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Nano-scale secondary ion mass spectrometry – a new analytical tool in biogeochemistry and soil ecology

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Abstract

Soils are structurally heterogeneous across a wide range of spatio-temporal scales. Consequently, external environmental conditions do not have a uniform effect throughout the soil, resulting in a large diversity of micro-habitats. It has been suggested that soil function can be studied without explicit consideration of such fine detail, but recent research has indicated that the micro-scale distribution of organisms may be of importance for a mechanistic understanding of many soil functions. Due to a lack of techniques with adequate sensitivity for data collection at appropriate scales, the question 'How important are various soil processes acting at different scales for ecological function?' is challenging to answer. The nano-scale secondary ion mass spectrometer (NanoSIMS) represents the latest generation of ion microprobes which link high-resolution microscopy with isotopic analysis. The main advantage of NanoSIMS over other secondary ion mass spectrometers is the ability to operate at high mass resolution, whilst maintaining both excellent signal transmission and spatial resolution (~50 nm). NanoSIMS has been used previously in studies focusing on presolar materials from meteorites, in material science, biology, geology and mineralogy. Recently, the potential of NanoSIMS as a new tool in the study of biophysical interfaces in soils has been demonstrated. This paper describes the principles of NanoSIMS and discusses the potential of this tool to contribute to the field of biogeochemistry and soil ecology. Practical considerations (sample size and preparation, simultaneous collection of isotopes, mass resolution, isobaric interference and quantification of the isotopes of interest) are discussed. Adequate sample preparation avoiding biases in the interpretation of NanoSIMS data due to artefacts and identification of regions-of interest are of most concerns in using NanoSIMS as a new tool in biogeochemistry and soil ecology. Finally, we review the areas of research

most likely to benefit from the high resolving power attainable with this new approach.

1. Introduction

Soils are highly complex porous media that are structurally heterogeneous across a wide range of spatio-temporal scales (Tisdall and Oades, 1982; Young and Ritz, 1998). Their organisation at the micro-scale results in a range of micro-habitats that exert differential selection pressures on microbial communities, both governing and sustaining the huge microbial diversity in soil (Ranjard et al., 2000b; Treves et al., 2003; Mummey and Stahl, 2004; Long and Or, 2005; Nunan et al., 2006). Micro-organisms mediate a vast range of reactions in soil, and fine-scale interactions between micro-organisms and the physical, chemical and other biotic components of the soil environment control or modulate these reactions (Sierra et al., 1995; Strong et al., 1997; Chenu et al., 2001; Ranjard et al., 2000a; Young and Crawford, 2004). Understanding of these relationships is complicated by the fact that interactions among the various components of the soil system are often scale-dependent (Ettema and Wardle, 2002), meaning that factors that greatly influence soil micro-organisms and soil function at a given scale may be of lesser importance at other scales. Soil biologists are therefore confronted with the issue of how to deal both conceptually and experimentally with such a high degree of diversity and array of interactions.

There are cogent arguments that suggest reductionist approaches that explicitly accommodate the inherent complexity of soils are not necessary to understand the controlling factors of many soil functions, nor to predict their magnitude and behaviour. So-called 'averaging engine' approaches have been successful, showing that it is possible to model and understand overall function without resorting to fine

84 detail; an analogy is the gas box where the pressure a gas exerts can be accurately
85 predicted without knowledge of the trajectory of every atom (Andrén et al., 1999).
86 Likewise, gross process rates arising from community-level activity in soil can be
87 predicted (Hart et al., 1994; Bengtsson et al., 2003; Herrmann et al., 2004). However,
88 more sophisticated predictions, for example where a number of environmental, soil
89 physical, chemical and biotic factors change simultaneously are considerably less
90 reliable. The crucial difference between the constituents in the soil biota and a gas is
91 that the component parts in soil are *individually adaptive* (over time-scales ranging
92 from instantaneous to evolutionary), and the interactions between them are likely to
93 be complex rather than just following 'simple' physical laws such as Brownian
94 motion. Interactions among constituents may therefore have important consequences
95 for function at larger scales that cannot be inferred from a mere inventory of the
96 constituents and integration of their individual properties. Large scale properties
97 relevant to soil function at field, catchment or regional scale may arise from
98 interactions among individual parts, a phenomenon termed emergent behaviour. For
99 example, a process such as horizontal gene transfer (van Elsas and Bailey, 2002;
100 Sørensen et al., 2005) cannot be easily explained by gross process-level phenomena
101 and there are examples in the literature where averaging approaches do not perform
102 well (e.g. ammonium oxidation; Darrah et al., 1987). In other words, the origin,
103 evolution, maintenance and control of function in soils as well as their capacity to
104 adapt is likely to depend upon mechanisms and interactions that *fundamentally occur*
105 at size scales of the range from molecular to microbial (Crawford et al., 2005).

106 An important challenge for soil research is to establish both (i) how the hierarchy of
107 processes and mechanisms that occur contribute to ecosystem function and (ii) the
108 scales at which these operate. The question 'How important are the various processes

109 acting at different scales for ecological function in soils?' cannot be answered in most
110 cases with any degree of certainty. A major obstacle to progress is the lack of
111 techniques with adequate sensitivity for data collection at appropriate (i.e. microbial)
112 scales. For example, most biochemical-based techniques for studying nutrient cycling
113 and micro-organism:plant nutrient transfers are applied at scales several orders-of-
114 magnitude greater (i.e. cm and mm, grams of soil) than at the cellular scale at which
115 the processes actually occur (Figure 1). For example, the average concentration of a
116 heavy metal in a 100-g soil sample may bear little relation to the concentrations of the
117 metal that micro-organisms may experience at the micro-scale, which could range
118 from effectively zero in some micro-sites, to very high in the proximity of metal
119 particles.

