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in Ecosystem Research

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**Abstract:**

This report presents technical improvements in terms of sample preparation for applying nanoSIMS (secondary ion mass spectrometry) approaches to the study of soil aggregation by specific organic matter components. Furthermore, it gives details about the combined analysis of secondary ions indicative of organo-mineral associations (C, N, Fe, Si, Al) in soil systems affected by water-stress and the use of AFM (atomic force microscopy). The combination of these sophisticated laboratory techniques and long-term field experiment for elucidation of ecosystem processes is presented.

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## Annex

Deliverable checklist

## 1 Executive summary

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This report presents solutions for applying nano-scale secondary ion mass spectrometry (NanoSIMS) approaches to the study of soil aggregation by specific organic matter components. Furthermore, it gives details about the combined analysis of secondary ions indicative of organo-mineral associations (C, N, Fe, Si, Al) in soil systems affected by water-stress and the use of atomic force microscopy (AFM). The combination of these sophisticated laboratory techniques and long-term field experiment for elucidation of ecosystem processes is presented.

## 2 In-situ exploration of micro-scale soil processes

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### 2.1 The advantages of nanoscale techniques for determination of soil processes

The nanoscale analysis of soil particles can provide more precise information on the occlusion of soil organic matter (SOM) in aggregates as well as the formation of organo-mineral associations than conventional techniques, which are addressing larger scales. To investigate the evolution and composition of submicron-sized organo-mineral associations and aggregate interiors the aim of EXPEER was to test novel micro-analytical techniques allowing the simultaneous analysis of the spatial distribution of elements involved in stabilization processes of soil organic matter (for example C, N, Si, Al and Fe). This is a major step forward in the understanding of soil formation with significant implications for our conceptual understanding of the soil C and N cycles, structural stability and sorptive properties (von Lützwow et al., 2008; Totsche et al., 2010). This is also a vital prerequisite to understand processes at larger scales up to the ecosystem level.

Until today, the NanoSIMS technique has been mainly applied in the field of material science, cosmochemistry, geology, mineralogy and biology (Hoppe, 2006; Lozano-Perez et al., 2008; Orphan and House, 2009; Wagner, 2009). In the field of soil science, the possible applications of NanoSIMS for the study of biogeochemical processes were reviewed by Herrmann et al. (2007). They described the great potential of submicron studies of soil samples with a special focus on soil fauna, but also mentioned the potential for the study of soil organic matter stabilization. In the

frame of ExPeer the main focus is on the application and development of this technique to study soil hydrological processes including diffusion and sorption of organic matter.

A fundamental objective was the establishment of measurement techniques with respect to research questions in soil science. Furthermore the elucidation of the elemental/isotopic composition and simultaneous analysis of the characteristic elements in organo-mineral associations (C, N, Si, Al and Fe).

The availability of a NanoSIMS microprobe adapted to soil science research was also considered as an initiative for further international activities and collaborative efforts for soil science and related disciplines. The technique was intended to add a new dimension to tackle research questions in several coordinated research efforts, where the soil science group from TU München is involved. It was considered to stimulate the collaboration between soil chemists, microbiologists but also plant physiologists. In addition, the proposed NanoSIMS facility at Freising-Weihenstephan was designed to be open for other research groups, which should on the longer term lead to additional research questions and approaches.

## **2.2 Progress in the establishment of micro-scale techniques and method development**

The official opening of the Cameca NanoSIMS was held on October 13th and 14th 2010. It was intended as one measure to announce the availability of the instrument to the science community.

