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Probing the nature of soil organic matter

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ABSTRACT

Soil organic carbon management is a nature-based carbon dioxide removal technology at the same time contributing to soil health and agricultural productivity. The soil science communities are refuting the traditional assumptions of the nature of soil organic matter (SOM) as based on 'humic substances' that are operationally-defined and have not been observed by contemporary, *in situ* spectromicroscopic techniques. Instead, new theories suggest that the interactions between molecular diversity of organic compounds, their spatial heterogeneity and temporal variability controls the formation and persistence of SOM. A mechanistic understanding of these processes occurring within organo-mineral and organo-organic assemblages requires non-invasive techniques that minimize any disturbance to the physical and chemical integrity of the sample. Here, we present a theory-driven review where a combination *of in situ* methods serve as potential solutions to better understand the persistence and dynamics of SOM and its effects on nutrient distribution at a micro- and nano-scale. We explore underlying theories in light of advances in available methodologies, their historical development and future opportunities. Examples of interdisciplinary approaches that have been utilized in other areas of science but not in soils offer both deductive and inductive analytical opportunities. We show how different conceptual methods across scales inform each other, and how important and indispensable high-resolution investigations are to resolving next-generation questions.
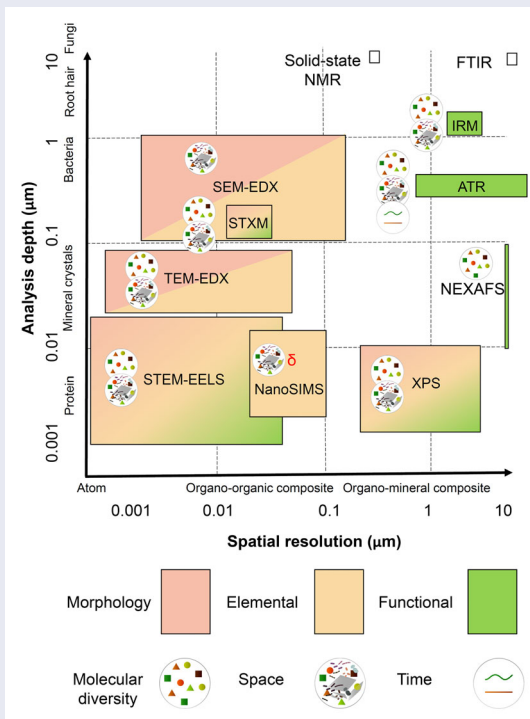
1. Introduction

Soil organic matter (SOM) plays a vital role in maintaining soil health, sustainable crop production, water quality (e.g. sorption of contaminants and minimized nutrient transport), human health (contaminant immobilization and increased micronutrient availability) and climate change mitigation (carbon sequestration) and adaptation (Kramer & Chadwick, 2018; Minasny et al., 2017; Rumpel et al., 2020; Sanderman et al., 2017; Schmidt et al., 2011). Soils contain more organic carbon (OC) than the atmosphere and vegetation combined, with global soil OC (SOC) stocks estimated to be around 1,500 G t to 1-m depth (Arneth et al., 2019; Minasny et al., 2017). Historically, conversion of natural ecosystems into ca. 5 G ha of farmland has caused loss of around 150 G t of C, with agricultural land currently losing an average of 2 t OC ha^{-1}yr^{-1} (Arneth et al., 2019; Minasny et al., 2017; Ogle et al., 2005; Sanderman et al., 2017). However, this has created an opportunity to rebuild SOC stocks, with potential to sequester up to 2.5 G t of CO_2-C per year globally (Smith et al., 2020). SOC derives almost entirely from photosynthesis through input of plants, and the products from faunal and microbial processing of these inputs. Therefore, retaining even a small portion of these photosynthetic C inputs could sequester 1.5–3.4 G t C yr^{-1} (Lal et al., 2018; Minasny et al., 2017). Building SOC is a key strategy to regenerate soil health which in turn helps to sustain productivity and resilience of agroecosystems to extreme conditions (IPCC, 2019; Rumpel et al., 2020). A recent global meta-analysis estimated the potential yield increase of wheat to be $10 \pm 11\%$ whilst that for maize to be $23 \pm 37\%$ with increasing SOC (Oldfield et al., 2019). Hence, building SOC offers multiple benefits and deserves high-level policy initiatives to support this outcome.

To optimize opportunities for C sequestration, a more detailed understanding of the mechanisms associated with SOC dynamics is required. Traditional wet chemistry approaches require processes which destroy the spatial architecture of the soil (Hayes & Swift, 2020). However, recent analytical advances have made it possible to measure the complex molecular structures of SOM without an analytical process that itself changes the structure of the OC (Kelleher & Simpson, 2006; Kleber & Johnson, 2010; Lehmann & Kleber, 2015; Schmidt et al., 2011). The *in situ* approaches examined in this review focus on methods that avoid chemical extraction or sample treatment that generates artifacts (Sec. 2). These approaches include spectromicroscopic techniques that can provide *in situ* spatial information of OC speciation in soil.

This review presents significant new syntheses and insights and extends upon previous reviews, including Stuckey et al. (2017), who examined scanning transmission X-ray microscopy (STXM) for studying submicron soil biogeochemical processes; Chenu et al. (2015), who synthesized fractionation methods with detailed molecular measurement of SOM composition and dynamics; Hoppe et al. (2013) and Mueller et al. (2013), who advanced the application of nanoscale secondary ion mass spectrometry (NanoSIMS) in biological geochemistry and soil microenvironments; Parikh et al. (2014), who summarized infrared spectroscopy for soil chemical insights; Lehmann and Solomon (2010), who reviewed the studies of SOC chemistry using synchrotron-based spectroscopy, by bringing a broad range of emergent methods in alignment with recent theory of SOM composition and formation.

Here, we present a theory-driven review where the methods are discussed to solve key fundamental questions about temporal changes in spatial assemblage of SOM composition in relation to microbial processes, aggregation, organo-mineral and organo-organic interactions. This review focuses on the deductive and inductive aspects of the dynamics and behavior of SOM that require knowledge about: (1) molecular diversity of OC functional groups; (2) spatial heterogeneity of OC species; and (3) the interaction and temporal influences between molecular diversity and
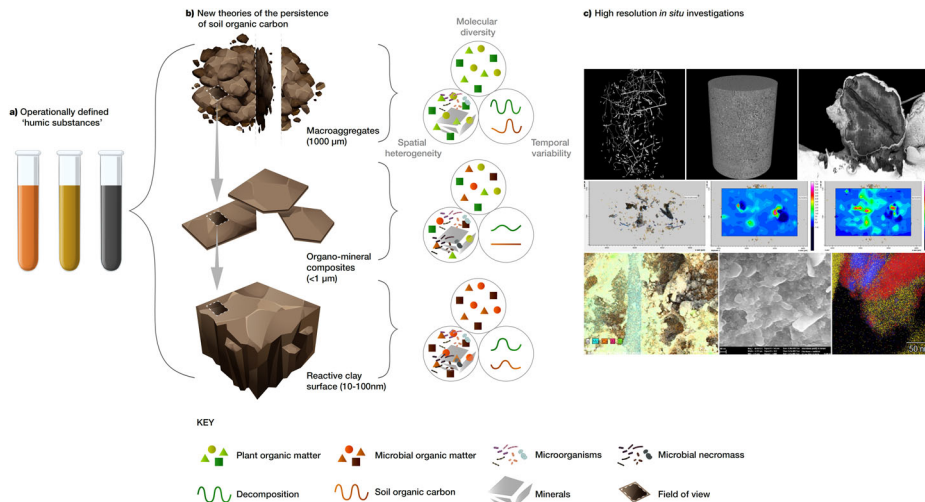
Figure 1. *In situ* approaches for studying soil organic matter (SOM). (a) The prevalent assumptions of 'humic substances' which were operationally-defined and have not been observed by contemporary, *in situ* spectromicroscopic techniques. Bulk analytical techniques generally require extraction (given the low abundance of SOC) followed by analyses; (b) emerging theories suggest that the interactions between molecular diversity of organic compounds, their spatial heterogeneity and temporal variability controls the SOM persistence; (c) this theory-driven review presents a combination of *in situ* methods which can serve as potential solutions to better understand the persistence and dynamics of SOM and its effects on nutrient distribution.

spatial heterogeneity (Figure 1). We recognize that there are also challenges and tradeoffs for each analytical approach and issues where we do not yet have an analytical solution, and those where we do not even have a theoretical solution.