120 Soils predominantly function by virtue of their spatial organisation. This has been,
121 and often still is, ignored in their study, where experimental approaches seek to
122 homogenise the 'inconvenience' of heterogeneity. But this is a wilful avoidance of a
123 crucial feature, which was eloquently articulated some seven decades ago by Kubiena
124 (1938), who stated '*Take, for instance, a city. If it were put in a large glass vessel with*
125 *water or hydrochloric acid, as we do with the soil, and shaken for twenty-four hours,*
126 *one would not then be able to reconstruct streets or buildings, or to find out what kind*
127 *of goods are found in the large warehouse. The first thing to know, in order to get an*
128 *idea of the city, is not much the nature of its chemical composition as a whole, but*
129 *how it looks in detail as a structural entity*.' Other authors have since reiterated this
130 rather obvious point (e.g. Harris, 1994; Wardle and Giller, 1996; Young and Ritz,
131 2005). But whilst soils function by virtue of their architecture, across scales from
132 nano- to mega-metres, study at the smallest scales is hampered by available
133 technology and methodology. Following Kubiena's pioneering work on soil micro-

morphology and that of soil ultra-structure using electron microscopy by Foster in the 1970's and 1980's (Foster and Rovira, 1973; Foster and Martin, 1981; Foster et al., 1983), there have been continued technological and methodological advances involving optical microscopy (e.g. Nunan et al., 2001), scanning (e.g. Chenu and Tessier, 1995) and transmission electron microscopy (e.g. Kilbertus, 1980; Chenu and Plante, 2006), X-ray tomography (e.g. De Gryze et al., 2006; Feeney et al., 2006; Nunan et al., 2006), and spatial statistics and modelling (e.g. Young et al., 2001; Grundmann et al., 2001; Wu et al., 2004).

A new generation of ion microprobes, nano-scale secondary ion mass spectrometers (NanoSIMS) is emerging, which allows precise, spatially-explicit, elemental and isotopic analysis at the nm scale. These instruments have been applied to studies of presolar materials from meteorites (For reviews, see Hoppe et al., 2004; Hoppe, 2006), in material science (e.g. Kailas et al., 2006), geology and mineralogy (e.g. Stern et al., 2005) as well as biology (For reviews, see Guerquin-Kern et al., 2005; Grovenor et al., 2006), and offer many exciting opportunities for potential application within the field of biogeochemistry and soil ecology. This paper describes the principles of such an instrument, provides an overview of NanoSIMS applications, and reviews the challenges and further opportunities for the application of NanoSIMS as an analytical tool to increase resolution and understanding of microbial processes in soil.

2. Principles of NanoSIMS

Secondary ion mass spectrometry (SIMS) is an ion microprobe technology linking high resolution microscopy with isotopic analysis, providing spatially resolved information on the molecular and isotopic compositions of materials (Pacholski and

158 Winograd, 1996). The basis for the technique was introduced in the 1960's by
159 Castaing and Slodzian (1962), and two types of SIMS are available, defined as static
160 and dynamic. Static SIMS is typically used to attain molecular and fine surface
161 information (less than 1 nm depth) whereas dynamic SIMS is routinely used to
162 acquire elemental and isotopic information from the upper few nm of the sample (for
163 further details see Pacholski and Winograd, 1999; Adams et al., 2005). The Cameca
164 NanoSIMS50 ® (Slodzian et al., 1992) currently represents the latest generation of ion
165 microprobes designed for dynamic SIMS and its advantages over other SIMS
166 instruments are given in Table 1. The prototype instrument was installed at Harvard
167 Medical School and Brigham and Women's Hospital (Boston, USA) in early February
168 1999. By mid-2006 another 14 instruments have subsequently been installed around
169 the world. An overview of the development of SIMS instruments is given in
170 Guerquin-Kern et al. (2005).

171 NanoSIMS is a destructive process that involves continuous bombardment of a
172 sample with an energetic ion beam (either a Cs^+ or O^- primary beam to enhance
173 negative or positive ion formation, respectively), which results in sputtering of the
174 upper sample surface and the consequent liberation of secondary ions (Figure 2).
175 These secondary ions are sorted on the basis of their energy in the instrument's
176 electrostatic sector before being dispersed in a mass spectrometer according to their
177 mass-to-charge ratios. By acquiring a series of spatially-referenced spectra, via a
178 raster-scanning process, a map can be produced for nearly any selected atomic mass,
179 and information of isotopic ratios in the form of regions-of-interest, line scans and
180 depth profiling can be obtained. The system is maintained permanently under ultra-
181 high vacuum to prevent atmospheric interference with primary and secondary ions
182 (typically 10^{-10} Torr in the analysis chamber).

3. Applications of NanoSIMS

3.1. Previous NanoSIMS applications

To date, NanoSIMS has been principally applied to the study of presolar material from meteorites, using trace element analysis and natural isotopic abundances (e.g. C, N, O, Mg/Al, Si and S), in order to determine the physical and chemical conditions of processes in the early solar system (e.g. Messenger et al., 2004; Floss et al., 2004; Hoppe et al., 2004; Bradley et al., 2005; Floss et al., 2006). NanoSIMS has also been used with some success to study the surface morphology and composition of thin film polymer systems (Kailas et al., 2005; Kailas et al., 2006) and in studies in biology (Guerquin-Kern et al., 2005; Grovenor et al., 2006). Specifically in biology, NanoSIMS has been used to detect both natural and isotopically-enriched elemental and isotopic variations in coral (Meibom et al., 2004; Sano et al., 2005; Clode et al., 2007) and hair melanin (Hallegot et al., 2004) and to study sub-cellular uptake of an ^{125}I-labelled drug by cancer cells (Guerquin-Kern et al., 2004). NanoSIMS has also provided information on C and N metabolism in cultured cells using ^{13}C and ^{15}N as isotopic tracers (Peteranderl and Lechene, 2004; Kleinfeld et al., 2004). More recently it has been used to study the chemical composition of lipid membranes (Kraft et al., 2006). Earth scientists have also successfully utilised the technique to study lead geochronology in minerals such as xenotime, zirconlite and uraninite (Stern et al., 2005), isotope exchange between feldspar and aqueous chloride solution (Labotka et al., 2004) and trace element distribution in peridotites (Hellebrand et al., 2005).

