, which can then be analyzed in a
mass-spectrometer. With high resolution SIMS instruments (Cameca NanoSIMS 50, 50L,
Gennevilliers, France), the distribution of elements and isotopes can be visualized with up to
50-150 nm lateral resolution within soil samples ranging from primary particles to sub-regions
of intact soil cores. For this reason, NanoSIMS has the potential to provide quantitative
measures of organic matter-mineral-microbial interactions and biogeochemical processing at
the macro- and microaggregate or single-cell scale.

Relatively few SIMS experiments have been conducted to date in soil science. In one of the
first, Cliff et al., (2002b) used time of flight-SIMS (ToF-SIMS) and additions of $^{15}$N-labeled
and $^{13}$C-labeled compounds to study small-scale differences in N assimilation as a function of
C vs. N limitation. When they compared SIMS values with bulk-measured microbial biomass
N assimilation, they found substantial spatial heterogeneity in $^{15}$N distribution that was not
apparent through bulk analysis (Cliff et al., 2007). More recently, studies using SIMS and
NanoSIMS analysis have revealed effects at even finer scales within individual
microaggregates, mineral surfaces, microbes, and root hairs (Blair et al., 2006; Cliff et al.,
2002a; Clode et al., 2009; DeRito et al., 2005; Herrmann et al., 2007a; Herrmann et al.,
2007b; Keiluweit et al., 2012; Pumphrey et al., 2009). An early review paper by Herrmann
and co-authors (Herrmann et al., 2007b) focused on potential applications for soil ecology
and included the first application of the NanoSIMS technique with an intact soil
microaggregate. Subsequent publications have addressed the technical aspects (sample
preparation) and investigations of organo-mineral associations at scales ranging from clay size mineral grain to intact soil cores (Keiluweit et al., 2012; Mueller et al., 2012b; Remusat et al., 2012).

In this article, our goal is to provide insight into the range of potential NanoSIMS applications in soil system research, discussing technical capabilities and limitations, major sample requirements, and important complementary micro-spectrometry techniques. As NanoSIMS applications in closely related fields, such as plant science and microbiology, have been reviewed recently (Moore et al., 2011a; Musat et al., 2012), we focus on the use of NanoSIMS in soil research.

1.2. Fundamentals of Secondary Ion Mass Spectrometry

SIMS is a surface analysis technique for solid samples. Primary ions, with a kinetic energy ranging from a few hundred electron volts to tens of thousands of electron volts, are focused on the sample surface, ejecting atoms and molecules in a process called sputtering (see Figure 1). A small fraction of the ejected atoms and molecules are ionized, and can be extracted with an electrostatic field into a mass spectrometer. The fraction of the sputtered material that is ionized is determined by the ionization efficiency of the element in the sample matrix, and is referred to as the secondary ion yield. For different elements, secondary ion yields vary over many orders of magnitude, and also strongly depend on the physico-chemical nature of the sample (Storms et al., 1977; Wilson et al., 1989). Within the mass spectrometer, secondary ions can be separated according to their mass to charge ratio in a quadrupole, magnetic-sector, or time-of-flight (TOF) mass analyzer. These analyzers differ in terms of detectable mass range, sensitivity, ion transmission, and cost. As NanoSIMS has both high sensitivity and spatial resolution at high mass resolving power, this particular SIMS instrument meets many of the specific requirements for micro-scale elemental and isotopic mapping analyses in soil science.
1.2.1. NanoSIMS

The NanoSIMS is optimized for SIMS imaging with sub-micron lateral resolution. The NanoSIMS 50 and 50L instruments, conceived by George Slodzian (Slodzian, 1987; Slodzian et al., 1992) was designed by Bernard Daigne, François Girard and François Hillion (Hilion et al., 1993), and manufactured by Cameca France under a license from the Office National d’Études et de Recherches Aérospatiales at Université Paris-Sud (UPS ONERA).

There are now more than thirty NanoSIMS instruments installed world-wide, working on a wide range of applications ranging from geology and cosmochemistry (Floss et al., 2006; Hoppe, 2006; Stadermann et al., 1999; Wacey et al., 2010a), to biology (Finzi-Hart et al., 2009; Kraft et al., 2006; Lechene et al., 2006), material science (Valle et al., 2011) and soil science (Herrmann et al., 2007a; Keiluweit et al., 2012; Mueller et al., 2012b).

The key innovation of the NanoSIMS is the coaxial lens (Figure 1) which focuses the primary ion beam and extracts and focuses the secondary ion beam as well. This configuration minimizes the distance between the sample surface and primary focusing lens, allowing the primary beam to be focused to a much smaller diameter than in conventional SIMS instruments. In addition, the secondary mass spectrometer is optimized for high-transmission at high (>3000) mass resolving power. The NanoSIMS comes equipped with a Cs⁺ primary ion source for analysis of negative secondary ion species (e.g. $^{12}$C⁻, $^{13}$C⁻, $^{12}$C$^{14}$N⁻, $^{12}$C$^{15}$N⁻, $^{28}$Si⁻, $^{27}$Al$^{16}$O⁻ and $^{56}$Fe$^{16}$O⁻), and an O⁻ primary beam source, for analysis of positive secondary ions (e.g. $^{23}$Na⁺, $^{39}$K⁺, $^{44}$Ca⁺, $^{56}$Fe). Due to the coaxial lens setup (Figure 1 B), secondary ions must have the opposite charge from primary ions to enable extraction to the mass spectrometer. A ~150 nm diameter Cs⁺ primary ion beam with a beam current of 1 to 2 pico Ampere can routinely be achieved. While an even smaller beam diameter is possible, there are trade-offs between high-resolution (with reduced beam current) and secondary ion count rates. Higher currents and thus beam diameters are often crucial to yield significant amounts of secondary ions (e.g. $^{13}$C⁻, $^{12}$C$^{15}$N⁻, $^{56}$Fe$^{16}$O⁻) when analyzing soil samples. With an O⁻ beam, a diameter of ~400 nm is typical.
The advantage of the NanoSIMS instrument lies in the coupling of a continuous, high spatial resolution analysis beam with high mass resolving power, resulting in high sensitivity and specificity with relatively short integration times. Users should also be fully aware that the NanoSIMS 50 and 50L are both ‘dynamic’ SIMS instruments where the sample is actively eroded during the sputtering process and molecular bonds are broken by the primary ion beam. Up to five (NanoSIMS 50) or seven (NanoSIMS 50L) secondary ions can be detected simultaneously. Additionally, if operated in Cs\(^+\) mode, secondary electrons produced by the collision cascade can be detected by a photo-multiplier, providing a secondary electron image that can provide structural and textural information that is comparable to a low-resolution SEM micrograph.

1.2.2. Basic requirements for NanoSIMS samples

A wide range of solid samples are compatible with SIMS, provided they are dry and stable under high vacuum (< 10\(^{-9}\) mbar), relatively flat (< 2-30 microns of relief), and conductive. Sample out-gassing can be caused by absorbed water, other volatiles, hydrocarbons or samples prepared via resin embedding. Pre-treatment in a vacuum oven under low heat can reduce out-gassing within the analysis chamber. Out-gassing may degrade analysis conditions by elevating chamber pressure, reducing ion transmission and generating molecular interferences or physical contamination, which will all lead to poor analysis quality. Sample flatness is also important as any surface roughness may influence sample sputtering, ion extraction and mass spectrometer tuning. For natural abundance isotopic ratio measurements, 2-4 per mil level external precision can be achieved with repeated analyses of bacterial spores with ~1 micron of relief (P.K. Weber, unpublished results). For isotopic tracer experiments, more topographic relief (10 to 20 microns) can be tolerated (Woebken et al., 2012). Even higher topographic relief (~30 microns) may also be viable in some applications (P.K. Weber, unpublished results) but with significant loss in precision and the need for careful monitoring of measurement quality. Finally, because SIMS uses an ion beam to eject charged ions from the sample’s upper atomic layers, a mechanism to dissipate
charge from the analysis location is critical. In our experience, most soil samples are semi-insulating, and typically must be coated in an evaporator or sputter coater with a 2 – 20 nm layer of gold (or carbon, iridium, gold-palladium, or platinum) to minimize charging during analysis. Sample flatness can also interact with charge dissipation characteristics. As a general rule, the more topography a sample has, the thicker a conductive coat needs to be to bridge topographic gaps. Metal coating and sample flatness become particularly important if an electron flood gun is to be used for charge compensation (see NanoSIMS soil preparation details below).