2. Prevalent theories of soil organic carbon persistence

In recent times, the persistence of OC in soil has been described by three competing theories: *2.1 'Humification'* (Kögel-Knabner, 2000; Kononova, 1966; Rice, 2001); *2.2 'Selective preservation'* (Aber et al., 1990; Melillo et al., 1982; Mueller & Kögel-Knabner, 2009; Prater et al., 2020); and *2.3 'Progressive decomposition'* (Burdon, 2001; Cotrufo et al., 2013; Trumbore, 1997). These concepts have been extensively discussed in the review, here we only provide a summary in the supplementary information.

3. Emerging theories and hypotheses regarding soil organic matter

New theories of SOC persistence have reconciled the traditional, prevalent views and assumptions (Sec. 2), and suggest that the persistence of SOM depends on the molecular diversity of organic compounds (Sec. 3.1), the interactions between organo-mineral and organo-organic interfaces (Sec. 3.2), and the temporal changes of microbial access to SOM (Sec. 3.3) (Figure 1b). This shift in understanding of the nature of SOM provides opportunities for improved prediction and management of soil structure and health, water and nutrient balance, greenhouse gas emissions and temperature responses.

3.1. Molecular diversity not the quality of organic compounds controls decomposition

Different microbial uptake and metabolism processes result in a high diversity of molecular structures of SOM (Lehmann et al., 2020). OC input differs in composition and spatial distribution which can affect the nature of SOC turnover (Angst et al., 2019, 2021). Molecular diversity

determines the persistence of OC in solution (Roth et al., 2019) and the interactions with organo-mineral and organo-organic surfaces (Possinger et al., 2020a). In fact, the traditional understanding of SOM ('humic substances') was also based on the interpretation of the diversity of SOM. It is now apparent that the postulated large size and mass of molecules are not found in natural soils but occur as a result of self-assembly of small molecules during the harsh chemical extraction process (Myneni et al., 1999; Piccolo, 2001; Sutton & Sposito, 2005).

3.2. Organo-mineral and organo-organic interactions increase spatial heterogeneity

The role of organo-mineral interactions and aggregation in SOC protection has been demonstrated across widely varying agroecosystems, soil types and environments (Cotrufo et al., 2019; Hemingway et al., 2019; Kleber et al., 2015; Mikutta et al., 2009). Macroaggregates are continuously developed and destroyed, along with the adsorption and desorption of OC on to aggregates (Peng et al., 2017). Organo-organic interactions may also be important in altering SOC persistence but remain understudied. Irregular organic structures and the organo-organic interfaces have been observed at a nanometer scale which contradicts traditional assumptions of ordered and unidirectional layered organization of SOM (Possinger et al., 2020a; Vogel et al., 2014). Further, the co-location of enrichment of N with oxidized C species at the mineral interface, in contrast to alkyl C at the organo-organic interfaces, supports such a notion (Possinger et al., 2020a).

3.3. Temporal variability in spatial assemblage of organic matter in relation to microbial accessibility

To better understand and predict SOM turnover, it is therefore critical to determine its spatial distribution at the micro- to nano-scale within the soil-mineral matrix and the interfacial interaction between microbial ecology and mineral surfaces over time. Current evidence suggests that temporal changes in the spatial assemblage of SOM in relation to microbes may be an important driver of SOC persistence (Lehmann et al., 2020). A new theory of SOC decomposition has been proposed which reflects the energy return on investment and adaptation by microorganisms and transport of organic matter over time. Accordingly, the interaction between molecular diversity of organic compounds, their spatial heterogeneity and temporal variability may dictate the persistence of SOC (Lehmann et al., 2020).

4. Probing the nature of soil organic matter based on the new theories

We have considered three broad categories of techniques that are of importance in understanding the persistence of OC in soil based upon the most recent theories and hypotheses (Sec. 3; Figure 1c). Firstly, we examine methods for the *in situ* assessment of C forms in the soil and their interactions with soil components, both in one dimension (i.e. bulk analyses) and in two dimensions (i.e. mapping), with such approaches providing important information on how the diversity of OC compounds influences their interactions with soil components, with each other, and with soil microorganisms. Secondly, we examine methods that allow elemental mapping of the distribution of C (plus other important elements, such as N, Fe and Ca) within the soil matrix at a micro- and nano-metre scale, both in two- and three-dimensions, with such approaches allowing an assessment of the mechanisms whereby C and other elements interact with the various soil components, the arrangement of C within the soil matrix, and how the arrangement of C within the soil influences its interaction with microorganisms. Finally, we examine *in situ* approaches that provide complementary and interdisciplinary information on the nature of SOM beyond C itself. We propose various investigations that can provide information to better understand processes

driven by the molecular diversity of OC functional groups, the spatial heterogeneity of OC species in soil, and the interaction between space-time-composition (Figure 1). Determining which method is most suitable for any given study depends upon a range of factors, including the nature of the information required for C itself (for example, C speciation mapping *vs.* total C mapping), the number of dimensions required [one-dimensional (bulk) *vs.* two-dimensional *vs.* three dimensional], the resolution required, and the sensitivity.

4.1. Assessing forms of carbon and their interactions with soil components in one- and two-dimensions

4.1.1. Solid state ^{13}C nuclear magnetic resonance (NMR)

Technical background: Nuclear magnetic resonance (NMR) spectroscopy can be used for bulk analyses to determine the form of OC functional groups but not the molecular diversity (Figure 2). In NMR, the sample is placed in a magnetic field with the signal produced by excitation of certain atomic nuclei (Knicker & Lüdemann, 1995; Kögel-Knabner, 1997; Smernik, 2005). Whilst liquid state NMR has traditionally been performed *ex situ* using soil extracts, solid state ^{13}C NMR can be used with minimal sample preparation (Wilson, 2013).

Information: Using this approach, it is possible to determine the forms of OC present in bulk soil or SOM fraction. Chemical shift ranges can be fitted to four spectral regions, labeled as (1) alkyl C (10–45 ppm; long chain polymethylene type structures, e.g. fatty acids, waxes and resins); (2) O-alkyl C (45–110 ppm; mostly carbohydrates); (3) aromatic C (110–160 ppm; protonated and C substituted aromatics and unsaturated C and oxygenated aromatics); and (4) carboxyl C (160–200 ppm; carboxylic C, esters and amides) (Baldock et al., 1992; Oades et al., 1987).