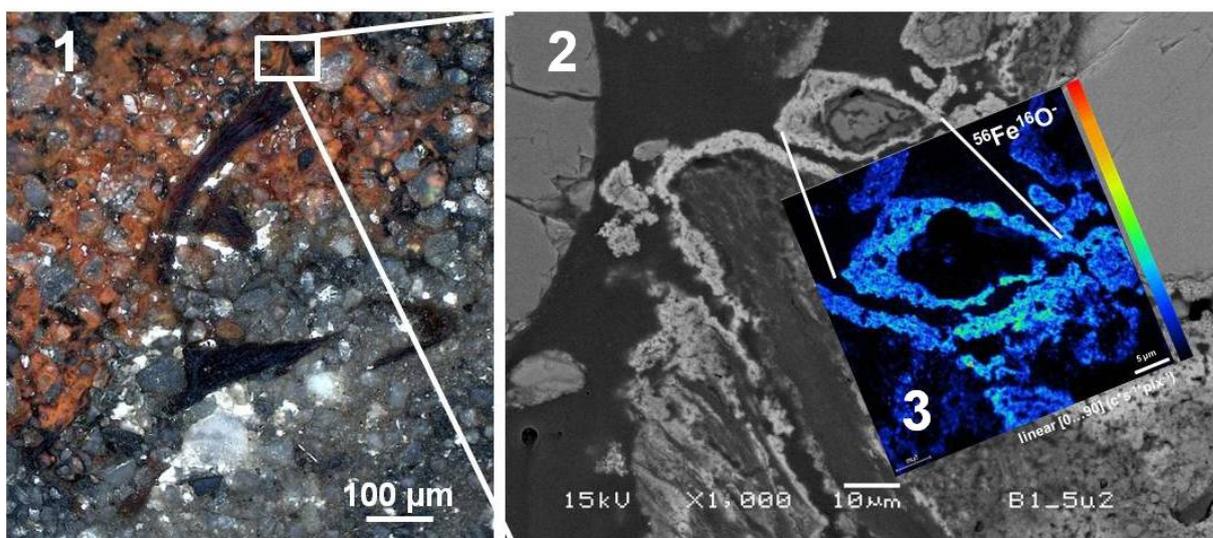
The first time period of operation was especially devoted to the development of operating skills and the establishment of the method for ecological research questions related to soil science and related fields.

This comprised:

- i) The development of different protocols for the embedding of soil aggregates in epoxy resin. This technique enables the in-situ measurement of intact soil structures and is crucial for the combined analysis of soil structure and function.
- ii) Testing different soil model compounds and systems as simple organic samples simulating organic matter and/or bacteria in soils. Here we used stems of *E.coli* bacteria which were labeled with  $^{15}\text{N}$  to establish the analyses of stable

isotopes at the micro-scale. As typical mineral particles different clay mineral mixtures were used. To simulate natural soils, mixtures of mineral and organic compounds were analyzed. This step was crucial to ensure the accurate measurement on complex soil systems.

- iii) The most complex step was to start with natural soil samples. We were able to show different zones of Mn coatings from aggregated soil structures, comprising important features of water saturated soils. This was done on embedded cross sections, indicating the great use of NanoSIMS for the study of elemental zonations due to changing redox conditions in water saturated soils. Furthermore we were able to track spots enriched in  $^{15}\text{N}$  in the centre of intact soil aggregates. By this approach we were able to show the feasibility of NanoSIMS to track specific organic substances in intact soil structures. In the future this allows for the determination of elemental transport and sorption at the micro-scale.
- iv) Another important step was the combination with other techniques as well as long-term field experiments. We tested the measuring of the same regions of interest by different techniques (optical micro-scope, atomic force microscopy (AFM), scanning electron microscopy (SEM)). To permit analyses of the same region of interest a coordinate transformation software was developed (example in Figure 1). A fundamental breakthrough was the correlation of AFM and NanoSIMS on goethite needles, this work was recently accepted for publication (Liu et al. 2013).



**Figure 1:** Example for the successful coordinate transformation in combination of different microscopic techniques (intact iron rich soil core, embedded in Araldite 502). Image 1

shows an iron oxide rich sphere on the polished sample disc, image 2 indicates the iron layers as light grey spheres in the backscattered electron image and image 3 shows the distribution of detected  $^{56}\text{Fe}^{16}\text{O}^-$  secondary ions on the particular field of view.

At the nanosims facility in Paris, CNRS tested its applicability for the study of interactions between iron and organic matter using samples from a field incubation experiment with  $^{13}\text{C}$  and  $^{15}\text{N}$  labelled root material. Nanosims in combination with SEM analyses demonstrated for the first time close interaction between  $^{15}\text{N}$  labeled microbial cells and metal oxides.