204 *3.2. Proof-of-concept: Application of NanoSIMS in soil*

205 As soil is a medium where geological and biological materials are combined
206 intimately, NanoSIMS potentially offers a range of advantages for biogeochemistry
207 and soil ecology (Table 1). Pioneering work in the application of SIMS to soils (Cliff
208 et al. 2002a) showed that it was possible to qualitatively describe the assimilation of
209 added ^{15}N and ^{13}C into soil micro-organisms *in situ*, using time-of-flight secondary
210 ion mass spectrometry (TOF-SIMS). Their results suggest that SIMS shows promise
211 as a tool for studying soil micro-habitat heterogeneity and microbial activity in
212 combination. While the advantages of TOF-SIMS include the ability to acquire
213 molecular and true isotopic surface information, these data cannot be acquired under
214 conditions suitable for obtaining both high mass (i.e. peak separation of elements with
215 similar masses) and high spatial resolution with adequate signal transmission. Any
216 attempt at increasing mass resolution to ensure separation of isobars or mass
217 interferences will result in a loss of spatial resolution and signal transmission.
218 Conversely, conditions designed to allow for increased signal transmission or
219 improved spatial resolution will result in a decline in the operating mass resolution of
220 the instrument. For example, Cliff et al. (2002a) used very high beam currents (600
221 pA) in order to obtain sufficient mass resolution and signal, which meant they could
222 not achieve a high level of spatial resolution (< 200 nm). The main advantage of
223 NanoSIMS over TOF-SIMS is the ability of NanoSIMS to operate at high mass
224 resolution, whilst maintaining both excellent signal transmission (i.e. increased
225 sensitivity) and high spatial resolution (Table 1).

226 A recent study by Herrmann et al. (2007) showed that NanoSIMS can be used to
227 detect isotopically enriched bacterial cells in the soil matrix. This was achieved by
228 adding ^{15}N enriched *Pseudomonas fluorescens* grown in a mineral salt medium

containing ^{15}N-ammonium sulphate to a coarse textured sand soil. The soil cores were embedded in Araldite resin and sectioned for NanoSIMS analysis. To allow the study of biophysical interactions in soils at relevant scales, ion distribution images of $^{28}Si^-$, $^{12}C^{14}N^-$ and the $^{15/14}N$ ratio data were superimposed using image processing software and mosaics of ion images were made. The mapping procedure, utilising secondary ion images of $^{12}C^-$, $^{28}Si^-$, $^{12}C^{14}N^-$ and $^{15/14}N$ ratios revealed the location of ^{15}N-labelled *P. fluorescens* in coarse textured sand (Figure 3; full details of the methods can be found in Herrmann et al., 2007). The resin distribution was revealed by the $^{12}C^-$ ion image (Fig. 3a) as the resin was inevitably carbon-based, while the $^{28}Si^-$ ion image provided information on the soil matrix (Fig. 3b). Nitrogen-rich organic matter was also clearly visible in the $^{12}C^{14}N^-$ ion image (Fig. 3c), and the distribution and level of ^{15}N enriched *P. fluorescens* were revealed in the $^{15/14}N$ ratio image (Fig. 3d). When secondary ion images of $^{28}Si^-$, $^{12}C^{14}N^-$ and those of the $^{15/14}N$ ratio data were superimposed (Figure 4) the potential of the technique in enabling small-scale study of bacteria in soil and their biophysical interactions is apparent (Herrmann et al., 2007).

4. Practical considerations in the use of NanoSIMS for soil studies

Despite recent technological progress, there are several practical issues to be considered if NanoSIMS is to be used as a component method in a study of biogeochemistry or soil ecology. Key issues include sample size and preparation, simultaneous collection of isotopes, mass resolution, isobaric interference and quantification of the isotopes of interest.

4.1. Sample size and preparation

Samples presented for analysis by NanoSIMS must be dry, stable, conductive and tolerant of ultra-high vacuum (10^{-10} Torr). In addition, soil samples should ideally be flat and highly polished with no more than nm-level variations in surface topology as charging effects (i.e. obscuring the boundaries between mineral and organic particles) are likely to occur when analysing soil particles without specific sample preparation (Figure 5a). Gold coating in combination with the use of the electron flood gun can lessen such charging effects (Figure 5b). In this example, whilst regions of higher C enrichment are evident, the nature of this material (minerals, soil organic matter or micro-organisms) cannot be identified due to charging effects. As such, it appears critical to produce embedded soil sections that can be polished and made conductive. Usually sample preparation involves stabilisation of biological components (fixation), removal of water (dehydration) and resin-embedding of soil. These requirements therefore prohibit the study of material in any aqueous phase and hence restrict application of imaging ion mass spectrometry outwith dynamic *in vivo* studies, as preparation of samples for analysis is necessarily destructive. However, resin-based techniques for preparing undisturbed soil samples are well characterised and proven, and have been routinely used to study the small-scale distribution of micro-organisms in soils (Postma and Altemüller, 1990; Tippkötter and Ritz, 1996; Fisk et al., 1999; Nunan et al., 2003; Harris et al., 2003; Bruneau et al., 2005).

Fixation and dehydration of biological tissues is typically carried out either by chemical means (fixation followed by dehydration with acetone; Tippkötter and Ritz, 1996; Nunan et al., 2001) or low temperature methods (rapid freezing followed by freeze drying or substitution; Chandra et al., 1992; Echlin, 1992). Chemical fixation was shown to be a suitable method for studying ^{15}N accumulation in *P. fluorescens*

276 mixed into a coarse textured sand (Herrmann et al., 2007). However only 35% of
277 photosynthetically fixed ^{13}C was retained as protein in symbiotic algae, following
278 chemical fixation in a glutaraldehyde:paraformaldehyde mixture (Clode and Marshall,
279 unpublished data). In studies where significant migration of the element(s) of interest
280 is likely to occur during sample preparation, low temperature methods such as freeze-
281 drying offer a more promising solution. This method has been reliably used to study
282 ^{13}C and ^{15}N metabolism in cultured cells using NanoSIMS (Peteranderl and Lechene,
283 2004). There are, however, several limitations to cryo-techniques, particularly in
284 relation to soils. Of most concern is the satisfactory freezing of biological material
285 within bulk soil samples. Adequate quality of freezing only extends to depths
286 typically in the order of µm, beyond this, damage induced by ice crystals is severe
287 (Echlin, 1992). Thus, sufficient preservation of soil samples and their associated
288 micro-organisms is unlikely to be routinely achievable using cryo-techniques.