		Section 3.1 Molecular diversity			Section 3.2 Spatial heterogeneity				Section 3.3 Temporal changes		Limitations	
	Linking with theories	Functional groups	Multi-element analyses	Range of detectable elements	Dimension of analyses	Maximum lateral resolution (µm)	Analysis depth (µm)	Spot size (µm)	In vivo analyse	Upscaling	Accessibility of the technology	Difficulty of sample preparation
Solid-state NMR	2.2 & 2.3; 3.1 & 3.2	Yes	No*	N/A	1	N/A	N/A	N/A	No	N/A	Difficult	Dehydrated, ball-milled
FTIR	N/A	Yes	No**	N/A	1	N/A	N/A	1000	No	1D->2D	Easy	Dehydrated, ball-milled
IRM	3.2 & 3.3	Yes	No	N/A	2	6 (lab.) 3 (synch.)	N/A (fixed angle)	50 (lab.) 3-10 (synch.)	Possible	2D->3D	Difficult	Dehydrated, microtomed ***
NEXAFS (fluorescence, electron yield)	3.1	Yes	No	Be-Os	1	N/A	fluorescence 0.1; electron yield 0.01	1000 high throughput 50-150 spectroscopy	No	1D->2D	Difficult	Dehydrated, ball-milled
NEXAFS-STXM	3.1 & 3.2	Yes	No	B-Y	2	0.02-0.03	0.1-0.3	0.03	No	2D->3D; modelling	Very difficult	Dehydrated, microtomed
XPS	3.1, 3.2 & 3.3	Yes****	Yes	Li-U	1-2	7.5 (lab.), 0.2 (synch.)	0.005-0.01	200	Possible	2D->3D; modelling	Difficult	Dehydrated, ball-milled
STEM-EELS	3.1 & 3.2	Yes	Yes	B-U	2	0.0005	0.05	0.05-0.2 ×10⁻³	No	2D->3D	Very difficult	Dehydrated microtomed FIB
SEM- and TEM-EDX	3.2	No	Yes	B-U	2-3	0.002 (SEM) 0.001 (TEM)	0.1-2 (SEM) 0.06-0.1 (TEM)	1 (SEM) 0.001-0.02 (TEM)	No	2D->3D	Easy	Dehydrated, microtomed
NanoSIMS	3.2	No	Yes	H-U	2	0.05	0.005-0.02	0.15 (O⁻) 0.05 (Cs⁺)	No	2D->3D; modelling	Difficult	Au or Au/Pd coating
CT	3.3	No	No	N/A	3	80 (lab.) 1-40 (synch.)	100,000	5	Possible	modelling	Difficult	None
XFM	3.2	No	Yes	Al-Mo (lab.) P-Ag (synch.)	2	5 (lab.) 0.015 (synch.)	10-10,000	1-10 (microprobe) 0.05-0.5 (nanoprobe)	Yes	modelling	Difficult	None

Nuclear magnetic resonance (NMR); Fourier-transform infrared spectroscopy (FTIR); Infrared microspectroscopy (IRM); Near edge X-ray absorption fine structure (NEXAFS); Soft X-ray scanning transmission X-ray microscopy (STXM); X-ray photoelectron spectroscopy (XPS); Scanning transmission electron microscopy-based (STEM) electron energy loss spectroscopy (EELS); Scanning/transmission electron microscopy-based (SEM/TEM) energy-dispersive X-ray spectroscopy (EDX); Nanoscale Secondary Ion Mass Spectrometry (NanoSIMS); X-ray tomography (CT); X-ray fluorescence microscopy (XFM).

*NMR only measure one at a time with the exception of double CPMAS ^{13}C and ^{15}N NMR; **FTIR reflects multiple elements, but only provides information of bonds; *** With the exception of macro-ATR where only pelleted samples are required for surface analyses; **** XPS has only low sensitivity to changes in speciation

Figure 2. Comparison of 11 broad techniques used for probing the nature of soil organic matter. All values are indicative of typical systems.

Limitations and potential solutions: A limitation of NMR is the difficulty in identifying specific compounds (Kögel-Knabner, 2000; Oades et al., 1987; Smernik & Baldock, 2005). Furthermore, the presence of paramagnetic and ferromagnetic minerals in soils can potentially pose a challenge for solid state NMR (Oades et al., 1987). However, with recent advances in NMR, these two limitations have been addressed, and in some cases, used as an advantage (Supplementary information).

Linking with theories: NMR has been used to study the theories of selective preservation (Sec. 2.2) and progressive decomposition (Sec. 2.3). It was suggested that the increasing aromatic- and alkyl-C contents in the 2–20 μm fractions of soil were entirely caused by selective preservation using NMR (Baldock et al., 1992). However, this notion is largely refuted by ^{14}C dating techniques, demonstrating that supposedly recalcitrant molecules are not specifically preserved in SOM (Amelung et al., 2008; Dignac et al., 2005; Gleixner, 2013). The chemical structure of decomposed natural SOM characterized by NMR showed that certain chemical moieties increase with ongoing decomposition, with some being assimilated faster than others, leading to a distinctively different chemical composition of SOM (Baldock et al., 1992; Sollins et al., 1996). This direct evidence explained the chemical composition of 'humic substances' as the mixture of known plant and microbial compounds (Kelleher & Simpson, 2006).

NMR has also been deployed to validate emerging theories regarding molecular diversity (Sec. 3.1) and organo-mineral interactions (Sec. 3.2). Spectral resolution can be improved for solid samples when combining cross-polarization with the magic angle spinning technique (CPMAS). Double CPMAS ^{13}C-^{15}N NMR spectroscopy can be used to detect ^{13}C nuclei close to ^{15}N nuclei which show the coupling between amide-C and N-substituted-alkyl-C atoms (Knicker et al., 2002) and immobilized fertilizer N in decomposed wheat straw residues in soil (Chen et al., 2021). This supports the notion that the prevalent N from inorganic sources is immobilized into peptide-like structures during microbial degradation (Sec. 3.1). For SOC stabilized via organo-mineral-metal interactions NMR has shown the preferential association between O/N-alkyl C and Fe oxides in clay fractions (Schöning et al., 2005). NMR has also been applied to explain the effect of soil order and particle size on the decomposition of SOM which indicated the proximity of mineral and C forms but not the spatial distribution in a range of mineral soils (Angst et al., 2017; Baldock et al., 1992; Kögel-Knabner, 1997; Paetsch et al., 2017). Spin relaxation in NMR can be used to probe C-Fe interactions where Fe^{3+} proximity of carboxylic and aromatic C can be identified (Possinger et al., 2020b).

4.1.2. Infrared spectroscopy and microspectroscopy (IRM)

Technical background: Fourier-transform infrared (FTIR) spectroscopy can provide diagnostic information of the principal chemical groups and mineral components of soil, with these measurements generally performed using conventional globar-sourced infrared light. An FTIR spectrum in the mid infrared region, produced when infrared radiation is absorbed or reflected by a soil sample, provides information on the overall chemical composition of the soil. Here we focus on the most common benchtop approaches: transmission and attenuated total reflectance (ATR) modes. Infrared microspectroscopy (IRM) can provide laterally resolved (two-dimensional mapping) information on the molecular composition of biological material or complex heterogeneous soil at a micrometre scale (Johnston & Aochi, 1996; Lehmann et al., 2007; Marinkovic et al., 2002). Measurements can also be performed using ATR, with this relying on a form of internal reflection to enable the analysis of samples that do not adequately reflect IR or cannot be microtomed for transmission measurements (Vongsvivut et al., 2019). The total internal reflectance occurs when IR radiation travels through a high refractive index crystal onto the sample surface. This spares ATR from water interference which makes it a useful technique for studying solution-solid interfaces.