### **2.3 Technical challenges, solutions and applications**

Many mineral particles show charging effects, thus working with the e-gun to compensate this effect is necessary. But the tuning of the e-gun is not trivial and still needs training to gain constant and reproducible results. The very heterogeneous mixture of mineral (charging) and organic (no charging) particles makes the focusing of the ion beam difficult. Furthermore, some particles, such as charcoal or biotite do not attach well to the polished sample holders. Here, new approaches for sample preparation have to be tested in the future.

#### **2.3.1 Optimizing and establishment of protocols for sample preparation**

The high sensitivity and lateral resolution of the NanoSIMS instrument can only adequately be used with appropriate sample preparation. In particular topography, outgassing and charging effects are of major concern to achieve highly precise analyses (Winterholler et al., 2008). Different sample preparation techniques for soil particles and soil aggregates were evaluated and tested for their suitability with soil materials. Two different sample preparation techniques (i) wet deposition and (ii) the sectioning of epoxy resin embedded samples were developed. Wet deposition is used for primary soil particles, i.e. mineral particles and particulate organic matter (see also Figure 2). The epoxy resin embedding technique is used for intact soil aggregates up to intact soil core sections. Both techniques allow to produce samples that meet the

requirements of the instrument, i.e. vacuum stability at  $< 10^{-9}$  mbar, flat and polished surface and sample sizes fitting exactly into the sample holders.