289 To date, the epoxy resin Araldite 502 has proven to be the most suitable resin-
290 embedding medium among three different resin brands trialled (Herrmann et al.,
291 2007), as it gave the most rapid outgassing (i.e. trapped and adsorbed gas in the
292 samples has to be released, to enable pumping to the high vacuum required for
293 NanoSIMS analysis). This resin contains carbon with ^{13}C at natural abundance
294 (ProSciTech, Australia), therefore $^{13/12}C$ ratios may not be indicative of true ratios of
295 ^{13}C enriched material in the sample. However, the ratio provides a semi-quantitative
296 indication of the level of enrichment above natural levels, and accounts for any
297 variation in ion yield due to topographical and matrix effects. Furthermore, ^{12}C
298 distribution can also be used to visualise resin distribution (Herrmann et al., 2007). An
299 alternative could be the use of elemental sulphur as an embedding medium. However,
300 only very small samples can be prepared and analysed by this means. Lehmann et al.

(2005) restricted study of biomass-derived black C particles to those with a diameter of 5-80 μm, as the optimum consistency of the sulphur for embedding lasts for only 10-30 seconds.

In the ultra-high vacuum environment of the NanoSIMS, Herrmann et al. (2007) found that samples must be < 4 mm thick in order to avoid outgassing issues (see above). Furthermore, the most suitable NanoSIMS sample mounts for soil analysis appear to be the 10 mm diameter mounts as up to eight samples can be placed into the analysis chamber at any one time. A larger (25 mm diameter) mount could also be used, but very thin samples are needed to avoid outgassing issues and it must be borne in mind that only one sample can be placed into the analysis chamber at any one time. The most appropriate sample preparation method will always be dependent upon the sample size and type, the level of retention and migration of the element(s) of interest during sample preparation together with the specific question to be addressed by the NanoSIMS analysis.

4.2. Simultaneous collection of isotopes

The NanoSIMS is able to detect up to five ion species at one time (Table 1), allowing simultaneous measurement of two to five isotopes from the same micro-volume of sputtered material. This is particularly important in samples that are susceptible to damage from the primary ion beam, where low concentrations of ions may be rapidly destroyed in a small volume of material. As mentioned above, negative secondary ions are sputtered using a Cs^+ primary ion beam (lateral resolution = of 50 nm), and positive secondary ions are sputtered using an O^- primary ion beam (lateral resolution = 150 nm). Nitrogen ions, as well as elements in Group VIII of the Periodic Table, do not ionise easily and therefore do not produce enough secondary ions to be detected.

However, ejected N ions combine with C ions to form cyanide ions (CN^-), which can be readily detected. These CN^- ions have extremely high electron affinity (3.9 eV; Bradforth et al., 1993), thus the yield of secondary CN^- is particularly high.

Simultaneous analysis of ion species is, however, limited. The physical separation of the detectors is limited by the radius of secondary ion trajectories (R) (Figure 2), which is dependent on the magnetic field. Up to mass 30, one mass interval between the detectors can be analysed simultaneously, i.e. it is possible to analyse $^{12}C^-$ and $^{13}C^-$ or $^{16}O^-$, $^{17}O^-$, $^{18}O^-$ or $^{26}CN^-$, $^{27}CN^-$ or $^{28}Si^-$, $^{29}Si^-$, $^{30}Si^-$ isotopes simultaneously. Above mass 30 it is not possible to analyse one mass intervals between the detectors; for example, $^{31}P^-$ and $^{32}S^-$ cannot be analysed simultaneously. In addition, the radius of secondary ion trajectories (R) is only a window in the mass range, and the size of the window is dependent on the magnetic field. For example, when the magnetic field is set to look at mass ^1H on Detector 1 then the maximum mass to be simultaneously analysed on Detector 5 is mass 11; therefore it is not possible to look at H and C simultaneously.

4.3. Mass resolution and isobaric interference

The main advantage of NanoSIMS over other SIMS ion microprobes is the ability to operate at high mass resolution, whilst maintaining both excellent signal transmission and high spatial resolution. Analysis conditions have to be optimised to obtain satisfactory separation of isobars (i.e. other isotopes and molecular complexes with the similar mass) that may interfere with the ion species of interest. For example, C isotope measurements require a mass resolving power of ~3000 to separate the $^{13}C^-$ peak from the overlapping $^{12}C^1H^-$ peak. Similarly, a mass resolving power of ~7200 is necessary to separate $^{13}C_2^-$ from $^{12}C^{14}N^-$ on mass 26 (Clode et al., 2007). This high

mass resolution is achieved through the use of slits at the entrance to the mass spectrometer. The geometry of the NanoSIMS, however, minimises the loss of signal at the slits, thus maintaining high transmission, and therefore sensitivity. In addition, Cliff et al. (2002) reported isobaric interference of $^{27}Al^-$ with $^{13}C^{14}N^-$ and $^{12}C^{15}N^-$ when analysing soil using a Ga^+ primary ion probe with TOF-SIMS. Such interferences are not an issue in NanoSIMS analysis as $^{27}Al^-$ ions do not ionise very easily in the negative polarity (i.e. using a Cs^+ primary ion beam), thus the yield of secondary Al^- is very low and interferences with CN^- ions are negligible.

4.4. Quantitative analysis of isotopes

Quantitative SIMS analysis is difficult because although the secondary ion intensity of a particular element is proportional to the concentration of the element in the sample the proportionality factors are not readily obtained (Morrison et al., 1994). The latter include the practical ion yield and the total sputtering yield. These vary with variation in the matrix of the sample. Matrix effects in resin-embedded tissue (Brenna and Morrison, 1986) and freeze-dried cells (Chandra et al., 1987) appear to be small or negligible. This means that relative ion intensities from compartments in the same sample can be obtained by normalising to an ion such as ^{12}C that is representative of the total mass of the analysed compartment. Matrix effects, however, have not been checked to determine the inhomogeneity that can now be resolved at the μm scale using NanoSIMS. The most promising approach is based on the use of matching standards in which the analyte of interest is dispersed in a matrix mimicking the composition of the sample matrix. However, when working with soils containing a diverse mixture of micro-organisms within a heterogeneous soil matrix that is embedded in resin or sulphur, the preparation of representative standards becomes challenging. Nevertheless, isotopic ratios can be readily obtained, providing a semi-

quantitative analysis of the isotopes of interest, independent of matrix effects and variations in topography etc. From this, levels of isotopic enrichment in comparison to natural terrestrial values can be accurately measured and statistically analysed.