2. Experimental approaches for the study of soil microenvironments using NanoSIMS

2.1. Lessons learned from geology and microbiology

Perhaps one of the most appealing aspects of NanoSIMS analysis for many soil scientists is the instrument’s potential to quantitatively localize stable isotopes at a previously unresolved spatial scale. Since the fields of geology, cosmochemistry, and geomicrobiology have a more extensive tradition with SIMS and NanoSIMS applications, that literature is a logical source of illustrative models for soils research. Cosmochemists were first to use NanoSIMS for isotopic measurements, taking advantage of its high spatial resolution and simultaneous imaging capabilities. Using NanoSIMS, Messenger et al. (2003) located rare isotopically anomalous (> 100 ‰) micron-sized pre-solar grains within meteoritic samples that are too small to have been analyzed by bulk measurements or even conventional SIMS (e.g. Cameca SIMS 1280). NanoSIMS has also been used to measure large isotopic fractionation in biominerals (e.g. carbonates from mollusk shells and corals) and deduce metabolic pathways such as methanotrophy (Rasmussen et al., 2009) or sulfate reduction and sulfur disproportionation (Philippot et al., 2007; Wacey et al., 2010b). In geologic samples, NanoSIMS imaging was employed to support a microbial origin for Ooids - concentrically-laminated sedimentary grains found in turbulent marine and freshwater environments (Pacton et al., 2012), and a
microbial role in Fe-mineralization within spheroidal iron oxide concretions associated with palaeoaquifers (Weber et al., 2012).

All of the studies mentioned above capitalized upon natural isotope fractionation effects that can shift natural abundance values by 10s to 100 per mil (e.g. for biological sulfur cycling - 8.5‰ to +19‰ δ34S_{CDT} (CDT – Canyon Diablo troilite); for methanotrophy -55‰ to -43‰ δ^{13}C_{PDB} (PDB – Pee Dee belemnite) (Rasmussen et al., 2009). These effects are in many cases significantly larger than those found in soil/sediment systems, where prominent isotopic effects range from ~ 1 to 11‰ δ^{13}C (litter decomposition, C3-C4 plant shifts, (Ehleringer et al., 2000)), ~7‰ δ^{15}N (soil depth gradients, (Billings and Richter, 2006)) or as little as 0.8‰ δ^{56}Fe (soil iron pools, (Kiczka et al., 2011)). One exception is the work of Orphan and colleagues (2001) who have used SIMS and NanoSIMS to image isotopic fractionation in modern anoxic sediment cores of the Eel river basin in the Pacific Ocean. There, the authors report isotopic fractionations of δ^{13}C of up to -96‰ in the interior of bacterial aggregates, indicating consumption of isotopically light methane by methanotrophic bacteria.

For most soil process-level questions, the best approach may be to use stable isotope labeling as a way to document transformation pathways in soil micro-environments over time. This approach, like many of the published examples in cosmo- and geo-chemistry, would take full advantage of the NanoSIMS’s spatial resolution while improving detection of the isotopic species of interest. In the past decade, stable isotope labeling (often with ^{13}C or ^{15}N) and NanoSIMS analyses have been widely used in environmental microbiology, supporting research on the metabolism of single microbial cells (Pett-Ridge and Weber, 2012) both in pure culture and in natural environmental samples ranging from marine bacterial and archaeal communities (Dekas et al., 2009; Halm et al., 2009; Musat et al., 2008; Ploug et al., 2010), to acid mine drainage biofilms (Moreau et al., 2007), to ^{13}C and ^{15}N fixation in diazotrophic cyanobacteria (Finzi-Hart et al., 2009; Popa et al., 2007; Woebken et al., 2012) and eukaryotes (Lechene et al., 2006; Lechene et al., 2007).
An idealized stable isotope labeling experiment in soil might proceed as follows: (1) add an organic compound of interest, labeled for instance with $^{13}$C or $^{15}$N, to a model system that contains reactive mineral surfaces and an active microbial decomposer community; (2) incubate under controlled conditions, varying an edaphic variable of interest (moisture, temperature, pH); (3) prepare and analyze samples via NanoSIMS imaging to determine the physical fate of the target compound – whether it becomes metabolized (and the label is transferred to microbial decomposers) or whether it is adsorbed to mineral surfaces. Enrichment of the label above a background value could then be used to support inference about the fate of the compound of interest. This type of application could potentially contribute to both studies of soil carbon turnover dynamics as well as investigations of contaminant fate in soils.

Previous NanoSIMS studies of biotic (microorganisms and plants) and abiotic materials (minerals, fossils) represent endmember models of the soil system, with its inherent combination of geologic and microbiological aspects. This substantial literature serves as a valuable resource for soil scientists interested in designing micro-scale soil research, particularly as a resource for experimental concepts and sample preparation protocols. In the following section we discuss examples from soil science where NanoSIMS has been successfully applied, as well as additional areas where soil interface research could significantly benefit from high-resolution isotopic imaging in the future. As we point out potential applications, we also mention pitfalls and methodological constraints.

### 2.1.1 Investigating mineral-organic associations

Historically, studies of mineral-organic associations have employed bulk analysis procedures performed on operationally defined physical fractions (Balesdent et al., 2000; Christensen, 2001; Eusterhues et al., 2005; Schöning et al., 2005). The goal of such procedures is to isolate mineral-organic associations of given characteristics, such as an increasing proportion of microbially processed organic matter (OM) in fractions of increasing density (Derrien et al., 2006; Grandy and Neff, 2008; von Lützow et al., 2007). In contrast to bulk
analysis, NanoSIMS offers the possibility to examine organo-mineral assemblages in the context of intact spatial structures. If a stable isotope labeling experiment (see above) is used, NanoSIMS images can potentially reveal the spatial distribution and dilution of a tracer material as it moves into the soil matrix. They can also reveal whether preferential associations of certain OM types predictably associate with certain mineral phases. This is possible because of three main advantages NanoSIMS has over other microscopic techniques: (1) elemental mapping can be done with better lateral resolution, (2) the low depth penetration (~ 10 to 20 nm) of the NanoSIMS primary beam allows thin surface layers to be examined, (3) highly accurate isotope detection allows the operator to track OM\(^{13}C\) and OM\(^{15}N\) onto distinct minerals in an intact micro-environment, and thus enables process-level studies. In a proof-of-concept example, these three advantages were exploited by Heister et al. (2012), who showed that in artificial soil mixtures, soil minerals and organic materials can be distinguished in NanoSIMS images, using the distinction between organic material derived ions (\(^{12}C^-\) and \(^{12}C^{14}N^-\)) and mineral derived ions (\(^{28}Si\), \(^{27}Al^{16}O^-\), and \(^{56}Fe^{16}O^-\)). The authors used NanoSIMS in this study as a tool for micro-scale elemental mapping of organic matter on mineral surfaces. They showed that organic matter tended to be attached to phyllosilicate clays in the form of isolated patches, while continuous coatings of organic matter enveloped small ferrhydrite particles. Such micro-scale heterogeneities could not have been resolved by SEM/EDX measurements. In another example, Mueller et al. (2012b), working with resin-embedded soil macroaggregates, found heterogeneous isotopic enrichment at the micro-scale following the application of a \(^{13}C/^{15}N\) label (amino acid mixture of algal origin) to natural soils. They speculated that microbial activity may have lead to the increased utilization of freshly added organic matter, or that soil components have different sorption capacities. The NanoSIMS's unique capacity to detect stable isotope tracers at the micro-scale enabled both these studies to confirm the predicted physical dimensions of organo-mineral associations.

By combining isotopic and elemental imaging, NanoSIMS analysis can also reveal whether certain OM types predictably associate with certain mineral phases. This particular capacity
of the NanoSIMS was used by Keiluweit et al. (2012) in a study where $^{13}$C/$^{15}$N enriched fungal hyphal extracts were incubated with organic horizon soil. NanoSIMS images of $^{15}$N enrichment and iron distribution suggest that nitrogen from fungal cell walls was rapidly and preferentially deposited as thin organic coatings onto Fe (hydr)oxide surfaces (Keiluweit et al., 2012). Further analysis of these samples by scanning transmission X-ray microscope in combination with near edge X-ray absorption fine structure spectrometry (STXM-NEXAFS) revealed these soil microstructures were enriched in aliphatic C and amide N, suggesting that a concentration of microbial lipids and proteins had quickly become associated with Fe (hydr)oxide surfaces. Remusat et al. (2012) used a similar approach to image intact soil particles with low levels of isotopic enrichment sampled 12 years after a $^{15}$N litter labeling experiment in a temperate forest. They describe microsites of isotopic enrichment ("$^{15}$N hot spots") on mineral surfaces, and in one microsite, suggest that $^{15}$N enrichment was also linked to the presence of microbial metabolites. This kind of combined approach, NanoSIMS image analysis joined to complementary microscopy (SEM-EDX, STXM/NEXAFS), may be a particularly profitable means to infer the molecular and spatial fate of labeled organic materials in a mineral matrix, and has the potential to contribute to a mechanistic understanding of sorption, occlusion, and decomposition processes that operate at fine spatial scales.

A recent quantitative analysis of organo-mineral assemblages by Hatton et al. (2012) used a combination of macro- and micro-scale analyses for an internal calibration of C/N and $^{15}$N/$^{14}$N ratios in sequentially separated soil density fractions. This approach is based on the assumption that macroscopic features, visible under reflectance light microscope and analyzable by elemental analyzer isotope ratio mass spectrometry (EA-IRMS), are also found at the micro-scale as detected in SEM or NanoSIMS images. The authors collected NanoSIMS images over 500 µm$^2$ for each density SOM fraction, and corrected these using EA-IRMS data from macroscopic features. Because matrix-matching SIMS standards for soil organic matter do not yet exist, this calibration approach is a promising step towards a better quantification of data derived from SIMS images. While significant procedural challenges
remain, the examples presented above illustrate how well-designed experiments can benefit from NanoSIMS information to help decipher chemical and microbiological processes in soil microenvironments.