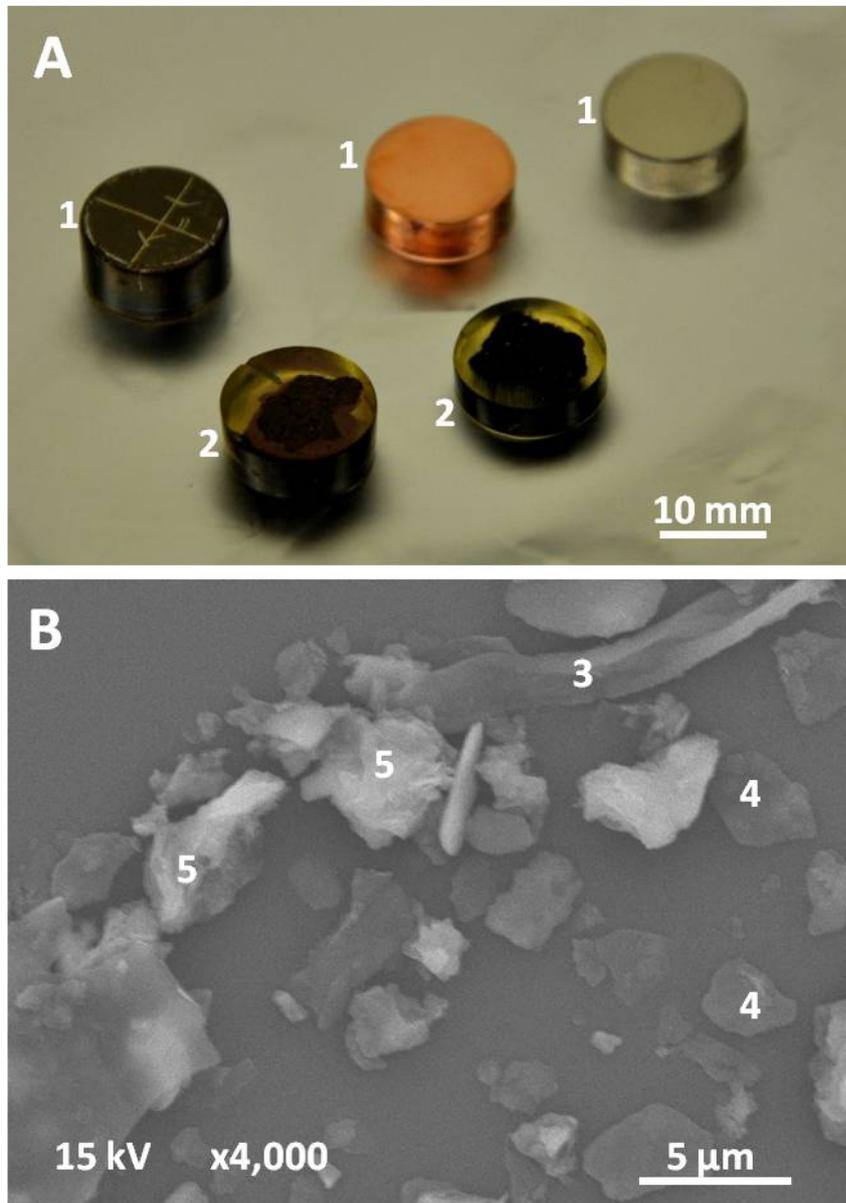


Figure 2: Examples for sample preparation: Image A shows polished metal stubs (1) for preparation of fine powdered samples (e.g. oxides, clay minerals) and intact aggregate cross sections (2) embedded in Araldite 502. SEM image B shows a fine powdered sample (SOM fraction) on a polished Al-stub ( $\varnothing$  10 mm), indicating particulate organic matter (3), primary mineral particles (4) and microaggregates (5).

The incubation of intact soil aggregates with an isotopic label and the subsequent resin embedding of whole soil aggregates was demonstrated to be adequate for the study of biogeochemical processes in situ within intact soil spheres. Nevertheless,

the preparation of resin embedded samples has to be done with great caution, as the study of cross sections by NanoSIMS is strongly affected by the sample preparation. Differences in density between quartz grains, clay minerals and particulate organic matter lead to clearly different abrasion rates in the course of the sample polishing. Therefore, a major point is the sample polishing, as high differences in topography on the sample surface may cause problems for the later focusing of the ion beam. On the other hand, the embedding agent is of high importance, as it determines the isotopic background composition (e.g.  $^{13}\text{C}/^{12}\text{C}$  for Araldite) and may also cause charging effects due to insulating properties.

### **2.3.2 Analysis of model systems of increasing complexity**

We produced artificial soils in a long-term biogeochemical laboratory incubation experiment to create soil material with known properties to simulate natural soils. Starting from simple systems, the complexity of the artificial soil systems was increased, until they mimic natural soils as good as possible. The individual compounds were incubated together with manure for at least 18 months to produce the artificial, well-defined soil material. In a study using these materials of known composition (Heister et al., 2012), we were able to demonstrate that it is possible to differentiate the major components of soils by NanoSIMS. Samples were prepared from particle size and density fractions and investigated with reflected light and electron microscopy in combination with energy dispersive X-ray spectroscopy prior to NanoSIMS analysis. For the study of organo-mineral associations, the use of  $\text{Cs}^+$  primary ions enables the simultaneous detection of organic material derived ions like  $^{12}\text{C}^-$  and  $^{12}\text{C}^{14}\text{N}^-$  and ions like  $^{28}\text{Si}^-$ ,  $^{27}\text{Al}^{16}\text{O}^-$ , and  $^{56}\text{Fe}^{16}\text{O}^-$  originating from minerals. Differentiation between highly condensed charcoal and soil organic material was achieved by their different amounts of  $^{12}\text{C}^-$  and  $^{12}\text{C}^{14}\text{N}^-$  secondary ions emitted and the resulting  $^{12}\text{C}^-/^{12}\text{C}^{14}\text{N}^-$  ratios. Moreover, two types of attachment of organic material to minerals were discovered. Attachment of organic material to mineral surfaces was predominantly found to occur in patchy structures on the clay minerals (Figure 3), whereas only little sorption of homogeneously distributed organic material onto Fe oxides occurred (Fig. 5). Although there are several reasons conceivable why we did not detect more sorption of organic matter to Fe oxides, it is

likely that this is caused by the neutral pH of the soils (pH 7.5), hampering sorption to the variable-charged surface sites of the Fe oxide. But it is also possible that the secondary ion yield of  $^{12}\text{C}^-$  and  $^{12}\text{C}^{14}\text{N}^-$  is reduced, when the organic material is directly bound to the Fe oxide.