Information: For bulk analyses (Sec. 3.1), FTIR vibrational bands associated with SOC can be assigned whereby absorbance: (1) at 3630 cm^{-1} corresponds to the stretching vibrations of O–H groups possibly assigned to clay minerals; (2) the peak at 2920 cm^{-1} corresponds to the C–H stretching vibrations of aliphatic biopolymers; (3) the peak at 1600 cm^{-1} corresponds to C=C stretching of aromatic C or N–H deformations; and (4) the peak at 1035 cm^{-1} corresponds to C–O stretching vibrations of polysaccharide C (Parikh et al., 2014). IRM analyses can be undertaken using either synchrotron-based sources or a bench-scale (globar) source. Both approaches are similar except for the source of the photons, with the higher brightness (flux) of synchrotron sources (100–1000 times brighter) being especially useful when mapping C in soils due to the comparatively low C concentration in most soils. The lateral resolution is generally $3 \times 3 \mu m$ (synchrotron) and $6 \times 6 \mu m$ (lab).

Limitations and potential solutions: It must be acknowledged that FTIR is useful for rapid testing and bulk analyses of large sample number but not for speciation and SOC dynamics. Accurate identification of FTIR absorbance bands for SOC is further hindered by mineral interference (in the range of 1400–400 cm^{-1}) and the heterogeneity of SOM (e.g. overlaps between C=N amide II and aromatic C=C at 1530 cm^{-1}). The limitation for IRM is sample preparation that is markedly more complex than for bulk FTIR analyses (Figure 2). Soil aggregates are commonly premoistened and manually cryo ultramicrotomed to a thickness of $\sim$200 nm using a diamond knife preferably without embedding media (Hernandez-Soriano et al., 2018; Lehmann et al., 2007).

Linking with theories: IRM can be used to probe organo-mineral interactions (Sec. 3.2). Linear regression of the spectra collected for each IR map can be examined for organo-mineral protection (Hernandez-Soriano et al., 2018; Lehmann et al., 2007), i.e. the correlation between the amount of clay (absorbance at 3630 cm^{-1}) and either polysaccharides C (absorbance at 1035 cm^{-1}), aromatic C (absorbance at 1600 cm^{-1}), or aliphatic C (absorbance at 2920 cm^{-1}). Such analyses provide microscale maps revealing the lateral distribution of specific chemical compositions in soil that can be linked to their functions (Figure 3a). ATR is well suited for determining the sorption mechanism of organic (e.g. oxalate and maleate from root exudates) and inorganic molecules (e.g. carbonate and nitrate) to mineral surfaces (e.g. hematite, goethite and kaolinite) between the outer- and inner-sphere (Parikh et al., 2014). Although it has not yet been applied to soil, this approach can test the notion of the continuous sorption and desorption of OC on to mineral surfaces as discussed in Sec. 3.2 and temporal changes (Sec. 3.3).

4.1.3. Near edge X-ray absorption fine structure (NEXAFS) spectroscopy coupled with soft X-ray scanning transmission X-ray microscopy (STXM)

Technical background: Synchrotron-based soft X-ray or C (1 s) near-edge X-ray absorption fine structure (NEXAFS or XANES) spectroscopy is particularly suited for the characterization of C speciation in complex heterogeneous soils since absorption spectra depend directly on the local bonding environment of atoms (Lehmann & Solomon, 2010), being useful for characterization of surfaces and near-surface interfacial layers (analysis depth 100–300 nm). For NEXAFS, spectra are obtained by progressively increasing the energy of an incident photon (X-ray) beam across the absorption edge of the element of interest (in this case, C, with the corresponding K-edge being at 284 eV). As determined by the photoelectric effect, the precise absorption of the incident beam (and the associated X-ray fluorescence) as the energy is increased across the absorption edge depends upon the bonding environment (i.e. speciation), with differences in the spectra depending upon the forms of C present. Given that for NEXAFS analyses the energy of the incident X-ray beam is increased across the edge of interest, these analyses are element-specific (each element has a different energy), but this gives NEXAFS the advantage of being markedly more sensitive to the bonding environment of the element of interest. NEXAFS is used for bulk analyses of C speciation, with the spot size of the beam under the operating conditions often ranging from approximately 0.1–1 mm (Cowie et al., 2010; Torres-Rojas et al., 2020, Figures 2 and S1).
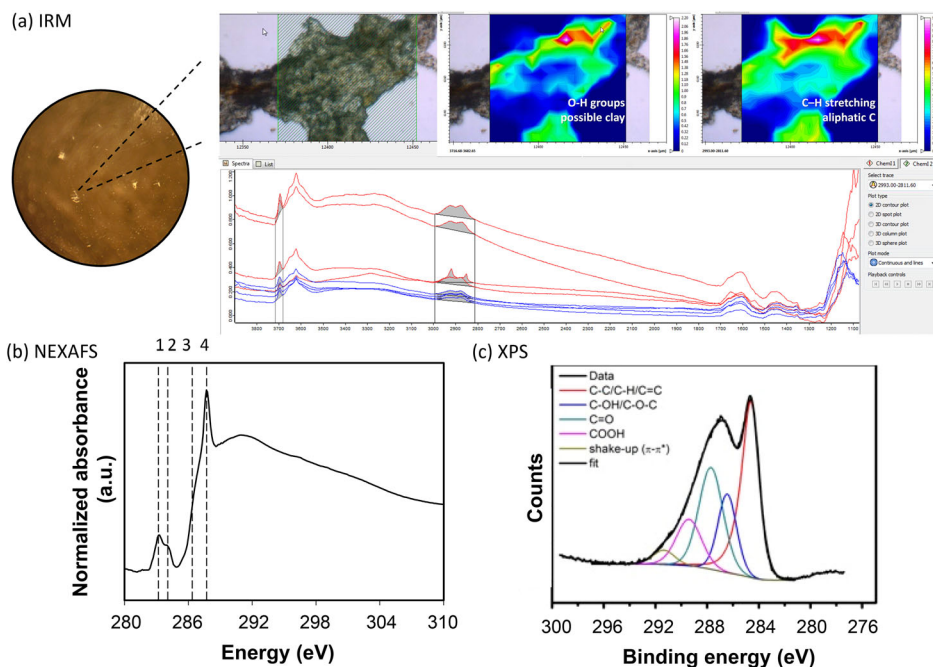
Figure 3. (a) Semi-thin (200 nm) sections of free water-stable microaggregates (53–250 μm) from a *Rhodic* Ferralsol under sub-tropical pasture analyzed using synchrotron-based IR-microspectroscopy (Australian Synchrotron). Spectral maps showing the distribution of aliphatic-C (2920 cm^{-1}), and mineral-OH (3650 cm^{-1}) were obtained from 64 coadded scans (4 cm^{-1} resolution), lateral resolution 5 μm (bars: 50 μm). (b) Average NEXAFS spectra of microaggregates (53–250 μm) showing (1) quinones (284.1 eV); (2) aromatic C (285.2 eV), and (3) aliphatic C/phenolic C–OH (287.3 eV), (4) carboxyl C–OOH (288.6 eV) (n = 9, CV% < 3%). (c) XPS spectra of the same sample showing common C 1s photoelectron peaks as: (1) C=C (284.6 eV); (2) C-OH (phenol or hydroxyl groups, 286.2 eV); (3) carbonyl groups (C=O) (287.5 eV); (4) carboxyl groups (COOH) (289.1 eV); and (5) shakeup satellite peak (291.4 eV).