2.1.2 Investigating intact three dimensional micro-structures

NanoSIMS imaging may also be profitable in studies of micro-scale soil architecture. The first systematic approach to the study of in-situ soil features was established by the micropedological work of Kubiena (1938). Whole intact soil clods were impregnated with epoxy resin, thin sections were produced and small scale pedological features were studied using transmitted light microscopy. A large range of soils have been studied using this technique, combining different light sources ranging from polarized light to fluorescent staining of microbial cells (Bullock and Murphy, 1980; Eickhorst and Tippkoetter, 2008; Fisk et al., 1999; Li et al., 2004; Pulleman et al., 2005). With the rise of analytical techniques that can resolve soil features at the nano- to micro-scale (e.g. TEM, AFM, NanoSIMS), the micromorphological examination of soils is experiencing a renaissance. However, mineral particles pose a challenge to elemental mapping and isotope tracing experiments in intact soil matrices because they make embedding and thin-sectioning more difficult, and can cause electrical charging effects (Cliff et al., 2002b; Pett-Ridge et al., 2012). Still, a number of proof-of-concept studies have successfully shown that \( ^{15}\text{N} \) and \( ^{13}\text{C} \) isotope additions can be imaged by NanoSIMS in two dimensions within a natural or synthetic soil matrix (Herrmann et al., 2007b; Keiluweit et al., 2012; Mueller et al., 2012b; Pett-Ridge et al., 2012; Remusat et al., 2012).

Sample preparation is the most important issue to be resolved prior to micro-scale studies of soil three dimensional structures. This is particularly true for soil macroaggregates (> 250 \( \mu \text{m} \) in diameter) which have topography too large for reliable NanoSIMS measurements. To maintain adequate flatness and integrity in friable samples, larger soil aggregates and intact soil cores will typically require embedding and subsequent sectioning. However, simply cutting large aggregates into sections can affect structural integrity. One solution is thin
sectioning, although the approach used must be chosen with the target ions in mind. The most important considerations include:

- Does the sample contain both mineral and organic phases?
- Might the embedding medium dilute the signal of the target species (e.g. $^{13}$C)?
- Is \textit{in situ} hybridization to be used, and are diffusible ions or molecules of interest?

We discuss the finer details of these concerns in Sections 3.3.2 and 3.3.3. In general, our experience has shown that for smaller macroaggregates (~250 µm) cryosectioning is a laborious but worthwhile technique to obtain cross sections while avoiding contamination with any artificial C or N sources. For examination of whole intact soil cores or macroaggregates (several mm in diameter) resin embedding is currently the best option, although it introduces an artificial C and N source, which can interfere with both isotopic analyses and techniques to determine the chemical structure of OM (e.g. STXM). If target ions include C and N, resin embedding should thus be used only for larger volume soil specimens consisting of a friable porous network of organic and mineral particles that have to be tightly bound together in order to allow cross sectioning and polishing. However, for some resins (e.g. Araldite 502) the $^{12}$C/$^{14}$N/$^{12}$C ratio allows to distinguish between sample OM and embedding agent (Weber et al., 2012). The resin embedding approach has been used to prepare slices of intact soil cores for elemental mapping of in-situ interfaces in a buried Oa horizon originating from a permafrost-affected soil in Northern Alaska (Typic Aquiturbel, coastal plain near Barrow) (Figure 2). In this case, NanoSIMS imaging was used for elemental mapping of natural micro-scale features at a scale which could not be resolved by comparable techniques such as SEM-EDX. This example shows how NanoSIMS can illustrate the patterning of distinct phases (organic matter ($^{14}$C/$^{14}$N), organo-mineral interfaces, plant cells) via elemental mapping of such friable and highly heterogeneous intact soil structures.

2.1.3 Investigating plant – soil processes

The interfaces between plant roots and soil (rhizosphere) or fungal hyphae and minerals (hyphaesphere) are extremely biologically active and important sites for mineral weathering
(Finlay et al., 2009). Hinsinger et al. (2009) suggest that a lack of suitable observational tools stands in the way of a better understanding of micro-scale elemental distributions in the rhizosphere. Here NanoSIMS might well fill the gap between reflectance light microscopic (e.g. epifluorescence, polarized light) and x-ray techniques (e.g. x-ray tomography) to trace C, N and nutrient transfers between roots, microbes and soil. For the biotic side of the plant-soil system, Gea et al. (1994) showed the utility of SIMS by imaging Ca in ectomycorrhizal fungi (*Hebeloma cylindrosporum*) associated with pine trees (*Pinus pinaster*). Figure 3 is a proof-of-concept of how NanoSIMS may be used to explore an intact plant-soil system at the micro-scale. In this example, a French oak (*Quercus robur*) seedling was grown in a vermiculite / soil mixture with a mycorrhizal fungi *Piloderma croceum* in order to track interfaces between mineral constituents and the plant root. NanoSIMS images of an embedded oak root tip illustrate that clay minerals may be distinguished from root cells and mycorrhizal cells within the vermiculite layers, revealing the interfaces between the mineral soil compartment, roots, and mycorrhizal fungi. This example demonstrates how NanoSIMS images might contribute to the exploration of intact plant-soil-microbe interfaces.

Part of the difficulty associated with attempts to image the interfaces between plants, microbes and mineral particles has to do with preparing samples in a manner that adequately preserves these interfaces. A challenging but promising approach to preserve intact soil architecture was demonstrated by Clode et al. (2009) who prepared 100 nm thick cross-sections of $^{15}$N labeled wheat roots and associated bacteria by slowly infiltrating with araldite epoxy over the course of several days. The resulting epoxy blocks were thin-sectioned and then observed by both TEM and NanoSIMS at the University of Western Australia. The TEM images clearly identified bacteria attached to the cortical cell walls, while NanoSIMS imaging revealed that not all of the bacteria had incorporated the $^{15}$N label (Figure 4). While it is possible that some cells were not metabolically active or dead, it is equally possible that some of the $^{15}$N 'hotspots' were actually remnant effects of salts derived from the enriched precursor material ($^{15}$NH$_4$)$_2$SO$_4$). This is a case where a complementary technique, for example fluorescent in situ hybridization (FISH) or a live/dead or DAPI stain (see section
might be useful to corroborate whether enriched features in NanoSIMS images truly are bacterial cells.

2.1.4 Tracking organic and inorganic pollutants

Organic and inorganic pollutants span a wide range of molecular properties and may be involved in a host of mechanistically different interactions with soil solids. Important inorganic pollutants are metals and metalloids (e.g. Pb, As) (Bradl, 2004; Wilson et al., 2010; Zimmer et al., 2011), including radioactive particles from nuclear accidents (Carbol et al., 2003; Spezzano, 2005). Organic pollutants are inherently more diverse, encompassing the full range from nonpolar polycyclic aromatic hydrocarbons to relatively polar chlorinated hydrocarbons and polychlorinated biphenyls. To date, SIMS has been used to study the micro-scale distribution of metals (e.g. Cd, Cr), metalloids (e.g. As), and halogens and organic pollutants in microbial cells (Eybe et al., 2008), plants (Lombi et al., 2011; Mangabeira et al., 2006; Martin et al., 2001; Migeon et al., 2009; Moore et al., 2010; Moore et al., 2011b; Tartivel et al., 2012), animals (Eybe et al., 2009) and human tissues (Audinot et al., 2004). NanoSIMS has been used to examine plutonium transport in the subsurface of heavily contaminated sites (parts per million levels) (Kips et al., 2012; Novikov et al., 2006). When there is substantial contamination, Pu can be directly imaged in situ and the association of Pu with specific minerals can be determined to constrain transport mechanisms. An intriguing example of a system comparable to primary soil particles (e.g. clay minerals, OM particles) was presented by Krein et al. (2007), who located heavy metal accumulation in aerosols using NanoSIMS by imaging $^{63}$Cu$^+$, $^{75}$As$^+$, $^{118}$Sn$^-$ and $^{123}$Sb$^-$. This work suggests that it is possible to determine spatial dependencies between organic matter and inorganic pollutants and evaluate ‘hot spots’ on micron-scale particles.

The primary limitations for NanoSIMS studies on organic pollutants are the vapor pressure of the target pollutants (e.g. non-volatile organic compounds), the concentration of the target, and incorporating a tracer for the target. Eybe et al. (2008) embedded *Anabaena* sp. cells grown on the pesticide deltamethrin in an epoxy resin. To trace the pesticide within the
embedded cells, $^{81}\text{Br}$- was imaged in the NanoSIMS, illustrating how halogen containing pollutants may be used as tracers within biological samples. Another example is the work of Tartivel et al. (2012), who traced bromotoluene by the imaging of $^{81}\text{Br}$- in chemically fixed plant roots (*Hedera helix*) and resin embedded soil cross sections.