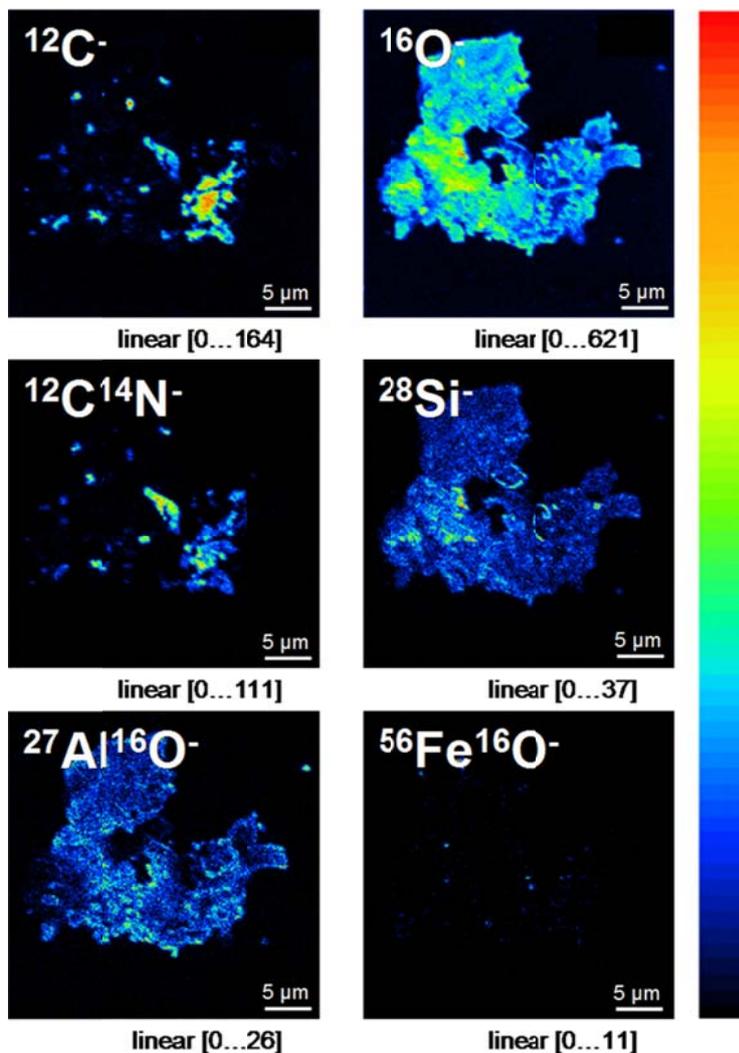


Figure 3: NanoSIMS images (256×256 pixels) of the  $^{12}\text{C}^-$ ,  $^{16}\text{O}^-$ ,  $^{12}\text{C}^{14}\text{N}^-$ ,  $^{28}\text{Si}^-$ ,  $^{27}\text{Al}^{16}\text{O}^-$  and  $^{56}\text{Fe}^{16}\text{O}^-$  distribution acquired on clay particles (30×30 μm) with a  $\text{Cs}^+$  primary ion beam of a current of ~1 pA with a dwell time of 1 ms pixel<sup>-1</sup> using the electron flood gun. The hue scale indicates the counts per second and pixel of the secondary ions. All images are in linear scale, and the values in parentheses below each image specify the range of detected counts per pixel (from Heister et al., 2012).