STXM is a form of X-ray microscopy in which a zone plate focuses an X-ray beam onto a small spot (30–50 nm), with a sample scanned in the focal plane of the zone plate and the transmitted X-ray intensity being recorded as a function of the sample position (Urquhart & Ade, 2002). By itself, STXM can be used to obtain elemental distribution maps, but when the STXM is coupled with NEXAFS it can produce quantitative two-dimensional maps of the various C species at 50 nm scale (Stuckey et al., 2017; Urquhart & Ade, 2002). Furthermore, three-dimensional imaging can be achieved via tomography by rotating samples. Transmission X-ray microscopy-based tomography has been used to reveal the three-dimensional structure of kaolinite and smectite clay aggregates (Zbik et al., 2008) and microbial cells (Guttmann et al., 2013).

Information: The NEXAFS spectra can probe the molecular diversity of SOM with prominent resonances assigned to (1) quinones (284.1 eV; aromatic quinones); (2) aromatic C (285.2 eV, 1 s-π* transitions of conjugated C=C; aromatic ring structures of hydrocarbons, amino acids and nucleobases), (3) aliphatic C/phenolic C–OH (287.3 eV; microbial-derived C), (4) carboxyl C–OOH (288.6 eV), and (5) C–O (289.3 eV) intensities of (hemi)cellulose and lignin (Lehmann & Solomon, 2010; Solomon et al., 2012). NEXAFS has been applied to study the structural composition of pyrogenic organic matter, soil colloids, dissolved organic matter, bulk soil, and biopolymers such as amino acids and peptides (Lehmann & Solomon, 2010; Torres-Rojas et al., 2020).

Limitations and potential solutions: Sample preparation for bulk NEXAFS analysis through grinding soils to fine powder can mask subtle changes in C functional groups which can be complicated by the small quantity of soil being investigated (∼<1 mg). Although NEXAFS can be

performed as a bulk analysis (one-dimensional), it can also be performed as a laterally-resolved analysis (two-dimensional mapping) when coupled with STXM.

Linking with theories: NEXAFS-STXM has been used to provide evidence for molecular diversity (Sec. 3.1) and organo-mineral interaction (Sec. 3.2) that dispute 'humification' (Sec. 2.1). Bulk NEXAFS analysis can capture changes in the inherent molecular structures of SOM which may impact biogeochemical C cycling and bioavailability of nutrients associated with SOM. For instance, the increasing intensity of the aliphatic C peak may indicate the retention of rhizodeposits (Figure 3b; Weng et al., 2017). Direct observation by *in situ* imaging and spectroscopy using NEXAFS-STXM has revealed great heterogeneity in OC speciation within soils at the nanometer scale (Lehmann et al., 2008; Schumacher et al., 2005). This validated the existence of simple biomolecules in soil but not the operationally defined 'humic substances' obtained by alkaline extraction (Sec. 2; Kelleher & Simpson, 2006). In addition to laterally-resolved information of C functional groups, NEXAFS-STXM can also be applied to study the K-edge or L-edge of several elements associated with organo-mineral assemblages, such as N, Al, Si, Ca, and Fe (Chen et al., 2014; Chen & Sparks, 2015; Solomon et al., 2012). The interactions between SOC and Al and Fe reactive phases in acidic soils has been a focus for understanding SOC persistence (Keiluweit et al., 2012, 2015; Kramer & Chadwick, 2018; Rumpel et al., 2015).

4.1.4. X-ray photoelectron spectroscopy (XPS)

Technical background: X-ray photoelectron spectroscopy (XPS) is a quantitative spectroscopic technique based on the photoelectric effect that can identify the elemental composition, chemical state, and the overall electron status of elements within a material or on its surface. In a manner similar to NEXAFS, for XPS the sample is irradiated with an incident photon (X-ray) beam. XPS analysis can be performed on both crushed and intact soil samples (Gerin et al., 2003; Mikutta et al., 2009).

Information: For molecular diversity, XPS can quantitatively detect the relative abundance of different C species (Briggs & Seah, 1990). Common C 1s photoelectron peaks can be classified as: (1) centered in 284.6 eV, typical of electrons in carbon sp^2 bounds (C=C), *i.e.*, delocalized sp^2 electrons; (2) centered in 286.2 ± 0.2 eV may be attributed to C-OH (phenol or hydroxyl groups), ether (C-O-C) or pyrrolic groups (C-N); (3) attributed to carbonyl groups (C=O) centered in 287.5 ± 0.4 eV; (4) attributed to the carboxyl groups (COOH) centered in 289.1 ± 0.3 eV; and (5) in 291.4 eV is attributed to the shakeup satellite peak, characteristic of $\pi \rightarrow \pi^*$ transition of electrons delocalized sp^2 (Figure 3c; Gerin et al., 2003). This approach can be effective for probing mineral surfaces (Sec. 3.2) with an analysis depth between 5–10 nm (Amelung et al., 2002; Woche et al., 2017).

Limitations and potential solutions: Air-dried soils are required for traditional XPS analyses that are often performed in an ultra-high vacuum station lower than 10^{-9} mbar, usually using Al or Mg X-ray sources. This prevents the measurements of temporal changes in SOC dynamics. Recently, the development of ambient pressure XPS, often using synchrotron X-ray sources, offers new possibilities to study soils in the presence of gas and water. However, it has not been widely applied in the study of SOC likely due to limited beam-time availability. This is despite its wide application in surface science and nanotechnology (Salmeron & Schlögl, 2008).

Linking with theories: XPS can be used to study theories regarding molecular diversity (Sec. 3.1), organo-mineral interactions (Sec. 3.2) and temporal changes (Sec. 3.3). XPS can measure the elemental content and functional groups of SOC in 3D as a function of depth (i.e. depth profiling) by sputtering deeper over time and accurately aligning with particular areas within the sample. The depth profile can be linked with a zonal structure of adsorbed SOM. This is evident with high aromatic C near mineral surfaces and high amide C in the outer regions of mineral organic associations via angle sputtering (Mikutta et al., 2009). This could indicate greater adsorption of metabolites and/or proteinaceous matter with increasing amino functional groups. Through ion

sputtering analyses by layers, the spatial and temporal behavior of SOM in soil-water interfaces (Sec. 3.3) can also be investigated using soil microarrays incubated with a predefined solution (Huang et al., 2020). Layers of nanoscale organic film have been characterized as the self-organised product of microbial biomass C (>130 nm) and mineral-organic associations (20–130 nm).

4.1.5. Transmission electron microscopy (TEM) with electron energy loss spectroscopy (EELS)

Technical background: Transmission electron microscopy (TEM) is another microscopic technique in which an incident beam of electrons is transmitted through an ultra-thin (ca. 100 nm) sample to produce an image. A TEM can be modified into a scanning transmission electron microscope (STEM) by the addition of a system that rasters a convergent beam across the sample to form the image, when combined with suitable detectors. For TEM/STEM, electron energy loss spectroscopy (EELS) can be used to provide laterally resolved (two-dimensional mapping) information on OC distribution and speciation in soil samples. In EELS, a sample is exposed to a beam of electrons with a narrow range of known kinetic energies. Some electrons will lose energy and have their paths deflected due to inelastic scattering. The amount of energy loss during the inelastic scattering is then measured by electron spectrometry and interpreted.