3. NanoSIMS requirements for soil related studies

3.1. Technical considerations for soil samples

With its improved primary ion optics and secondary ion transmission at high mass resolving power the NanoSIMS 50 and 50L enable SIMS analysis at the nanometer scale. However, there are specific technical limitations that the potential user must consider, especially for soils applications. While primary beams smaller than 50 nm are possible with idealized samples, the number of ions collected from the impacted volume starts to fall below the useful level in soils. NanoSIMS is a high vacuum ($\sim 10^{-10}$ Torr) instrument that requires samples to be dehydrated, conductive and have low topography (ideally submicron for natural abundance and < 30 micron-scale for isotopic enrichment experiments). As a result, live microbial cells cannot be tracked, and to measure process effects over time, one must rely on sub-sampling and replication. Samples should be fixed and can be thin-sectioned to achieve a flat surface, ideally without re-arranging the locale of target elements or molecules. While not currently available, a cryogenic stage might allow frozen samples to be analyzed, thereby preserving them in a more natural state.

Though analyses of natural abundance $^{13}\text{C}$ and $^{15}\text{N}$ are widely used in soil science, the NanoSIMS is capable of measuring isotope ratios with a precision of $\sim 1\%$ only in very favorable cases, and even a level of $\sim 10\%$ precision is likely to be very challenging to achieve in most complex soil samples. To obtain such a high precision ($<1\%$), relatively large amounts of material must be extracted from the sample surface (nanograms), requiring a large primary spot size $\sim 20 - 30$ μm. Such a spot size might itself exceed the micro-scale structures of interest (e.g. bacterial cells, clay minerals). Also, the much smaller primary beam used in NanoSIMS imaging generates a smaller number of secondary ions,
necessitating the use of electron multiplier (EM) detectors. EMs have a faster response time than faraday cup detectors (FC) (commonly used in larger beam SIMS instruments), and are thus both fast enough and provide sufficient dynamic range for imaging. EM detectors are, however, subject to a number of artefacts (limited count rates and detector aging) that effectively limit the precision to about 1‰. On top of this, the extraction conditions from location to location can be hard to maintain at a level that yields precision better than one part in a thousand, especially with heterogeneous samples such as soils. The limitations on precision mean that in most soil systems, natural abundance measurements will not produce useful data. Isotopic measurements with higher precision can be achieved using the Cameca IMS1280, a large-geometry magnetic sector ion probe, combining high transmission, high abundance sensitivity and high density of the primary beam with thermally insulated FC electronics. This instrument could potentially be used in complementary studies with a NanoSIMS to record images of high spatial resolution as well as per mil level precision.

In our experience, tracking of isotopically labeled tracers is probably the most practical way to explore micro-scale soil processes using NanoSIMS (Herrmann et al., 2007a; Keiluweit et al., 2012). As C and N are the key elements in organic matter studies, substrates enriched in $^{13}$C and/or $^{15}$N are regularly used for general investigations of microbial metabolism in soils as well as for the more specific purpose of following the fate of organic compounds as they cycle through soils (Kuzyakov et al., 2000; Ruetting et al., 2011). Of critical importance in tracer studies is whether the labeled substrate becomes chemically modified or is otherwise affected by the sample preparation. The potential user is reminded that NanoSIMS is not well suited for the identification of molecules or characteristic molecular fragments and so will rarely be able to address this kind of problem directly.

While quantitative NanoSIMS isotopic analyses are relatively straightforward, quantitative analyses of elemental abundances are considerably more challenging. In the fields of material science and mineralogy, SIMS users usually employ standards to correct for differences in yields and quantity for defined element-matrix combinations. The inherent
complexity and variability of soil matrices can complicate this approach, as appropriate standards are harder to obtain or manufacture. The C to N elemental ratio in soil OM, for example, is of general interest to soil scientists. The measurement of this ratio is inherently challenging because $^{12}\text{C}^{-}$ has a different formation mechanism than the $^{12}\text{C}^{14}\text{N}^{-}$ ion (N is detected as CN), as atomic N ionization is very poor. As a result, the yield of the two species can change relative to each other during the course of an analysis. C to N ratio measurements are therefore very challenging, requiring matrix-matched standards and method optimization, and ultimately may result in measurements of low accuracy and precision. It is an open question whether the heterogeneity of soil material makes such measurements additionally challenging.

3.2. Sample documentation

To facilitate the analyses, it is best to determine regions of interest on the sample prior to performing NanoSIMS measurements (Herrmann et al., 2007a; Moore et al., 2011a; Weber and Holt, 2008). Sample mapping can greatly enhance the efficiency of the analyses and is often critical to interpretation of results. Most SIMS instruments have the equivalent of an epi-illumination microscope for sample navigation, and therefore epi-illumination micrographs provide the best reference images for general navigation. Electron microscopy can also positively identify targets for analysis, and these images are often easily comparable to the secondary electron or ion images generated during NanoSIMS analyses. Ideal mapping images range from the whole sample scale to the individual target scale, with reference points that can be used to translate from one image scale to the next.

3.3. Instrument tuning and quality control

Here we present a brief introduction to issues that may be encountered during the tuning of a NanoSIMS 50 or 50L; more detailed instructions have been previously published (Pett-Ridge
and Weber, 2012). The central aspects of SIMS instrument tuning are mass selection, resolving isobaric interferences and peak shape.

a) **Mass selection**: To obtain accurate measurements, the secondary ion mass spectrometer must be tuned and aligned to collect ion masses of interest. Choosing masses will depend entirely upon the question being asked, what isotopically labeled compounds have been added, and whether operation is proceeding in Cs⁺ or O⁻ mode. Often this necessitates choosing between analyzing common bio-elements (e.g. C, N, O, S, P) with a Cs⁺ beam, or metals/metalloids (e.g. Fe, Al, Mn, Mo) with a O⁻ beam. If the ion yield is sufficient, some creative solutions exist, for example Fe can be detected as FeO⁻ in Cs⁺ mode. Alternatively, subsequent O⁻ mode measurement of the same spot is possible.

b) **Isobaric interferences**: When selecting ion masses it is critical to consider and exclude isobaric interferences, which are species with near-identical masses to the species of interest. Isobaric interferences can be problematic for SIMS because the sputtering process generates molecules. Therefore, in addition to isotopes with the same mass (e.g., ⁴⁸Ti and ⁴⁸Ca), users must consider isobaric interferences that are clusters of atoms, many of which are artificial (e.g., ²³Na²⁴Mg¹H⁺). To identify potentially significant isobaric interferences, the first step is to determine the major elemental composition of the sample (here, previous SIMS studies may be helpful). Blanks and control samples can be used to determine if interfering molecules are produced at significant levels. If this turns out to be the case, the exact masses of significant isobaric interferences need to be calculated relative to the exact mass of the target species to determine difference in mass (ΔM) and the mass resolving power (MRP = M/ΔM) required to resolve the target species. MRP is a metric of peak shape and the tuning of the mass spectrometer.

c) **Peak shape**: The scan of the mass peak should be both flat-topped and steep-sided. The shape of the peak is the cumulative result of everything from the primary beam location and size to the gain on the detector. A tightly focused and well centered
primary beam reduces angular aberration and minimizes potential distortions. The secondary ion beam should be aligned relative to all the lenses, slits and apertures in the secondary mass spectrometer in order to maximize transmission and minimize distortion. The entrance slit effectively sets the nominal MRP of the mass spectrometer, and the aperture slit and energy slit are used to steepen the peak side slopes and reach the target MRP by reducing angular and chromatic aberrations. Peak top flatness is important to measurement stability and indicates that 100% of the target species is collected to the exclusion of isobaric interferences. In addition to tuning and alignment issues, peak top flatness is also affected by the electron multiplier gain, high voltage, threshold and deflector settings.

Standards are used during the process of setting up the mass spectrometer; they are used for mass selection, quantification, and session to session comparison of transmission, MRP, and elemental or isotopic ratios. Reference materials can be simple (e.g., iron), multi-element standards like the NIST glass standard NBS610 (500 µg/g of most elements), or generated/characterized ‘in-house’ by characterizing samples through bulk methods and verifying high resolution homogeneity by replicate SIMS analyses. One example is the Bacillus subtilis spore sample used by the LLNL NanoSIMS group as a reference standard for C and N isotopic measurements (\(^{13}\text{C}/^{12}\text{C} = 0.0110;\) \(^{15}\text{N}/^{14}\text{N} = 0.00370\)). For this standard, isotopic enrichments were determined by bulk analysis at the University of Utah (Finzi-Hart et al., 2009); measurement precision, \(\sigma\) (internal), is \(\sim 4\%\) (2\(\sigma\)) for individual \(^{13}\text{C}/^{12}\text{C}\) and \(^{15}\text{N}/^{15}\text{N}\). For many soil science applications, the co-occurrence of both mineral and organic phases requires that both a multi-element (NBS 610) and organic phase standard be used. However, when studying complex organo-mineral interfaces, the aim should always be to simultaneously record mineral and organic derived ions to obtain a complete view on the studied mineral surfaces. Caution must also be exercised when applying SIMS to identify specific mineral phases. The count rate of an ion is not directly proportional to the concentration in the mineral. For example, the content of iron influences the secondary ion yield of other elements that are present in the sample. A linear interpolation based on the
Elemental concentration of the mineral is, therefore, not reasonable (Lehmann, 2003). It is thus advantageous to confirm mineral identity using X-ray techniques or other methods. In cases where quantification of an element is desired, mineral specific standards are necessary.