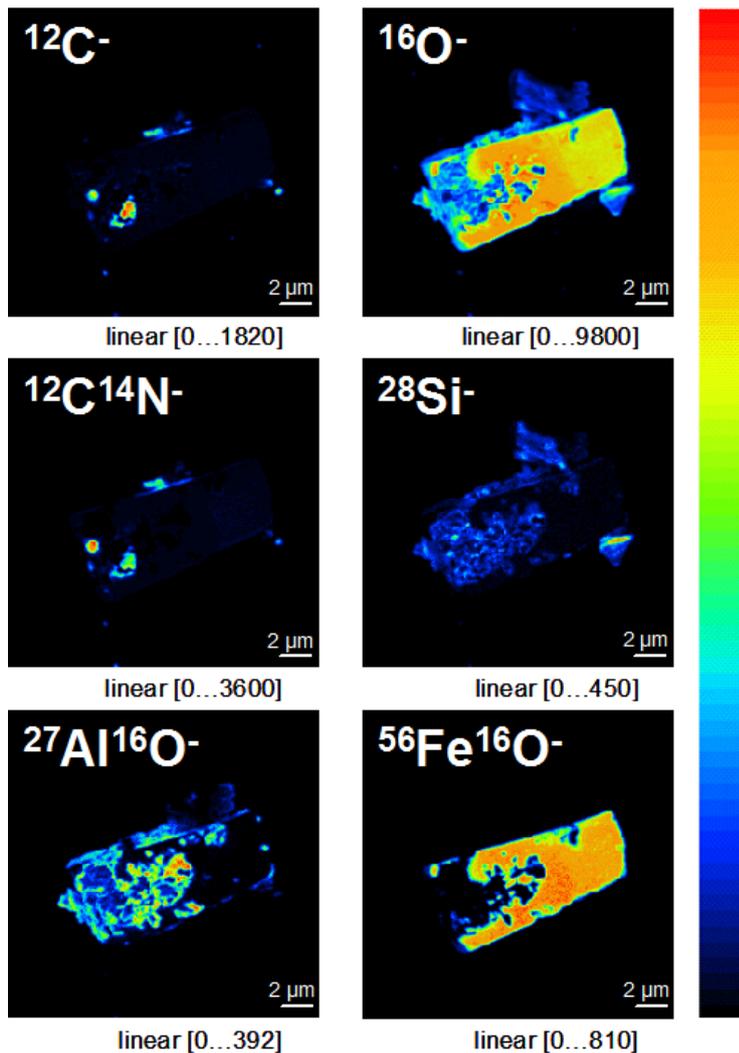


Figure 4: NanoSIMS images (256×256 pixels) of the  $^{12}\text{C}^-$ ,  $^{16}\text{O}^-$ ,  $^{12}\text{C}^{14}\text{N}^-$ ,  $^{28}\text{Si}^-$ ,  $^{27}\text{Al}^{16}\text{O}^-$  and  $^{56}\text{Fe}^{16}\text{O}^-$  distribution acquired on an agglomeration of ferrihydrite and illite (20×20 μm) with a  $\text{Cs}^+$  primary ion beam of a current of ~1 pA with a dwell time of 1 ms pixel<sup>-1</sup> using the electron flood gun. The hue scale indicates the counts per second and pixel of the secondary ions. All images are in linear scale, and the values in parentheses below each image specify the range of detected counts per pixel.

These results show that NanoSIMS enables the analysis of submicron processes in soil science-related research. However, the very heterogeneous matrix of soil particles, leading to various ionization rates, will make attempts for a quantitative analysis difficult, which is also due to a lack in the availability of suitable standards representing these complex matrices. Therefore, an important issue for further studies is the search for such standards, representing the large range of possible matrix effects in soil materials.

### 2.3.3 Labelling studies

To elucidate the power of the NanoSIMS technique for stable isotope studies in soils, we performed an incubation experiment with primary soil particles of the fine silt and clay fractions (Albic Luvisol), with occluded particulate organic material and intact soil aggregates (Haplic Chernozem), using a  $^{13}\text{C}$  and  $^{15}\text{N}$  labelled amino acid mixture as tracer. Results of this study were published by Mueller et al. (2012).