Information: EELS spectra in the energy range between 283–290 eV can be assigned to the following bonds and C forms: (1) $C=O$, quinine C (283.0–284.5 eV); (2) $C=C$, aromatic C (284.0–285.5 eV); (3) C-OH, phenolic C (286.0–287.4 eV); (4) C-H, aliphatic C (287.0–287.8 eV); (5) COOH, carboxylic C (288.0–288.8 eV); (6) C-O, O-alkyl C (289.0–289.5 eV); and (7) $C=O$, carbonyl (290.0–290.5 eV) (Figure 4d).

Limitations and potential solutions: A potential limitation of TEM-EELS is that the analyses themselves can cause radiation damage to organic components and C functional groups of samples. Furthermore, organic functional groups are difficult to detect in EELS, since many of the species are damaged or volatized by the high energy electron beam. The radiation damage in TEM can be managed through cryo-based sample holders and low dose illumination. To prepare the ultra-thin TEM lamellas, the samples must be thin-sectioned and the best-known methods to reduce sample damage are cryogenic focused ion beam (FIB) milling using an Au coating/cryo-
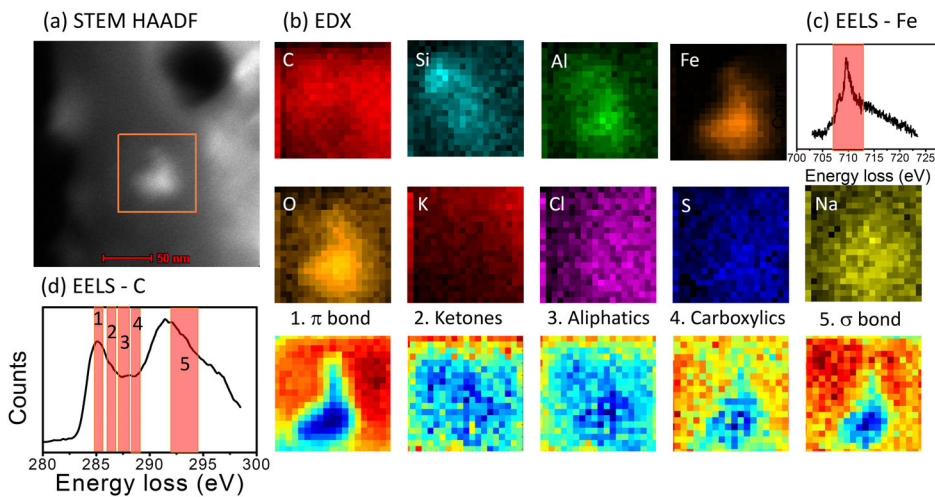

Figure 4. STEM nanoscale analyses of organo-mineral interface. (a) STEM high-angle annular dark-field image of the organo-mineral interface. (b) EDX maps were collected in the rectangular region (a) showing the elemental distribution of the C, Si, Al, Fe, O, K, Cl, S, and Na. (c) EELS Fe L-edge map. (d) EELS C K-edge spectra (left) and maps (right) can be assigned: (1) $C=C$, aromatic C (284.0–285.5 eV); (2) C-OH, phenolic C (286.0–287.4 eV); (3) C-H, aliphatic C (287.0–287.8 eV); (4) COOH, carboxylic C (288.0–288.8 eV); and (5) $C=O$, carbonyl (290.0–290.5 eV).

ultramicrotomy method (Archanjo et al., 2017; Possinger et al., 2020a). However, such methods are still under development and not readily available.

Linking with theories: TEM-EELS can be used to determine metal-mineral interactions (Sec. 3.2) and laterally quantify elemental speciation at nanoscale (Sec. 3.1; ca. <0.5 nm; Figures 2 and S1). Using cryo-STEM-EELS, enrichment of alkyl C and N at the organo-organic interfaces was first detected at < 10 nm (Possinger et al., 2020a). This finding has significance toward a better understanding of N-rich residues involved in C sequestration (Sec. 3.2). In addition, the FIB method can enable the original surface of the pyrogenic organic matter (Archanjo et al., 2017) and organo-organic and organo-organic interfaces (Possinger et al., 2020a) to be sectioned, identified and characterized. Therefore, choice of TEM sample preparation plays an important role for the detection of functional groups.

4.2. Elemental mapping of total carbon distribution within the soil matrix in two- and three-dimensions

The methods discussed in Sec. 4.1 provide information on the forms of C within soil, with some of these methods providing this information in two dimensions (i.e. maps of C and its forms). However, some methods can be simply used to provide maps of the total C distribution without differentiating between the different forms of C, with these methods being the focus of the present section.

4.2.1. Scanning electron microscopy (SEM) and TEM with energy dispersive X-ray spectroscopy (EDX)

Technical background: Scanning electron microscopy (SEM) is a form of electron microscopy that produces images of a sample by scanning the surface with a focused beam of electrons. The electrons interact with atoms in the sample, producing information regarding the surface topography. Energy-dispersive X-ray spectroscopy (EDX) is an analytical technique often coupled with SEM for the elemental analysis of samples based upon X-ray excitation by an energetic electron beam. Specifically, for EDX, the incident beam of electrons excites a range of different elements which can then be detected based upon the subsequent emission of characteristic fluorescent X-rays. EDX spectra can provide elemental composition of a sample, such as C, O, Si, Mg, Si, P, S, Cl, Ca, and Fe, but not the molecular diversity.

Linking with theories: SEM-EDX is broadly used for providing evidence for the organo-mineral interaction theory (Sec. 3.2). SEM-EDX can provide morphological and elemental information to probe mechanisms associated with mineral interactions (Archanjo et al., 2015; Schlüter et al., 2019) and microbe-driven biogeochemical processes involved in SOC cycling (Kaiser et al., 2002). SEM-EDX often complements other spectroscopic and microspectroscopic methods together to provide morphology and lateral distribution of molecular diversity and chemical speciation of SOC. Automatic sectioning by FIB (Lemmens et al., 2011) coupled with simultaneous SEM-EDX analysis can allow the study of SOM in 3D. Plant-microbe-soil C transfer in the rhizosphere can be linked with the soil physical structure in 3D via FIB-SEM-EDX (Vidal et al., 2018; Vogel et al., 2014). The interactions between roots, fungi and bacteria can transfer plant-derived OC from rhizosphere to bulk soil that can then interact with iron oxides, root and microbial metabolites in microaggregates (Vidal et al., 2018).

When coupled with TEM, TEM-EDX can improve the lateral resolution (Figures 2 and S1). High resolution TEM-EDX and STEM-EELS has confirmed the mineral coating surrounded by Al/Si, Fe nanoparticles (< 50 nm) at the surface of pyrogenic organic matter which may retain a range of C/O functional groups (aliphatic, ketones and carbonyl) in the micro-agglomerate (Figure 4b; Weng et al., 2017; Archanjo et al., 2017).

4.2.2. Nanoscale secondary ion mass spectrometry (NanoSIMS)

Technical background: Nanoscale secondary ion mass spectrometry (NanoSIMS) is an analytical technique that provides information of the microscale ($\sim$50–100 nm spatial resolution) elemental and isotopic composition of a material (Herrmann et al., 2007; Hoppe et al., 2013; Mueller et al., 2013). A primary ion beam (either Cs^+ or O^-) is accelerated onto the sample surface which releases secondary ion particles. These ions are separated according to their mass to charge ratio in a sector mass spectrometer. The primary ion beam can be focused to a spot of sample to achieve a lateral resolution of up to 50 nm, with scanned area typically between $5 \times 5\,\mu$m up to $30 \times 30\,\mu$m (Mueller et al., 2012; Steffens et al., 2017).