Minerals in soil pose a particular challenge because mineral grains may accumulate an electrical charge under sustained Cs⁺ primary ion beam sputtering. The phenomenon can readily be observed in the NanoSIMS because charged regions generate very low secondary electron yields. The area of charge accumulation may still yield secondary ions, but their trajectories through the secondary mass spectrometer will deviate from those of other ions when they have significantly different energy (>10 V difference). As a result, some species may not be detected with the same relative efficiency, resulting in inaccurate measurements. This is primarily an issue for the analysis of negative secondary ions because large numbers of electrons are extracted when the sample surface is bombarded by a beam of Cs⁺ ions. At the initiation of analysis, while the metal coating is still intact, charging may not be obvious. In some cases, minerals (e.g., magnetite) or organic material in the sample may provide sufficient charge conductivity to allow analysis. Most organic matter, while insulating in its natural state, becomes conducting under ion bombardment. However, soil particles, even those initially coated with Au, often begin to show evidence of sample charging after ~ 20 minutes of analysis, with significantly diminished total ion counts (Pett-Ridge et al., 2012). This effect has been observed in a NanoSIMS study of microaggregates deposited on silicon-nitride windows, where regions free of organic material appeared to undergo the most obvious charging (Remusat et al., 2012).

The best way to overcome sample charging is to use a device called an electron flood gun, commonly referred to as the e-gun. Tuning of the e-gun requires significant experience in aligning the electrons with the primary beam on the sample, and in adjusting the voltage to the charge status of the sample. Another challenge is that secondary electron imaging is not possible while the e-gun is in use. Often it is easiest to initially locate particles of interest with secondary electron imaging, and then turn on the e-gun for ion image data collection. When
the e-gun is tuned correctly, nonconductive minerals will not charge and the mass peaks will maintain the same level of mass resolution as achieved on a conducting sample. We note that the sample must be covered with a conductive coating for the e-gun to work; electrons that reach the sample outside the analysis area must have a path to ground. The e-gun is not likely to work with samples with significant topography and is not a solution in cases where topography is the proximal cause of charging and ion shadowing. In tests of different mineral types performed at LLNL, using the e-gun increased the ion yield for C and N by as much as a factor of 10. Additional tests showed that isotope ratios of standard reference materials analyzed both with and without the e-gun were not significantly different (Pett-Ridge et al., 2012). Caution is always advisable when interpreting results where charging is possible.

3.4. Sample preparation – from single particles to intact soil

In the next paragraphs we discuss possible ways to prepare soil samples ranging from primary particles and aggregates to complex intact samples containing microbial and plant tissues.

3.4.1. Direct deposition of individual particles and microaggregates

Individual soil particles (e.g. clay minerals, particulate OM) as well as microaggregates can be deposited directly onto an analysis support (e.g. Si-wafer, polished metal stubs) and imaged via NanoSIMS provided they have limited topography and remain adhered to the sample support under high vacuum.

Such materials may be derived from physical soil fractionation (Eusterhues et al., 2005) or taken directly from a soil suspension (Keiluweit et al., 2012). The application of ultrasound during soil fractionation may destroy natural soil structure and also re-distribute both organic compounds as well as individual mineral particles between physical fractions (Amelung and Zech, 1999; Mueller et al., 2012a). While pretreatments needed to isolate small particles may introduce experimental bias, it may still be desirable to attempt direct observations of small particles or microaggregates, especially when the nature of the research question prohibits the use of C and N-containing fixatives. Samples can be deposited on flat surfaces like
polished metal stubs, Si-wafers, gold foil or directly on SEM or TEM grids (Keiluweit et al., 2012; Mueller et al., 2012b). Alternatively, particles can be collected from soil suspensions on gold coated filter discs, a widely used preparation technique in microbiology (Musat et al., 2012). For obvious reasons, the sample support must be chosen with the elements of interest in mind (Si-wafers are not advisable when Si is a target element). In the direct deposition preparation technique, a single layer of small particles/microaggregates should be deposited on the sample support to minimize topography. If the goal is to simultaneously explore both mineral and microbial constituents, fixation of the biological tissues will be necessary (see below for detailed description).

3.4.2. Fixation and preparation of organic materials

A variety of biological sample types may be of interest to soil scientists, ranging from root or hyphal tissues to bacterial and archaeal cells, protists and microfauna, or decaying plant litter. For all these sample types, preservation of cell structure is important for target identification and to contain mobile organic and inorganic constituents. Sample preparation typically involves stabilization of biological components (fixation), removal of water (dehydration) and salts (derived from growth media or sea/soil water), deposition of the sample on a conductive support (Si wafer, TEM grid) and subsequent whole cell analysis or thin sectioning after embedding. Fixation of biological tissues is a process used to preserve cell morphology from decay and immobilize analytes of interest by increasing molecular mechanical strength or stability. Chemical fixation for imaging analysis is typically performed with aldehydes (e.g., glutaraldehyde, paraformaldehyde, formaldehyde) that cross-link proteins to hold cell structure together during dehydration, (Kuo, 2007; Nunan et al., 2001; Tippkötter and Ritz, 1996). Low temperature methods like flash freezing and high pressure freezing (Chandra and Morrison, 1992; Dykstra and Reuss, 2003; Echlin, 1992) are an alternative in some circumstances if it is critical to preserve the distribution of small molecules and diffusible ions. For biological samples that are to be analyzed intact, collection on a filter is very efficient, and nucleopore or polycarbonate filters can be used as a sample
support for SIMS analysis if they are kept flat at the micron scale. Samples grown on a solid sample support can be immersed in fixative and gently washed by repeated immersion in deionized water.

If subcellular elemental or isotopic distributions are of interest in soil microbial or plant tissue samples, fixation is often followed by embedding (impregnating the sample with a hard, vacuum stable matrix) and sectioning (cutting the sample into cross-sections) prior to NanoSIMS analysis. Samples can be embedded in a number of polymers for room temperature sectioning (e.g., epoxy, acrylic, paraffin (Dykstra and Reuss, 2003)). Cryogenic sectioning can be performed with sucrose, OCT or similar compounds. Cryosectioning of frozen (water/ice embedded) samples is also an option, but is more technically challenging and requires much practice. Sectioning can be performed with an ultramicrotome, a standard histological microtome or cryostat, or even with a razor blade, depending on the type of pre-NanoSIMS imaging that is desired. An adhesive surface coating (e.g. poly-L-lysine, vector bond, egg white) can be used to retain sections during washing or staining. Such surface coatings can also be used for the fixation of other thin sections or micro-scale single particles on sample mounts (e.g. Me-stubs, Si-wafers). Contrast stains used for TEM imaging of biological ultrastructure (e.g. uranyl acetate) typically are compatible with SIMS because mass spectrometry can easily resolve the stain components from many species of interest.

However, there may be cases where contrast stains would be a source of unwanted background counts, particularly for metal analysis. Standard TEM-grade ultrathin sections (~100 nm) can be analyzed by high resolution NanoSIMS, but with a limited analysis time to avoid section breakage or consumption. To extend the SIMS analysis time and collect more data while still allowing correlated TEM-SIMS analysis, thicker sections (up to 500 nm) can be prepared, though with a loss in TEM image quality. Thicker sections are also desirable if large areas (mm²) need to be analyzed. Focused ion beam (FIB) milling can also be used as an alternative to embedding and sectioning (Weber et al., 2010), particularly where the user needs to have precise control over the location and orientation of the sample thin-section. Thin sections can be laid on a TEM grid or directly on a solid substrate prior to SIMS
For example, this technique was used by Bonneville et al. (2011) to prepare cross sections of a micro-scale fungal hyphae / biotite interface for subsequent TEM analyzes. If transmission light imaging is necessary for sample mapping, indium tin oxide (ITO) coated glass slides are preferable to uncoated glass slides because the ITO coating does not charge under ion bombardment.

3.4.3. Preparation of aggregated soil structure and intact plant-soil systems

Many soil researchers would like to be able to image cross-sections of both biological and mineral phases in intact plant-soil samples. However, three dimensional multiphase objects often require additional preparation steps that go beyond what is required for a purely organic or purely mineral sample. Essentially, the object of interest needs to be embedded in a solid, vacuum-compatible medium and subsequently cut and or polished to generate a flat surface. The primary distinction between preparation of organic materials (see above) and preparing soil samples, is that cutting heterogeneous samples must accommodate both 'hard' (quartz, Fe oxides) and 'soft' components (plant tissues).