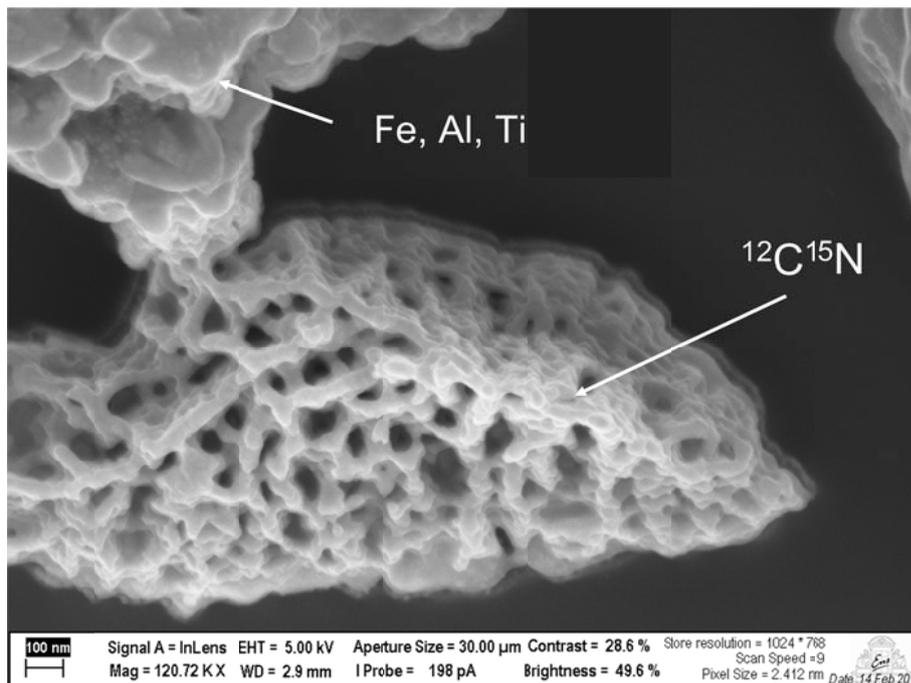
We chose the fine silt / clay mixture ( $<6.3\ \mu\text{m}$ ) from the Ah horizon of an Albic Luvisol for the investigation of micro-aggregates and the small occluded particulate organic matter (oPOM<sub>small</sub>,  $<20\ \mu\text{m}$ ) from the Ap horizon of a Haplic Chernozem for studying particulate organic matter in soils. The samples were chosen in such a way to represent two important SOM pools in relation to the long term stabilization of soil organic C. The association of soil organic matter (SOM) with the mineral phase represents the largest reservoir of soil organic carbon, stabilized by the interaction with clay minerals and pedogenic oxides on decadal to centennial timescales (Christensen, 2001; von Lützow et al., 2008), whereas the highly aromatic oPOM<sub>small</sub> (high content of black carbon) represents a SOM pool that is stabilized by its chemical recalcitrance (high aromaticity) but also by spatial inaccessibility (low bioavailability for microbes and enzymes) due to its occlusion in soil aggregates (Wagai et al., 2009). At the same time, clay sized minerals and small black carbon particles represent very important materials for the sorption of nutrients but also contaminants.

Before and during a 6-day incubation after the addition of the label, samples were consecutively prepared for NanoSIMS analysis. Single soil particles (fine silt / clay fraction) showed an enrichment of  $^{13}\text{C}$  and  $^{15}\text{N}$  after label addition which decreased over time. On aggregates of particulate organic matter, re-aggregated during the 6 day incubation experiment, we could show a spatially heterogeneous enrichment of  $^{13}\text{C}$  and  $^{15}\text{N}$  on the particle surface. The enrichment in  $^{15}\text{N}$  demonstrated the diffusion of dissolved organic matter into intact soil aggregate interiors. In addition, line scans and depth profiles revealed a heterogeneous pattern of the organic matter distribution.

The heterogeneous isotopic enrichment over a particulate OM cluster demonstrated the possibility to trace regions where microbial activity lead to the increased utilization of freshly added organic matter and/or different sorption capabilities. These results show for the first time that the NanoSIMS analytical technology can be applied to a broad range of soil individual particles or aggregates and enables us to locate the association of relevant elements/isotopes, such as  $^{12}\text{C}$ ,  $^{13}\text{C}$ ,  $^{12}\text{C}^{14}\text{N}$ ,  $^{12}\text{C}^{15}\text{N}$  and  $^{28}\text{Si}$  at the submicron scale. By comprising the topographic data from scanning electron microscopy (SEM) and atomic force microscopy (AFM) with NanoSIMS analyses in the future, the information on soil or sediment particle composition will be improved. An important task for the future is also the search for sufficient standards representing the wide array of possible matrix effects in soil materials, from microbial residues to plant derived organic matter and quartz, clay minerals and metal oxides.