Information: Besides Faraday cups to detect secondary ions with high yields, to also allow for the detection of ions with low yield, electron multipliers can be used to detect secondary ions such as $^{12}C^-$, $^{31}P^-$, $^{32}S^-$, $^{12}C^{14}N^-$, $^{31}P^{16}O^-$, and $^{56}Fe^{16}O^-$ according to the sample composition. The achieved mass resolving power enabled isotopic differences of ^{13}C and ^{12}C to be quantified. For the imaging of the spatial distribution of isotopic enrichment, for instance samples enriched with ^{13}C or ^{15}N, the enrichment should be above 1 atom%. Large geometry SIMS (e.g. Cameca SIMS 1280) applications can distinguish small isotopic differences but at much lower lateral resolution (down to 50 nm; Kollmer et al., 2020) where natural abundance differences are more important (Schopf et al., 2018).

Limitations and potential solutions: One challenge of the NanoSIMS is sample preparation. Flat sample topography is crucial for sufficient measurement results. Two methods are generally used to prepare soil samples, the embedding and sectioning of intact soil, and the direct analysis of microscale particles placed on flat sample substrates, e.g. Si wafers (Mueller et al., 2013).

Linking with theories: NanoSIMS is suitable for testing the spatial changes of SOC persistence. Whereas it is possible to measure isotopic ratios at natural abundance (e.g. Fike et al., 2008) using spot measurement, the major advantage of NanoSIMS for SOM research is the high resolution imaging of isotopically enriched samples (Figures 2 and S1). This allows the trace of isotopically labeled substrates at the actual process scale for plant-soil interactions, plant-microorganism interactions or on surfaces of micro and macroaggregates. The mechanisms of microbially regulated retention of SOC can be laterally resolved and tracked using NanoSIMS in the form of biologically relevant elements in the organo-mineral associations (Keiluweit et al., 2012). The use of NanoSIMS can enable 3D studies of stable ^{13}C and ^{15}N isotopes in organo-mineral associations by recording depth profiles of the submicron elemental composition of soil aggregates via ion sputtering (Mueller et al., 2012). It has been shown that N-rich compounds are critical for interfacial organo-mineral (Kopittke et al., 2018, 2020a) and organo-organic interactions (Possinger et al., 2020a, 2020b; Vogel et al., 2014) through microbial N assimilation. The development of SOM sequestered in new forming soils after glacial retreat are imprinted in the micro-spatial succession of SOM coatings of soil minerals as indicated by the increasing $^{12}C^{14}N^-$: ($^{12}C^{14}N^-$ + $^{12}C^-$) ratio, indicative of increasing microbial N assimilation (Schweizer et al., 2018).

NanoSIMS has also been used to test the mechanism of continuous adsorption-desorption of OC (Sec. 3.2). Combined NEXAFS-STXM and NanoSIMS investigations on extracellular polymeric substances suggests that fresh input of SOM does not adsorb to open mineral surfaces but rather onto existing native SOM and accumulates in crevices not pores (Liu et al., 2013). It is also possible to use NanoSIMS to analyze biogeochemical interfaces between plants, microorganisms, and soil minerals directly within the intact 3D soil structure (Figure 5; Kaiser et al., 2015; Vidal et al., 2018, 2019; Gorka et al., 2019). Thus, NanoSIMS facilitates the understanding of intact biogeochemical interfaces and the related formation of persistent SOM (Schlüter et al., 2019).

4.3. Complementary high-resolution techniques for studying the nature of SOM beyond C

4.3.1. X-ray tomography (CT) for 3D reconstruction of soil

X-ray tomography (CT) can provide a three-dimensional reconstruction of the physical and chemical properties of soil. CT uses X-rays to create virtual cross-sections of a physical object
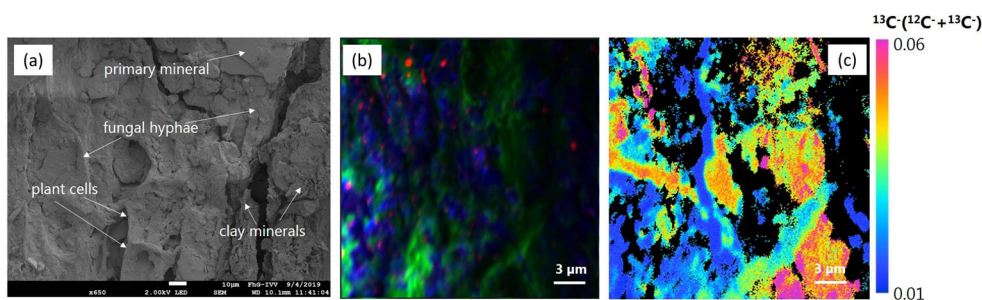
Figure 5. The formation of mineral associated OM (MAOM) on the surface of decaying particulate organic matter (POM) (Witzgall unpublished data). (a) SEM micrograph of the POM surface with still recognizable plant cells is covered by microbial residues (extrapolymeric substances), microbial cells (fungal hyphae), primary minerals and clay minerals. (b) Composite NanoSIMS image of elemental distribution of iron (red, $^{56}Fe^{16}O^-$), organic matter (green, $^{12}C^{14}N^-$) and silica (blue, $^{28}Si^-$). (c) Isotopic ratio map of $^{13}C^-/(^{12}C^- + ^{13}C^-)$ highlighting the clear enrichment of the microbial residues and hyphal structures on the POM surface (sample details in Witzgall et al., 2021).

that can be used to recreate a virtual model (3D model) without destroying the original object (Hou et al., 2021). Recent advances in CT enable the three-dimensional soil morphology characterization of intact samples using conventional (Dal Ferro & Morari, 2015) or synchrotron-based sources (Arai et al., 2019; Lammel et al., 2019; Peth et al., 2014; Quigley et al., 2018; Rawlins et al., 2016). 3D printing of soil coupling with conventional CT can reproduce the complex pore network at a similar scale in a silty-loam soil with a resolution of 80 μm (Dal Ferro & Morari, 2015). The *in situ* distribution of SOM in intact soil aggregates (5 mm) can be obtained using a combination of synchrotron-based X-ray CT (9.77 μm resolution) and osmium vapor as a staining agent for SOM (Peth et al., 2014). The application of osmium-thiocarbohydrazide-osmium (OTO) staining of unsaturated C in the solution has been well developed for electron microscopic observation of membrane structure since the 1960s (Seligman et al., 1966). The adaptation of OTO in combination with dual energy synchrotron-based μCT has been reported to improve the spatial resolution of C-enriched areas in the mineral associations (ca. 1 μm; Arai et al., 2019) by 4- to 10-fold compared with previous attempts (4–13 μm; Peth et al., 2014; Rawlins et al., 2016; Quigley et al., 2018). Combination of synchrotron- and conventional μCT may be adapted for studying microbial-driven processes in association with SOC dynamics (Sec. 3.3). The iodine treatment of soil samples offers a unique analytical opportunity to detect fungi (synchrotron K edge CT), soil fauna (conventional μCT; e.g. *Collembola*) and roots in undisturbed soil compared with alterative agents such as silver and eosin (Br based) (Lammel et al., 2019).