4.5. General practical considerations

The effective working field of view of the NanoSIMS instrument is necessarily restricted (usually 30-50 μm field of view). For example, in the study by Herrmann et al. (2007), the maximum workable field of view per ion image was approximately 30 x 30 μm^2 since beyond this there was notable distortion at the edges. A challenge arising from this constraint is that methods have to be devised for establishing the precise location to which to apply NanoSIMS in probing the sample. This can be achieved using microscopic visualisation at increasing resolution, but only if features being visualised by such microscopy are pertinent to locating regions-of-interest for NanoSIMS probing. This is particularly challenging at the very small spatial scales involved with nano-scale locations. The NanoSIMS has an optical microscope connected to a CCD camera, and a secondary electron detector (only available with Cs^+ primary beam) which assist in navigation (Table 1). Existing methods such as digital image analysis, transmission and scanning electron microscopy have been used to characterise samples in more detail and to identify potentially suitable areas for NanoSIMS analysis (Figure 6 and Herrmann et al., 2007). The cost and limitations of analysis of samples by NanoSIMS mean that the targeting of samples for NanoSIMS analysis needs to be carried out with great care across a range of scales e.g. the selection of sample sites and experimental treatments as well as identification of the most appropriate field of view. Thus, it is clear that the value of NanoSIMS is as a component of larger-scale integrated studies where a range of methods are combined (Guerquin-Kern et al., 2005).

There is however a severe constraint to the realisation of such goals, that is essentially scale-related. Location and visualisation of cells where the majority of such cells are duly labelled is relatively straightforward – hence the success of *in situ* mapping of bacteria and fungi using universal stains (Nunan et al., 2001; 2003, Harris et al., 2003), and the proof-of-concept study by Herrmann et al. (2007) where all bacteria were guaranteed to be labelled with ^{15}N. However, where specific labels are used, by definition only a subset of the total population will be labelled (and therefore potentially visualisable) there is soon an issue of locating cells within the areas defined by microscopic fields of view. For example, consider if 1% of the soil bacterial community were labelled, which would be an upper bound for even a relatively common property associated with soil micro-organisms such as nitrification. The frequency of occurrence of labelled cells, even if the property were evenly distributed throughout the community, would then be such that a very large number of fields of view would not contain a single instance of labelled cells. If the organisms were spatially aggregated, the problem would be exacerbated. These issues are related to the proportion of cells likely to be labelled, and hence the rarity of the prescribed organismal group or function. Techniques will therefore need to be developed to allow rapid screening of samples to determine their likelihood of containing target material.

5. Potential applications of NanoSIMS within the field of biogeochemistry and soil ecology

In the previous sections, we have highlighted the potential of NanoSIMS but also the challenges of the application of this method. The sample preparation methods prior to NanoSIMS analysis (described above), mean that the study of soluble soil

components not stabilised by fixation is not possible. Consequently, the technique is likely to be most suited to studying assimilatory rather than dissimilatory processes, the functional consequences of the spatial organisation of microbial activity and how these are affected by interactions with the local physical habitat (aggregate structure, mineralogical associations), with other micro-organisms (horizontal gene transfer, food web relations, inter-hyphal interactions) or environmental factors such as moisture content and temperature. In the following sections we discuss the current state-of-the-art in some of these areas and identify the areas in which integrated experiments including NanoSIMS analysis might be of significant benefit.

5.1. Biogeochemistry

5.1.1 Phosphatic fertiliser and organic amendments

The fixation of phosphatic fertiliser at soil mineral surfaces has long been known as a phenomenon, but the identification and spatial location of such fixation sites remains elusive. The role of soil organic matter and microbial activity in these processes is also recognised and has increasingly been elucidated. A variety of mechanisms has been proposed whereby increased soil organic matter and/or microbial activity reduces sorption of added P (Ayaga et al., 2005; Guppy et al., 2005). Use of organic amendments may reduce P sorption or simply increase P inputs (Iyamuremye and Dick, 1996; Haynes and Mokolobate, 2001). However, the precise mechanisms and reactions at soil surfaces and their controls are not well understood. NanoSIMS may offer an opportunity to visualise the soil surface:P interactions in new ways and together with radio-isotopic studies of P dynamics in soil may allow the controls over sorption reactions to be determined.

446 5.1.2. Stabilisation of soil organic matter

447 The mechanisms by which organic matter is stabilised in soils are still poorly
448 understood, and it is notable that some postulated mechanisms are currently only
449 weakly supported by data (von Lützow et al., 2006). Recently, Kleber et al. (2007)
450 presented a new conceptual model of the multi-layered structure of organo-mineral
451 associations in soils suggesting that organic matter sorbs to mineral surfaces in a
452 discrete zonal sequence (contact, hydrophobic and kinetic zones). This new model
453 sharply contrasts with the existing paradigm of organo-mineral interactions
454 (Stevenson et al., 1985) which were visualised as associations of large,
455 multifunctional polymers with mineral surfaces via a broad range of bonding
456 mechanisms (Stevenson, 1985; Leinweber and Schulten, 1998). The new conceptual
457 model (Kleber et al., 2007) has been derived from blending an earlier concept of
458 Wershaw (1993) with recent published evidence from empirical studies of organo-
459 mineral interfaces. There is certainly a need to experimentally validate this model.
460 NanoSIMS with its ability to simultaneously detect up to five ion species with high
461 sensitivity from the same micro-volume should allow the study of soil organic matter
462 stabilisation mechanisms (i.e. organic matter interactions with the soil matrix) as
463 never before.