The most useful approaches for NanoSIMS soil sample preparation fall into three general categories: 1) embedding with resin (epoxy, acrylic, polyester), sucrose or paraffin, 2) embedding in molten elemental sulphur (S), or 3) cryo-techniques. The appropriateness of these three techniques depends on the target elements which are to be analyzed and the size of the sample (microaggregate vs. intact soil core). In general, resin embedding is the most useful approach for larger aggregates and soil cores, whereas cryo-preservation/sectioning and S embedding are the best choices for small (~100 µm) microaggregates. Cryo and S embedding have the added benefit that no artificial C or N is added as background material. We note that it may be possible to subtract a background resin signal from the sample signal using the difference in the CN/C ratio, (e.g. the biological from the non-biological material). The CN/C ratio of resin is quite distinct from that of biological material, so regions representing resin within analysis images can be omitted at the data processing stage (Weber et al., 2012).
For larger specimens like macroaggregates (>2 mm) or intact soil cores containing both hard mineral constituents and soft tissues such as plant residues (e.g. roots in Figure 5), microbes and plant roots, resin embedded sections are typically created and then polished for further analyses. One epoxy resin approach was described by (Herrmann et al., 2007a), who developed a technique for the preservation of microbial communities in quartz-based soils that was later modified by Clode et al. (2009) to include plant roots. Infiltration of samples in acetone-epoxy resin mixtures was conducted over a period of days, with the concentration of ‘Araldite’ (epoxy resin) gradually increased until 100%. At a larger spatial scale, at TU München this procedure was successfully applied to prepare natural intact soil cores for NanoSIMS analyses from the permafrost layer of cryoturbated soils (see Figure 2). Previous studies show that the abundance of nitrogeneous compounds was not adversely affected by this procedure (Herrmann et al., 2007a; Peteranderl and Lechene, 2004).

An alternative to resin embedding is sulfur embedding (De Gregorio et al., 2010; Flynn et al., 2004; Lehmann et al., 2005) which has been successfully used to section heterogeneous soil aggregates (Herrmann et al., 2007a; Lehmann et al., 2005). The most significant benefit of S embedding is that aggregates can be embedded in a non-C based, room temperature sectionable medium. In the DeGregorio et al. (2010) approach, elemental sulphur is heated to its molten state (> 100ºC), then a soil aggregate is inserted and allowed to cool. The resulting material can be glued to a metal stub and sectioned using an ultramicrotome. Unfortunately, the sulphur may provide only limited structural integrity for the sections because it tends not to impregnate samples extensively. Also, the high temperature could potentially alter organic materials, particularly those on the surface of the aggregate. A low-temperature alternative, the S embedding approach described by Lehman et al. (2005), consists of S heated until molten and rapidly cooled in liquid N\textsubscript{2}. As the S is slowly warmed to room temperature, it goes through a phase of high viscosity and at this stage (~20 seconds) aggregates may be inserted into the S. The resulting S block remains amorphous or plastic for a limited amount of time, during which sectioning should be carried out.
A final option for fixation and sectioning is the cryopreservation/sectioning approach used by researchers at LLNL: high-pressure freezing (Leica EMPACT2) of soil aggregates surrounded by ultrapure H$_2$O in small copper tubes (16.6mm) with an internal diameter of 350µm, followed by cryosectioning and freeze drying. This approach results in samples of regular thickness (<400 nm) and minimal chemical changes (Figure 6). This method, though challenging, preserves the distribution of diffusible ions and molecules. Because it involves fast freezing but no cryo-protectant, no background material is introduced that might dilute the C or N isotopic signal (Figure 6).

Once a soil sample is embedded, it must then be cut to expose a cross-section for NanoSIMS analysis. The presence of mineral grains significantly increases the difficulty of sectioning samples. While ultramicrotomy with a diamond knife is possible, it is significantly easier to cut embedded soil aggregates and cores with a wafer saw and then polish them as per a geological sample. This is, however, not ideal for some softer biological materials and resins, which can abrade more easily, resulting in topographical differences across the sample surface. This abrasion effect can be particularly pronounced at the edges of mineral grains, where inorganic-organic interfaces are located.

Sectioning via ultramicrotomy is the primary alternative option to saw cutting, and has proven highly effective for combined TEM and NanoSIMS stable isotope measurements of plant-soil systems (Clode et al., 2009). In Figure 5, a SEM image of three embedded roots illustrates how the heterogeneous distribution of soft and hard soil compartments can both be preserved in a thin section (Clode et al., 2009). In this procedure, both plant tissues and microorganisms appeared to be well preserved (Figure 4 and Figure 5). We note that the sectioning of embedded soil aggregates with heterogeneous density requires a highly skilled operator; it is easy to chip a diamond knife. A final option for sectioning fixed samples is FIB sectioning. This approach is a particularly good option for preserving the distribution of diffusible species because a fully dry sample can be sectioned, however the method requires
specialized equipment and limited sample material can be processed. If the samples are only
destined for SIMS analysis, top-cutting is a more rapid FIB option (Weber et al., 2010).

3.5. Data acquisition and analysis

There are two main data collection modes possible with the NanoSIMS 50; spot
measurements and ion images. The collection of secondary ions in the spot mode is usually
used for the direct recording of isotopic ratio data for a discrete location. However, the main
data collection mode for soil related studies is likely to be ion imaging because users working
with highly complex samples are usually interested in spatially explicit isotope ratios and
contextual information. NanoSIMS ion image files consist of individual images for each scan
(plane) for each given mass, along with metadata describing the conditions of the analysis.
There are a number of image processing software options available to analyze the ion image
data. WinImage is Cameca’s Windows-based image processing package that comes pre-
installed with the Windows-based NanoSIMS software. The previous Solaris-based
NanoSIMS software featured a somewhat rudimentary ion image processing application
called simply, Image. Another software package for SIMS image processing is the Windows
based LIMAGE program developed by Larry Nittler at the Carnegie Institution of Washington
(independent from Cameca). In addition to these commercial programs there are two open
source packages. The NRIMS (National Resource for Imaging Mass Spectrometry) group at
Harvard University has developed a plugin for the java-based freeware ImageJ. The so-
called MIMS plugin provides a number of specific data processing options in addition to the
full range of ImageJ capabilities. Most notable is the ability to express ratio images on a Hue-
Saturation-Intensity (HSI) scale, where the scale parameters can be set by the user. Finally,
a very recently developed software is the Matlab based application Look@NanoSIMS
developed by Lubos Polerecky at the Max-Planck Institute for Marine Microbiology in Bremen
(Polerecky et al., 2012). No matter what software is used there are some basic steps for the
processing of NanoSIMS images (the sequence of steps may vary slightly for some specific
data sets or research questions).
Briefly, the first step of image analysis is to examine all obtained images for possible flaws (e.g. spots of charging, unrecorded spots or lines within images). When multiple cycles are recorded, the analyst has to decide whether any cycles should be excluded from further analysis. Individual cycles should be aligned to correct for primary beam drift, and also dead-time corrected. This dead time is the period when the detector has counted a single secondary ion but is not ready to count another (usually tens of nanoseconds). Next, the species of interest are normalized to major element species (e.g. $^{13}\text{C}/^{12}\text{C}$, or $^{12}\text{C}/^{12}\text{C}^{14}\text{N}$); ratio images can be used efficiently to evaluate the quality of the ratio data and the distribution of normalized data. Typically, quantitative data are obtained by defining regions of interest (ROIs), which consist of a group of pixels bounding a particular feature. In most image processing applications a ROI can be drawn directly onto the image, and the software then records the sum of the counts from the pixels within the ROI and thus also ratio data. Defining ROIs is a critical step. Without objective criteria, it is possible to change the isotopic enrichments just by changing the size of an ROI. The best ROI selection criteria are external images (e.g. SEM image or fluorescence image) or single ion images (e.g. $^{56}\text{Fe}^{16}\text{O}^-$ for iron oxide particles). Alignment with an external image is possible when using the Look@NanoSIMS software. In many cases, it is necessary to visually evaluate the spatial distribution of several ions to interpret and properly define ROI’s. Unfortunately, there are no standard procedures for feature selection in complex matrices like soils.