4.3.2. X-ray fluorescence microscopy (XFM) for visualization of soil nutrients

X-ray fluorescence microscopy (XFM) can be used to visualize the lateral distribution of elements in plants and soil minerals (Kopittke et al., 2020b). XFM operates using the same principles as STXM, but operates at higher X-ray energies and thus allows detection of heavier elements (P to Ag at the K edge). Specifically, in XFM, high energy incident X-rays are used to produce characteristic fluorescent X-rays. When a sample is progressively raster scanned using a focused incident X-ray beam, the characteristic fluorescent X-rays can be determined at each pixel of the sample, yielding an elemental distribution map. XFM is generally used for elemental mapping (Kemner et al., 2004), but can also be used to determine elemental speciation through micro-XANES (i.e. point XANES analyses) or through XANES mapping (Etschmann et al., 2014; Kopittke et al., 2014) in a manner similar to combining STXM with NEXAFS (see Sec. 4.1.3).

Despite C being too light for XFM beamlines, it can be complementary by examining soil nutrients and other elements which play an important role in organo-mineral-metal interactions for SOC persistence (Sec. 3.2). Generally, XFM is synchrotron-based, with resolution being in the

range of ∼1 µm to ∼10 nm. There are also bench-scale XFM systems, with these generally having a resolution of >∼5 µm. These bench-scale systems are also considerably slower (longer dwell times) than synchrotron sources.

As a recommendation of future research, we suggest to explore coupling XFM with XANES to spatially resolve the role of Al and Fe-mineral interactions in acid soils and Ca-mediated mechanisms in alkaline calcareous soils for SOC persistence (Sec. 3.2). Another possibility for understanding the role of SOM in nutrient cycling is to analyze the spatial distribution of available nutrients in the form of cations (e.g. Zn, Ca, Fe etc.) and anions (e.g. P) in soil.

5. Combined *in situ* approaches to study the space-time-composition continuum

Given the strengths and weaknesses of the various individual approaches, there is an increasing need to compare (Supplementary information) and use a combination of *in situ*, high-resolution, complementary techniques for the study of SOM. Such interdisciplinary research will advance nano- and microscale understanding of the nature of SOM, and mechanisms like mineralization or the retention and protection of SOC, which are critical to mitigate climate change.

Complementary use of techniques can potentially provide important information for understanding C behavior in soils. For example, combining µCT, SEM-EDX, and NanoSIMS, the spatial distribution of bacteria in soil indicates a preferential foraging close to macropore surfaces and fresh particulate organic matter (Schlüter et al., 2019). At a microorganism level, a shift from plant litter to mineral associated organic matter in earthworm casts was observed using TEM and NanoSIMS (Vidal et al., 2019). Observations such as these support that spatial assemblage of SOM in relation to microbial accessibility is key to SOC persistence (Sec. 3.2). Furthermore, the role of Al and Fe-mineral interactions for SOC persistence in acid soils has been visualized using complementary *in situ* approaches. For example, submicron-scale investigations using NanoSIMS, XPS and NEXAFS reveal the mineral protection of SOC by Al and Fe minerals with swine manure fertilization over 24 years compared with inorganic fertilization (Xiao et al., 2016).

Upscaling of fine-scale processes requires statistical and graphical presentation of approaches that demonstrate that random sampling of a small spot has high probability of capturing a relevant process (Figures 2 and S1). One possibility is to use geospatial techniques from larger scales that have not been sufficiently utilized at the nano- and micro-scale. Application of remote sensing science has been trialed to upscale nanometer NEXAFS SOC data to micrometre-scale aggregates (Milne et al., 2011). Furthermore, the application of visible to near infrared laboratory imaging spectroscopy has been used to evaluate permafrost soil complexity (Mueller et al., 2021) and characterization, classification and mapping of elemental concentrations in soil profiles (Steffens & Buddenbaum, 2013). These techniques can provide possible upscaling. High-resolution investigations discussed in this review can confirm a mechanism hypothesized at one scale and can then generate new hypotheses to be tested at another. Factors such as elemental speciation, level of oxidation, and organo-mineral interaction complicate the measurement of relevant molecular characteristics of SOM. The quantification of such relationships is important to explain substrate preference by microbial communities and to predict the persistence of individual compounds. It remains unknown how to characterize or calculate molecular diversity that is relevant for OC persistence (Lehmann et al., 2020). The challenge lies in how to link the concentrations of particular molecular groups with functional composition and spatial distribution. Another possibility for potential upscaling is the use of 'models with intent'. Predictive models have been used to simulate climate sensitivity (Lugato et al., 2021) and soil C storage capacity (Cotrufo et al., 2019) of particulate and mineral-associated SOM, microbial feedback of SOC dynamics (Woolf & Lehmann, 2019), continental molecular composition of SOC (Hall et al., 2020), and regional rhizosphere priming (Keuper et al., 2020). There are opportunities to incorporate high-resolution *in situ* measurements in those models from microscopic understanding to macroscopic implications.

6. Recommendations and conclusions

This review critically examines how state-of-the-art technologies can be employed to explore the current theories and hypotheses regarding the behavior of SOM. This review addressed new theories regarding the SOM persistence, such as the importance of the interactions between the molecular diversity of organic compounds, and their spatial heterogeneity and temporal variability. We highlighted that a mechanistic understanding of these processes occurring within organo-mineral and organo-organic assemblages requires noninvasive techniques that minimize any disturbance to the physical and chemical integrity of the sample.

It is clear that there are a multitude of opportunities for further studies, especially for complementary techniques, such as:

- The combined spectroscopic- and microspectroscopic techniques discussed in the current review can determine 2D surface chemistry and/or 2D projections of a 3D soil (Figure S1). In this regard, it is important to develop 3D techniques to understand the biogeochemical SOC cycling in a physically representative environment to preserve its spatial structure. Of interest, it must be noted that many previous studies state that they discuss 'spatial' information but only report two-dimensional information (strictly speaking, 'spatial' refers to three dimensional analyses).
- Mechanisms associated with organo-mineral and organo-organic interactions need to be differentiated from spatial co-location between decomposer and substrate. High-resolution investigations can confirm a mechanism hypothesized as a result of an investigation at a coarser scale, such as through incubations or batch adsorption experiments. At the same time, high-resolution investigations can generate hypotheses that must then be confirmed at a coarser scale. For example, organo-organic interactions observed at a sub-nanometer scale (Possinger et al., 2020a) need to be tested at a microscale (Figure 1).
- Few measurements exist at 20–30 nm x-y resolution with a depth of 100–300 nm (thickness of the section) using NEXAFS-STXM, and very rarely with STEM-EELS at 0.1 nm x-y resolution with 50 nm depth. That means we do not know the interface well. Future studies should consider approaches with a range of spot size and analysis depth and explore approaches to capture all three dimensions (lateral resolution *vs.* depth; Figure S1).
- We need tomography of EELS, NEXAFS, and even XPS to improve our understanding of the bonds at the submicron and nanoscale interfaces which can test the theory of the continuous adsorption-desorption of OC onto mineral surfaces. Recently, atomic force microscopy has been shown to directly measure the binding of C functional groups at the organo-mineral interface (Newcomb et al., 2017) with the potential to create a 3D tomography.

We encourage further research to better understand the complex behavior of C in soils, with this being required in order to resolve the interactions between the molecular diversity of organic compounds, and the importance of their spatial heterogeneity and temporal variability. By better understanding the nature of SOM, it will allow the optimization of SOC sequestration to meet the 4 per mille (4 per 1000) initiative. This information is critical in developing broad-scale decision-making frameworks to understand how the soil type is expected to influence the stabilization of OC following changes in either land management practices or changes in land use.

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