464 Physically uncomplexed organic matter (isolated on the basis of particle size or by
465 density fractionation techniques) has an important role in soil nutrient supply and
466 structure formation. Natural abundance studies of fractionated organic matter,
467 following the differential fractionation of ^{13}C by C4 and C3 plants, have revealed
468 much about the kinetics and turnover of physically uncomplexed organic matter in
469 soil; results that are important in the management of C sequestration (Gregorich et al.,
470 2006). However, the range of physical fractionation methods commonly used to

measure the pools of physically uncomplexed organic matter do not allow the importance of the spatial arrangement of micro-organisms, soil organic matter and primary particles to be studied since they are necessarily destructive of soil structure. The potential of synchrotron-based X-ray computed tomography, near-edge X-ray absorption fine structure (NEXAFS) spectroscopy, scanning transmission X-ray microscopy (STXM), Fourier-transform infrared spectroscopy-attenuated total reflectance (FTIR-ATR) and X-ray micro-fluorescence have all been used to map the physical and chemical make-up of soil at the micro-scale (Lehmann et al., 2005; Solomon et al., 2005; Nunan et al., 2006; van Oort et al., 2006). Such approaches have the potential to shed light on the functional significance of interactions among the various components of soil. When coupled with the targeted application of NanoSIMS, this is likely to lead to increased understanding of the importance of physical location and biophysical interactions as a key constraint in the turnover of organic matter in soil. More proof-of-concept work is needed with NanoSIMS to establish whether natural isotopic fractionation, such as occurs during the contrasting routes of photosynthesis in C3 and C4 plants, can be detected. Nonetheless NanoSIMS offers opportunities to add value to studies, for example such as Devevre and Howarth (2001) by allowing focussed study of organo-mineral associations and uncomplexed organic matter within the soil matrix following the use of isotopically-enriched tracers in fertilisers or plant materials.

5.1.3. Spatial distribution of gross N assimilation processes within the soil matrix

Kirkham and Bartholomew (1954; 1955) first formulated differential equations to estimate gross N processes in soils that form the basic concepts of the ^{15}N isotope dilution technique. Dissimilatory processes such as gross N mineralisation and nitrification processes are estimated by enriching the product pool with ^{15}N and

measuring the changes of the product pool size and dilution of ^{15}N in this pool over time. The ^{15}N isotope dilution technique has been widely applied to the study of N (Murphy et al., 2003; Booth et al., 2005) and has revealed complex interacting processes at the heart of the soil N cycle (e.g. Schimel et al. 1989; Davidson et al., 1992; Hart et al., 1994; Cookson et al., 2006). Gross N assimilation processes, usually termed gross N immobilisation, by the microbial biomass in soil is a critical process in the regulation of the soil internal N cycle (Murphy et al., 2003). However, gross N immobilisation rates in soils are difficult to estimate at a meso-scale and studies are fraught with difficulty. Gross N immobilisation rates are estimated indirectly by measuring ^{15}N tracers into the microbial biomass using the fumigation-extraction method (e.g. Ledgard et al., 1998; Hatch et al. 2000) or by determination of residual ^{15}N in soils after KCl extractions in combination with numerical modelling of N processes (e.g. Mary et al., 1998; Recous et al., 1999; Andersen and Jensen, 2001). The ^{15}N isotope dilution approach indicates the importance of gross N immobilisation process, but gives relatively little insight into the major controlling factors at micro-scale as both approaches treat the microbial biomass as a black box. In addition, there are several assumptions inherent in ^{15}N isotope dilution technique (Murphy et al., 2003) and violation of the assumption of equilibrium and identical behaviour of added and native N has been reported to significantly impact estimates of gross N transformation rates (Monaghan, 1995; Watson, et al. 2000; Cliff et al., 2002; Luxhøi et al., 2004; Herrmann et al., 2005).

Spatial distribution of gross N immobilisation processes could potentially be quantified by superimposing maps derived from digital image analysis of soil thin sections (i.e. distribution of both active and non-active micro-organisms; see below) and NanoSIMS images examining the spatial distribution of ^{15}N immobilising micro-

organisms (i.e. active ^{15}N immobilising micro-organisms). Because soil thin sections are often prepared on glass slides which do not allow NanoSIMS analysis due to mounting issues (Section 4.1.), there is still a need to employ a method to couple digital imaging of biological soil thin sections with NanoSIMS image analysis. However, given the high degree of spatial resolution of NanoSIMS, this method may have the potential to quantify the spatial distribution of gross N immobilisation and may give new insights of the major controlling factors of this process (e.g. environmental factors such as moisture content and temperature) at the micro-scale as well as validating the assumption of equilibrium and identical behaviour of added and native N.

5.2. Soil ecology

5.2.1. Association of micro-organisms with particular minerals within the soil matrix

Work by Gleeson et al. (2005; 2006) has shown particular relationships between micro-organisms and minerals during weathering of exposed rock surfaces. Bacterial and fungal community structure was driven by the chemical composition of the mineral *in situ*. Biological breakdown of minerals has been shown to be an important process during micro-scale weathering in aquatic and soil environments (Brehm et al., 2005). Scanning transmission X-ray microscopy and spectromicroscopy has been used at the sub 40-nm scale to study bio-weathering products following microbial interaction with a Fe-Mg-orthopyroxene (Benzerara et al., 2005). It has also been postulated that low pH and bacterial rich environments within the guts of worms promote biological weathering; new weathering products were detected by X-ray diffraction and Fourier transform infrared spectroscopy after a mineral mud was ingested and excreted by worms (Needham et al., 2004; Needham et al., 2006).

545 However, these techniques have a limited elemental range. The capability of
546 NanoSIMS to measure light elements, particularly C and N and their isotopes, should
547 allow increased understanding of the microbial:mineral interactions at rock surfaces
548 and within soils.

549 5.2.2. Spatial distribution of active micro-organisms at the micro-scale

550 Determining the spatial location of particular micro-organisms within the soil matrix,
551 and especially their actual or potential functional capabilities, is a desirable goal in
552 soil ecology. There are many hypothesised reasons why the precise location of cells is
553 pertinent to soil function. For example, Grundmann and Normand (2000) found that
554 the genetic distances of the genus *Nitrobacter* at a local scale (< 3 cm) were as large
555 as those among reference strains from a range of geographical areas, suggesting that
556 the biological and physical processes regulating diversity occur at much finer scales.
557 Others have suggested that the activity of microbial cells can be affected by the
558 proximity of other active cells (Darrah et al., 1987; Strong et al., 1997), that the
559 response of microbial communities to external stresses is modulated by the micro-
560 scale location (Ranjard et al., 2000a) and that the spatial spread of cells has an impact
561 on overall activity (Pallud et al., 2004).