After ROIs are defined, the data can be extracted and interpreted. One promising way to evaluate micro-scale ion images might be use of geostatistics, which are often used for larger plot or ecosystem analyses (Steffens et al., 2008). Geostatistics can be used to identify dependencies within any spatial data (e.g. NanoSIMS ion images). For example it might be possible to use this approach to evaluate the spatial distribution of Fe within a complex soil matrix. Figure 7 illustrates the application of a statistical approach (R 2.13.1 (R Development Core Team, 2011) in combination with the g-stat 2.4.0 package (Pebesma, 2004) to evaluate spatial dependencies between organic matter ($^{12}\text{C}^{14}\text{N}$) and particles containing iron ($^{56}\text{Fe}^{16}\text{O}$). The analysis is based on data extracted from a 32 µm line scan.
The sample is a Cambisol soil aggregate embedded in Araldite, originally from Höglwald, Bavaria, Germany. Both semivariograms for $^{12}\text{C}^{14}\text{N}$ and $^{56}\text{Fe}^{16}\text{O}$ (Figure 7 C and D) show a comparable periodic behavior, where the semivariance is oscillating (y-axis). The semivariance is a measure of spatial dissimilarity. Thus the wave like appearance of the semivariance demonstrates a distinct spatial pattern of similar ion counts ($^{12}\text{C}^{14}\text{N}$ and $^{56}\text{Fe}^{16}\text{O}$) and thus a regularity in the aggregate architecture (organic and mineral spheres). But as the spatial frequency (x-axis) of the maxima and minima of the semivariance is different between $^{12}\text{C}^{14}\text{N}$ and $^{56}\text{Fe}^{16}\text{O}$, this indicates differences in spatial structures and different pattern sizes. This analysis indicated that iron clusters ($^{56}\text{Fe}^{16}\text{O}$) are spatially independent from organic matter ($^{12}\text{C}^{14}\text{N}$). Similar analyses may be particularly useful to reveal spatial inter-dependencies between mineral and organic soil constituents or even between areas enriched or depleted in specific stable isotopes. Another approach to NanoSIMS data analysis is the upscaling approach used by Fike and colleagues (Fike et al., 2008). They collected numerous single spot (6 µm x 6 µm) NanoSIMS measurements (>300) and measured the isotopic variability of sulfide within the oxycline of a cyanobacterial mat (sampling grids: 3500 µm by 450 µm and 3000 µm by 500 µm, spatial increment of 50-200 µm). Analyzing the $\delta^{34}\text{S}$ abundance and isotopic fractionation, they observed fine scale laminations (1 to 400 µm) and clear zonation. This work suggests that single spot NanoSIMS measurements, collected on up to a millimeter scale and linearly interpolated, could be applied for upscaling micrometer spot data in soils.

4. Combination with other micro-scale techniques
4.1. Scanning and transmission electron microscopy

Prior to the development of NanoSIMS, electron microscopy was the approach of choice for the observation of soil particle arrangements (Gillott, 1970; Gray, 1967; Howard et al., 1996; Kowalkowski and Mycielskadowgiallo, 1985). Electron microscopy has evolved to achieve very high resolution, touching on 1 nm in scanning electron microscopy (SEM) and 0.05 nm in transmission electron microscopy (TEM). Similar to NanoSIMS, electron microscopy
techniques can be used to obtain information about the material properties of a sample, in addition to providing an image. In a scanning electron microscope (SEM), a high energy focused beam of electrons is produced. The electrons interact with electrons in the sample, producing secondary electrons, back scattered electrons (BSE), and characteristic X-rays (Energy-dispersive X-Ray Spectroscopy EDX/EDS/EDXS) that can be detected and that deliver information about the sample's surface topography and composition. An SEM-BSE image is a good tool to differentiate between organic and mineral spheres. This can be useful in samples without topography such as polished soil cross sections. The crystalline state of a sample can be determined by recording patterns of the diffracted backscattered electrons (EBSD) and matching them with a data base for crystallographic structures. An EDX scan yields information on the localization of minerals versus organic matter and is useful for identifying regions of interest within the sample.

It should be noted however, that in contrast to NanoSIMS, where information is gathered only from secondary ions sputtered from the uppermost atomic layers, the interaction volume of secondary ions in SEM extends in a pear shaped fashion between 100 nm and 5 µm deep into the sample (depending on the energy of the electron beam, atomic number and density of the specimen). EDX is therefore not a surface technique sensu strictu and it has been shown that TEM maps do not always compare well with subsequently obtained NanoSIMS images (Badro et al., 2007).

4.2. Synchrotron based techniques

To study soil process dynamics as a function of location within aggregates and microaggregates, simultaneous information on (a) localization, (b) identification and (c) transformation of organic matter and mineral phases is required with very high spatial resolution. Conventional electron microscopes can visualize basic elemental composition but are unable to speciate carbon compounds. A number of techniques can yield data on C composition (ion microprobe laser desorption, laser ionization mass spectrometry, Raman microscopy, $^{13}$C-NMR, FTIR (Lehmann et al., 2005; Lehmann et al., 2009), though few can
provide molecular or elemental characterization with the sub-micron resolution necessary to study mineral-organic interactions in microaggregates. These goals can be approximated with the combination of NanoSIMS and Scanning Transmission X-ray Microscopy, Near Edge X-ray Absorption Fine Structure Spectroscopy (STXM/NEXAFS). The resolution of synchrotron based X-ray microscopes can approach 50 nm, comparable to the NanoSIMS spot size. Raster scan images can be obtained at energy spacings of ~0.1 eV across 100's of eV energy ranges. The spatially resolved NEXAFS spectra extracted from the respective image ‘stacks’ reveal the bonding environment of the element of interest, allowing the speciation of organic matter forms and elemental redox states. STXM/NEXAFS has been used successfully to describe spatial patterns and speciation of soil organic matter associated carbon (Bardgett et al., 2007; Cheng et al., 2008a; Cheng et al., 2008b; Lehmann et al., 2005), and nitrogen (Gillespie et al., 2009; Kögel-Knabner et al., 2008; Sleutel et al.) in a broad range of environments, including marine systems (Brandes et al., 2004). A combined ‘STXM-SIMS’ approach allows precise, quantitative measurement of molecular and isotopic patterns in undisturbed samples, at high resolution (Keiluweit et al., 2012; Pett-Ridge et al., 2012). By combining NanoSIMS with STXM/NEXAFS it is possible to map organic C distribution, to image associations of organics with specific mineral types, and to trace organic matter of variable origin into the soil matrix (Lehmann et al., 2007; Wan et al., 2007). If combined with isotope tracer experiments, the images acquired through this approach can document the forms of C that become stored in soil aggregates and simultaneously track microbial debris and other organic polymers into the soil matrix.

Technical challenges to the combined application of STXM and NanoSIMS have recently been summarized by Pett-Ridge et al. (2012). They point out that experimental activities have to be planned in a way such that STXM analysis precedes NanoSIMS, as the latter method has a much greater potential for destructive interference with the sample. Sample holders commonly used in synchrotron spectroscopy may contain N (Si₃N₄) or C (TEM Cu grids with C lacey) and must therefore be chosen to avoid unwanted secondary ion species.
(e.g. $^{12}\text{C}$, $^{12}\text{C}^{14}\text{N}$) in subsequent NanoSIMS applications. Samples cannot be significantly thicker than 150-200 nm for STXM/NEXAFS or they will be impenetrable for soft X-rays. This limits the type of microstructures that can be observed with both methods at the same time to thin platelet–like objects. Taking these restrictions into account, SEM, NanoSIMS and STXM may be applied to the identical soil sample specimen, providing information that is complementary: SEM may be used to generate a mesoscale surface image of the region of interest (ROI), STXM/NEXAFS imaging can obtain information about whole sample chemistry, and NanoSIMS analysis can yield elemental/isotopic data on either surface characteristics or from a depth profile (Pett-Ridge et al., 2012) (Figures 6, 8). To allow for high-resolution SIMS imaging and STXM/NEXAFS spectromicroscopic analysis of the same sample, a specimen must be prepared with limited topography, able to withstand high vacuum, be dry, conductive, thin enough to allow photon transmission (<1 µm), and prepared without carbon-based reagents.

An example for the synergistic application of NanoSIMS and STXM/NEXAFS was recently presented by Keiluweit et al. (2012). These authors employed NanoSIMS to follow the fate of isotopically labeled amino sugars from fungal cell walls as they became metabolized or bound to minerals and SOM. Concurrently, STXM/NEXAFS spectromicroscopy was used to determine the chemical transformations of substrate C and N functionalities during the process (Figure 8). The authors determined that $^{15}\text{N}$-labeled amide N derived from fungal cell wall material preferentially associated with Fe (hydr)oxide surfaces or Fe-OM coprecipitates on the surface of other minerals. Through the combination of NanoSIMS and STXM/NEXAFS it was further possible to determine that amide N found on mineral surfaces originated from bacterial protein rather than from the original amino sugars or from nucleotides. This example illustrates that combined applications of synergistic imaging techniques such as NanoSIMS and STXM have the potential to provide information about organic matter-mineral-microbial relationships while avoiding the artifacts inevitably generated by chemical or physical fractionation procedures.
4.3. Atomic force microscopy

The atomic force microscope (AFM) is another tool for imaging, measuring, and manipulating matter at the nanoscale. An AFM consists of a cantilever with a sharp tip that is used to scan the specimen surface. When the tip is brought into proximity of a sample surface, forces between the tip and the sample lead to a deflection of the cantilever. The deflection of the cantilever is measured and converted to an image of the sample surface. Reports on AFM applications in soil science are increasing (Cheng et al., 2008c; Rennert et al., 2012; Schaumann and Mouvenchery, 2012; Totsche et al., 2010) and typically provide information about local topography and properties of minerals and soil aggregates surfaces at very high spatial resolution. To date, the focus of soil related AFM work has been on interactions between microorganisms and minerals, mainly iron oxides and hydroxides (Maurice, 1996; Maurice et al., 2000). Balogh-Brunstad et al. (2008) combined SEM and AFM to investigate fungal weathering of biotite in a batch liquid culture.