#### **2.3.4 Combining long-term field experiments, compound specific isotope analysis and nanoscale techniques to elucidate SOM stabilization**

Long-term field experiments are absolutely necessary to understand the timescale of SOM stabilization processes. We used samples from a three year field incubation carried out at the EXPEER TA site Lusignan of  $^{13}\text{C}$  and  $^{15}\text{N}$  labeled root litter at three soil depths. These samples were analysed by a combination of compound-specific molecular analyses as well as nanosims. To elucidate organic matter interactions with metal oxides, we isolated a density fraction  $> 3 \text{ g cm}^{-3}$ . Our results showed that pedogenic parameters at different soil depths are crucial for the fate of root derived organic matter. Whereas lignin may contribute to SOM storage at specific soil depths, we also demonstrated that metal-SOM associations in the deepest soil layer (90 cm) concern mainly microbial material (Fig. 5).



**Fig 5:** SEM analysis of a density fraction ( $> 3 \text{ g cm}^{-3}$ ) after Nanosims measurement.  $^{15}\text{N}$  labelled microbial cell attached to a metal oxide. Note that organic material is characterised by holes created by the nanosims beam

### 3. Publications in the frame of task 7.2

Müller C. W., Weber P. K., Kilburn M., Hoeschen C., Kleber M., Pett-Ridge J. (2013): Advances in the Analysis of Biogeochemical Interfaces: NanoSIMS to Investigate Soil Microenvironments. *Advances in Agronomy*, 121, **in press**

Liu X., Eusterhues K., Thieme J., Ciobota V., Hoeschen C., Mueller C.W., Kuesel K., Kögel-Knabner I., Roesch P., Popp J., Totsche K. (2013) STXM and NanoSIMS investigations on EPS fractions before and after adsorption to goethite. *Environmental Science and Technology*, **accepted**

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Heister K, Hoeschen C, Pronk GJ, Mueller CW, Kögel-Knabner I (2012) NanoSIMS as a tool for characterizing soil model compounds and organomineral associations in artificial soils. *Journal of Soils and Sediments*, 12, 37-45.

Herrmann AM, Ritz K, Nunan N, Clode PL, Pett-Ridge J, Kilburn MR, Murphy DV, O'Donnell AG, Stockdale EA (2007) Nano-scale secondary ion mass spectrometry – A new analytical

tool in biogeochemistry and soil ecology: A review article. *Soil Biology and Biochemistry*, 39, 1835-1850.

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Wagai R, Mayer LM, Kitayama K (2009) Nature of the "occluded" low-density fraction in soil organic matter studies: A critical review. *Soil Science and Plant Nutrition*, 55, 13-25.

Wagner M (2009) Single-cell ecophysiology of microbes as revealed by Raman microspectroscopy or secondary ion mass spectrometry imaging. *Annual Review of Microbiology*, 63, 411-429.

Winterholler B, Hoppe P, Foley S, Andreae MO (2008) Sulfur isotope ratio measurements of individual sulphate particles by NanoSIMS. *International Journal of Mass Spectrometry* 272, 63-77.

## Annex

### Deliverable Check list

To be completed by Deliverable leader

	Check list	√	Comments
BEFORE	I have checked the due date and have planned completion in due time	√	Please inform project management team of any foreseen delays
	The title corresponds to the title in the DoW (Description of Work)	√	<i>One additional author has also contributed (Eric Allan)</i>
	The contents corresponds to the description in the DoW (Description of Work)	√	
	The dissemination level corresponds to that indicated in the DoW (Description of Work)	√	
	The contributors (authors) correspond to those indicated in the DoW (Description of Work)		
	The Table of Contents (ToC) has been validated with the WP Leader	√	Please validate the ToC with the WP leader before drafting the deliverable
	I am using the ExpeER deliverable template (title page, styles etc)	√	Can be found in the intranet
AFTER	The deliverable has been reviewed internally in my organization	√	Please ask colleagues to review the deliverable for its scientific content
	The deliverable has been reviewed by all contributors (authors)	√	Make sure all contributors have reviewed and approved the final version of the deliverable. You should leave sufficient time for this validation.
	I have done a spell check and had the English verified	√	Ask a colleague with a good level of English to review the language of the text and do a spell-check too.
	I have sent the final version to the WP Leader for approval	√	Please send the final validated draft to the Coordinator (project management team) & ExC for validation before the submission to the EC.