562 Two methodological approaches have been developed for the quantification of spatial
563 patterns of micro-organisms at the micro-scale and their impact on microbial function.
564 The methods have inherent weaknesses most of which may be overcome with
565 NanoSIMS. The first method is a micro-sampling technique of specific active
566 microbial groups and it is based on the relation between sample size and the
567 frequency of occurrence of a process (Grundmann et al., 2001; Dechesne et al., 2003).
568 The advantage of this approach is that the three-dimensional spatial distribution of
569 bacterial activity and their functional significance can be studied but it is not possible

570 to quantify the spatial relationship between micro-organisms and soil structure. The
571 second is the use of universal fluorescent staining of soil bacteria combined with
572 preparation of biological soil thin sections to examine *in situ* spatial distribution of
573 micro-organisms at the micro-scale (White et al., 1994; Fisk et al., 1999; Nunan et al.
574 2001; Li et al., 2004). Digital image analysis of soil thin sections allows the
575 relationship between micro-organisms and the microbial habitat to be quantified but
576 does not distinguish between active and non-active micro-organisms and patterns are
577 measured in two dimensions. Consequently, the functional significance of a given
578 distribution is difficult to ascertain, specific functions cannot be attributed to bacteria
579 and a degree of extrapolation is necessary in order to account for three dimensions.

580 A comprehensive range of nucleic-acid based probes that enable the specific labelling
581 of organisms on a taxonomic or functional basis are now available (For reviews, see
582 van Elsas et al., 1998; Torsvik and Øvreås, 2002). These can be used to label
583 individual cells, and with appropriate epitopes attached, used to visualise the location
584 of such probes and the associated organisms. Fluorescently-labelled probes have wide
585 application in visualising cells using epi-fluorescence and confocal microscopy and
586 have been applied in environmental contexts, predominantly where cell
587 concentrations are relatively high and background matrices not overtly complex, such
588 as in biofilms (Neu et al., 2004) or rhizoplanes (Mogge et al., 2000; Eller et al., 2001).
589 The complex nature of soil matrices, resulting in non-specific binding of probes to
590 organic matter and the inaccessibility of target organisms to the probes means that
591 there is a significant risk of introducing spatial bias during labelling. This
592 consideration has effectively curtailed application of such probes to soil systems.
593 Whilst labelling cells with stable isotope probes may also result in spatial biases as

not all micro-organisms that have the capacity to use the substrate may be labelled, these are likely to be more accurate.

5.2.3. Horizontal gene transfer

There is a growing body of evidence to suggest that horizontal gene transfer has played an important role in shaping the evolution of bacterial communities and that it is an important mechanism in soil bacterial communities' capacity to adapt to external change (van Elsas and Bailey, 2002; Crawford et al., 2005). Although gene transfer has been detected in soil and in other environmental samples, the controls and triggers that operate *in situ* are still poorly understood (van Elsas and Bailey, 2002; Sørensen et al., 2005). The frequency of transfer of mobile genetic elements from donor to recipient cells occurs more readily in zones of high microbial density and metabolic activity such as the rhizosphere. The frequency is known to be affected by a range of factors such as soil type, moisture content, pH and temperature, though it has been postulated that this may be more to do with indirect effects on population density than on the frequency of transfer itself (Sørensen et al., 2005). The physiological status of donor and recipient cells and their ability to sense signal molecules may also be important determinants in the frequency of transfer (van Elsas and Bailey, 2002). In soil the impact of many of these factors is regulated by the nature of the micro-habitat in which the cells exist. By allowing the spread of an introduced trait such as the capacity to degrade an enriched organic molecule to be followed at the scale of individual cells, NanoSIMS provides us with the opportunity to investigate the micro-conditions that are conducive to horizontal gene transfer.

5.2.4. Fungi

Filamentous (eucarpic) fungi play many significant roles in mediating transport phenomena in soils, principally by virtue of the manner in which the fungal mycelium is a spatially-integrating structure (Ritz, 2006). Elements and compounds are mobilised within regions of the mycelial front and transported to distal regions, governed by source:sink relationships largely established by the spatial organisation of the mycelium in relation to the location of substrate resources and reproductive structures. As well as a huge range of saprophytic contexts, two out of three of all plant species (Trappe, 1987) are associated with arbuscular mycorrhizal fungi (AM fungi) and the extra-radical mycelia of AM fungi are powerful underground mediators of nutrient assimilation and transport to plants (Leake et al., 2004). Ectomycorrhizal fungi are also abundant, to the extent that the majority of roots in natural environments are not roots as such, but mycorrhizas. Experiments utilising isotopically labelled materials have shown the pathways and associated gene expression for uptake and transformation of N (Govindarajulu et al., 2005) and non-invasive techniques have been developed to study C (Tlalka et al., 2002) and P (Nielsen et al., 2002) transport within hyphae and mycelia. However, few studies of AM fungi and plant relationships are able to distinguish clearly between the role of the root and the fungal associates in the assimilation of nutrients (e.g. Hodge et al., 2001). The high spatial resolution of NanoSIMS offers many opportunities to understand more precisely the transformation and uptake of elements and compounds at the mycelial front (significantly at the intra-hyphal scale), and their subsequent location and transport through mycelia. Very little indeed is known about the fungal:soil interface at the hyphal scale, but NanoSIMS analysis has been shown to

putatively identify fungal hyphae (Figure 6) and therefore it may be feasible to study this interface in more detail.

5.2.5. N_2-fixing bacteria

The ability to fix atmospheric dinitrogen gas (N_2) is restricted to only a few prokaryotes which have an ecological advantage over other organisms that must rely on fixed sources to meet their cellular N requirements. Cyanobacteria are among the most abundant classes of micro-organisms and are one of the largest global contributors to atmospheric nitrogen fixation. Their evolutionary success and ecological importance is largely owed to their unique ability to reduce both C and N in aerobic conditions. Due to the irreversible inhibition of nitrogenase by free oxygen, various mechanisms of separating the oxygen producing (photosynthesis) and nitrogen reducing processes have evolved. Using 99.99 atom% $NaH^{13}CO_3$ and $^{15}N_2$ as cyanobacterial substrates, Popa et al. (unpublished data) and Pett-Ridge et al. (unpublished data) have demonstrated that NanoSIMS can be used to isolate regions of high N_2-fixation activity, as well as storage locations, mobilisation and utilisation rates of newly fixed N in these bacteria. As this work was carried out with pure cultures, the challenge ahead is to repeat this type of analysis in a more complex environmental matrix such as soil.