One potential combined AFM-NanoSIMS application could be the use of AFM to correct for topography effects in NanoSIMS analyses. Here AFM would be applied both before and after SIMS, allowing the user to convert SIMS data into a true three-dimensional representation of the analyzed species (Fleming et al., 2011). Wirtz et al. (2012a; 2012b) report the development of an integrated SIMS-scanning probe microscope (SPM). In this instrument, a specially developed SPM system was integrated in a Cameca NanoSIMS 50, allowing the user to record topographical in situ images of the sample surface before, in between and after SIMS analysis.

4.4. In situ single-cell labeling

Environmental microbiologists see particular value in the combination of in situ phylogenetic labeling and NanoSIMS in order to link metabolic function with taxonomic identity (Kuypers and Jorgensen, 2007). This capacity was first demonstrated using a combination of conventional SIMS and fluorescence in situ hybridisation (FISH) to analyze an archaeal-
bacterial consortium in anoxic marine sediments (Orphan et al., 2001). The FISH-SIMS combination allowed Orphan et al. (2001) to demonstrate that cell aggregates binding a specific archaean probe were strongly depleted in $^{13}$C, indicating a methane-based metabolism. In many natural microbial systems, this approach may be even better suited to NanoSIMS, as it can provide this information at the length-scale of an individual bacterium (~1 μm). Two variations of this approach are currently used: element-labeled fluorescent in situ hybridization (EL-FISH) where catalyzed reporter deposition FISH (CARD-FISH) is used to deposit high concentrations of fluorine-containing fluorophores in target cells (Behrens et al., 2008); or halogen-labeled in situ hybridisation (HISH) where a standard FISH protocol is used in combination with halogen (I, Br) tagged probes (Li et al., 2008). NanoSIMS can then be used to visualize the labeled cells by acquiring a signal for $^{19}$F, which is not naturally present at high concentrations in most environmental cells (Behrens et al., 2008; Halm et al., 2009; Musat et al., 2008). In these approaches, a phylogenetic probe is linked to a highly electronegative elemental label (fluorine, iodine, gold, selenium, or bromine) instead of the typical fluorophore, which can be detected in concert with $^{13}$C and $^{15}$N isotopes for functional characterization. These approaches enable simultaneous localization of the tag and chemical mapping in the NanoSIMS. It may even be possible to use FISH-SIMS approaches in embedded samples (Woebken et al., 2012). The work of Lemaire et al. (2008), where fixed samples were embedded in TissueTek® (Sakura Finetek Labware & Accessories) and then cryosectioned and FISH labeled, suggests this may be possible. However, its usefulness in soil may be limited unless a means to overcome soil’s natural background fluorescence is developed. Also, care must be taken with quantitative interpretation of FISH-SIMS results, since these approaches may reduce the original cell enrichment by 60-80% for $^{13}$C and 30-60% for $^{15}$N (J. Pett-Ridge and S. Behrens, unpublished data).

5. Conclusion

Over the last decades soil scientists have gained a working knowledge of fundamental soil processes ranging from the stabilization of organic matter to microbial diversity in soils. We
are now able to track the fate of specific molecular plant biomarkers into organo-mineral associations and to determine the microbial communities responsible for the turnover of specific organic compounds. However, commonly used bulk analyses average over a vast swath of microbial and mineral landscapes, and can miss micro-scale phenomena caused by specific micro-habitats or distinct spatial heterogeneities in the formation of organo-mineral assemblages. Thus, while our knowledge of bulk scale biogeochemical soil processes is expanding, we have lacked the high resolution techniques needed to illustrate the mechanistic underpinnings such processes in intact soil structures.

The emerging class of nano- and microscale spectroscopy and spectrometry techniques has opened a new frontier in the effort to develop a fundamental understanding of soil processes. Though the first review of NanoSIMS in soil ecology and biogeochemistry by Herrmann and colleagues was in 2007 (Herrmann et al., 2007b), this field is still in its early phases as soil scientists grapple with the many technical challenges. Looking forward, the challenge will be upscaling from micro-scale analyses to scales from the soil horizon or even to the pedon or ecosystem scale. These leaps may not come soon, but for now the ability to image elements and stable isotopes at a previously unresolved spatial scale permits the combination of established isotopic enrichment techniques with the description of their spatial distribution in soil microenvironments. Recently, several new NanoSIMS instruments have been installed and the access for soil scientists is increasing. NanoSIMS analyses have the potential to contribute to a fundamentally new understanding of soil processes, one that is rooted at the relevant scale of microbial, mineral and organic matter interactions.

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Figure captions

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**Figure 2:** Micrograph and microanalysis of an embedded cross section derived from a Cryosol soil core (Oa horizon, Typic Aquiturbel) from Barrow, Northern Alaska. A) back scatter electron image recorded with a SEM; B, C) NanoSIMS images ($^{12}$C$^{14}$N$^-$ and $^{56}$Fe$^{16}$O$^-$) recorded with a NanoSIMS 50 L at TU München. The back scatter SEM image shows collapsing plant cells of particulate OM in the center surrounded by mineral spheres. The red square in the SEM image indicates the area analyzed by NanoSIMS, the green line indicates the interface between particulate organic matter and mineral phase, the blue line depicts the boundary between totally and partly collapsed plant cell structures. The NanoSIMS images indicate the distribution of organic matter ($^{12}$C$^{14}$N$^-$) and the iron distribution ($^{56}$Fe$^{16}$O$^-$) within the plant cell region, and suggest organo-mineral interfaces in the early stages of formation. D) line scan data derived from analysis of NanoSIMS $^{12}$C$^{14}$N$^-$ and $^{56}$Fe$^{16}$O$^-$ secondary ion images. The line scans demonstrate the spatial distribution of both secondary ion species along a transect, illustrating the iron clusters within the organic matter region. An area of 0.5 to 0.5 µm (square #2 in images) in size was scanned along the transect (Mueller, unpublished data).

**Figure 3:** Back scattered secondary electron micrograph and NanoSIMS ion images ($^{16}$O$^-$; $^{12}$C$^{14}$N$^-$) of an embedded root tip cross-section prepared from a French oak root (*Quercus robur*, clone DF159 infected with mycorrhizal fungi *Piloderma croceum*, courtesy of F. Buscot, UFZ Halle, Germany and T. Grams, TU München, Germany) grown in a vermiculite / soil mixture. The root and adhering rhizosphere soil was fixed according to Karnovsky (1965), embedded in an epoxy resin, cross sectioned, polished and imaged via NanoSIMS. The $^{16}$O$^-$ NanoSIMS images illustrate thin clay mineral layers, whereas the $^{12}$C$^{14}$N$^-$ ion images
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Figure 4: TEM and NanoSIMS images of wheat roots (*Triticum aestivum*) exposed to $^{15}$N for 24 h. The samples were fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffered saline and dehydrated in a graded series of acetone. The roots were infiltrated in acetone araldite mixtures over several days, using a gradually increasing araldite concentration. Final embedding in Araldite 502 was done according to Herrmann et al. (2007a). Embedded samples were cut into slices, re-embedded in 10 mm mounts and polished using silicon carbide paper and finally diamond paste. TEM images (A, D) show the presence of microorganisms in the rhizosphere (rh) and extracellular mucilage matrix (e) adjacent to the root cells (c). NanoSIMS images (B, E) of the same regions show organic matter distribution recorded as $^{13}$C$^{14}$N. NanoSIMS ratio images (E, F) of $^{15}$N/$^{14}$N (natural abundance at 0.004), confirmed the $^{15}$N enrichment of some microorganisms. Linescan data from the regions between the arrows (in C, F) is shown in G (from C) and H (from F). (Figure adapted from Clode et al. 2009) (Copyright American Society of Biologists www.plantphysiology.org).

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**Figure 8:** Microstructures associated with fungal hyphae in an organic forest soil investigated by both NanoSIMS imaging and STXM/NEXAFS spectromicroscopy. A) STXM optical density map recorded at 300 eV; B, C) NanoSIMS isotope ratio images for $^{12}$C$^{15}$N/$^{13}$C$^{14}$N and Fe concentrations ($^{56}$Fe$^{16}$O/$^{12}$C, normalized to carbon) of the same feature shown in the STXM image. Brighter colors reflect high enrichment/concentration. D) Optical density map of hyphal-associated microstructures with colored regions of interest (ROIs) from which NEXAFS spectra at the C 1s absorption edge and the N 1s absorption edge were collected. ROIs are color-coded according to the spectral types extracted from them: intact fungal hyphae (grey), decomposing hyphal residue (brown), microbial residue (green) and mineral surfaces (blue). E) Average NEXAFS spectra representing the major carbon forms encountered in the regions of interest defined in D. Carbon 1s absorption edge peaks are identified as C=C 1s-π* transition of aromatic C at 285.1 eV (a), 1s-π* transition of C=C in ene-ketone at 286.7 eV (b), 1s-3p/σ* transition of aliphatic C at 287.4 eV (c), 1s-π* transition of carboxylic and/or amide C at 288.3 eV (d), the 1s-3p/α* transition of alcohol C-OH at 289.4 eV (e), and the 1s-π* transition of carbonyl C at 290.3 eV (f). Reproduced with permission from Geochimica and Cosmochimica Acta.
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