6. Conclusions

There are still many challenges for the application of NanoSIMS as a robust tool to improve understanding of microbial processes in soil at a micro- and nano-metre scale and inform studies of biogeochemistry and soil ecology. The method itself provides two main obstacles: (i) adequate sample preparation to avoid artefacts which may introduce a bias in the interpretation of NanoSIMS data and (ii) location of regions-of-

interest. The necessity of studies explicitly focusing on sample preparation and identification of region-of interest is therefore substantiated. In addition proof-of-concept for many of the areas of study discussed above is still necessary. Currently only *ex situ* labelled materials have been detected in the soil matrix using NanoSIMS – and in that instance the soil matrix used was relatively simple, being dominated by quartz sand. The application of NanoSIMS to studies within soil is still at a very early stage of development. Nonetheless, NanoSIMS provides one of the only current opportunities to study soil at levels of resolution and characteristic scales appropriate to the operational scale for micro-organisms. Where the method is applied within integrated studies and with appropriate care taken to ensure robust and relevant data collection, then we believe that NanoSIMS will allow access to minute universe which has previously eluded study, and interactions therein which may have profound implications for understanding soil processes at field, catchment and regional scales.

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Table 1: Advantages of Cameca NanoSIMS50 ®

Advantages
• Improved transmission of secondary ions at high mass and spatial resolution • Multi-collector: Simultaneous collection of up to five ion species[1] • Full periodic table (H-U) • Distinction between isotopes of elements • Increased sensitivity (ppm) • Improved resolution through co-axial optics (i.e. 90° incident angle), low pA beam currents and short working distance: Lateral resolution of 50 nm (Cs^+ primary ion beam) and 150 nm (O^- primary ion beam) Depth resolution of 1 nm • Navigation: CCD camera assists in navigation • Mini Scanning Electron Microscope (Cs^+ primary ion beam only): Secondary Electron collection and imaging; revealing surface details • Electron gun (Cs^+ primary ion beam only): Charge compensation

[1] The Cameca NanoSIMS 50L is capable of collecting seven ion species simultaneously.

Figure legends:

Figure 1: Biochemical processes versus techniques at different physical scales.

Figure 2: Schematics of NanoSIMS Ion Optics. R = Radius of the secondary ion trajectories (figure kindly provided by Frank J. Stadermann, Washington University, St Louis, Missouri, http://presolar.wustl.edu/nanosims/schematic.html).

Figure 3: Typical NanoSIMS images of a cross section of ^{15}N-labelled *Pseudomonas fluorescens* mixed in coarse textured sand and embedded in Araldite resin. (A) $^{12}C^-$ (grey); (B) $^{28}Si^-$ (blue); (C) $^{12}C^{14}N^-$ (green) and (D) $^{15/14}N$ ratio (red). Four electron-multiplier secondary ion detectors were used to simultaneously collect $^{12}C^-$, $^{12}C^{14}N^-$, $^{12}C^{15}N^-$ and $^{28}Si^-$ data with the nominal size of images between 12 µm field of view. The mass resolving power was ~5000 and spatial resolution was ~100 nm probe diameter. Maps representing $^{15/14}N$ ratios were obtained by dividing the $^{12}C^{15}N^-$ counts by $^{12}C^{14}N^-$ counts for each pixel, using the MIMS plug-in for the freeware package, Image J (image processing technique available at http://rsb.info.nih.gov/ij/).

Figure 4: Cross section of ^{15}N-labelled *Pseudomonas fluorescens* mixed in coarse textured sand: (A) Superimposed NanoSIMS images (blue = $^{28}Si^-$; green = $^{12}C^{14}N^-$ and red = $^{15/14}N$ ratio) (field of view = 12 µm) and (B) Mosaic of $^{28}Si^-$ ion images (blue) and $^{12}C^{14}N^-$ (green), superimposed with $^{15/14}N$ ratio images (red) (field of view = 30 µm for each ion image and step between images of 25 µm giving a total field of view of 105 x 55 µm).

Figure 5: Soil particles from a sandy soil amended with ^{13}C and ^{15}N-labelled *Pinus ponderosa* fine roots and needles (Bird and Torn, 2006). (A) NanoSIMS $^{12}C^-$ image of a 15 μm field of view of an uncoated soil particle, dried and pressed into an aluminium stub. (B) NanoSIMS $^{13/12}C$ image of the same region, coated with gold and using the electron flood gun. Image is an integration of 50 individual 256 x 256 pixel planes (scans). (Courtesy Drs Jennifer Pett-Ridge and Peter K. Weber, Lawrence Livermore National Laboratory and Dr Jeffrey Bird, University of California Berkeley, USA).

Figure 6: Soil particles from a sandy soil amended with ^{13}C and ^{15}N-labelled *Pinus ponderosa* fine roots and needles (Bird and Torn, 2006). (A) Montage of multiple transmission electron microscopy images (FEI Tecnai 12 120KV Transmission Electron Microscope) of a single soil particle, embedded in sulphur (Bradley et al., 1993) and microtomed to ~200 nm. (B) NanoSIMS image of $^{12}C^{14}N^-$ soil particle (16 μm field of view) of putative fungal hyphae (area is depicted as red box in (A)) and detailed NanoSIMS image of (C) $^{12}C^{14}N^-$ and (D) P^- (5 μm field of view, mass resolving power was >7000, integration of 20 individual 256 x 256 pixel planes; area is depicted as white box in (B)). Its relatively lower P content (Figure 6d) to the background suggests that this feature may be a 'ghost hyphae', i.e. the shell marking where live tissue once existed. (Courtesy Drs Jennifer Pett-Ridge and Peter K. Weber, Lawrence Livermore National Laboratory and Dr Jeffrey Bird, University of California Berkeley, USA).

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