Chapter 5
Exometabolomics for Linking Soil Carbon Dynamics to Microbial Communities

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1 Introduction

Microbial metabolism has helped shape the world into what it is today, and continues to drive biogeochemical cycles (Falkowski et al. 2008) including the carbon cycle. Soil microorganisms play a central role in the global carbon cycle, with an estimated soil carbon pool of 2500 Gt, over three times the size of the atmospheric carbon pool (Lal 2004). Inputs of organic carbon into soil is largely plant and microbial biomass-derived, and carbon is released from soil into the atmosphere mainly as CO_2, the product of plant root, and microbial respiration (Johnston et al. 2004). While we are able to measure emergent properties such as the total release of CO_2 from soil and total organic carbon in soil at a particular time, the underlying processes that occur between input and release are not well defined. This limits our ability to understand how human activities are altering the balance of the global carbon cycle, and how this will affect soil carbon dynamics (Lal 2004) that are mediated by soil microbial community metabolism.

The bulk of microbial community studies have been based on metagenomics approaches, where total genomic DNA from soil is sequenced (Delmont et al. 2011; Fierer et al. 2012; Roesch et al. 2007). This culture-independent approach yields insights into the microbial community structure (phylogenetic makeup), and has become a very active field of research due to advances in sequencing technologies (Franzosa et al. 2015). Besides community structure, metagenomes also reveal the complement of genes present in soil microbial communities, reflecting their potential metabolic functions which are inferences based on often poorly annotated

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genomes. These inferences of in situ metabolic processes can be strengthened through metatranscriptomics and metaproteomic studies of environmental samples, since these analyses enable correlation of genes actively transcribed and translated (respectively) in the community with environmental variables and stresses (Morales and Holben 2011).

Metabolomics is emerging as a very promising complement to soil metagenomics approaches as it can provide direct insights into the functioning of soil microbial communities in their environment. Exometabolomics, the study of how cells transform their extracellular small molecule environment (Silva and Northen 2015), is particularly relevant for studying soil metabolic processes and provides an experimental approach to link organic carbon in the soil to the metabolism of particular microorganisms or taxonomic group. Soils are complex mixtures of organic and inorganic components, and are estimated to contain more than two-thirds of the carbon in the terrestrial biosphere (Lal 2004). The organic components, known as soil organic matter (SOM), make up most of this pool, and thereby comprise the largest reservoir of carbon on Earth. While total pools of organic carbon in soil can be estimated, the form it takes has been contested (Lehmann and Kleber 2015). Most organic matter inputs to soil decompose within a year (Jenkinson and Rayner 1977). Initial degradation is performed by exoenzymes released by fungi and bacteria that break down organic matter into pieces small enough to be assimilated by microbial cells (Baldock and Nelson 2000; Weiss et al. 1991). A longstanding view was that some of the degraded organic carbon was assimilated into microbial biomass, and the rest was converted to large stable polymeric compounds called humic substances (Stevenson 1994). Their stability was thought to account for the large belowground pool of organic carbon. However, advances in analytical techniques in the last few decades revealed a lack of evidence for polymeric humic substances in soil (Piccolo 2002). Recent evidence suggests that soil organic matter is rather a continuum of progressively decomposing organic compounds (Fig. 1) (Lehmann and Kleber 2015). The new view suggests that much of the SOM exists in the form of lower molecular weight molecules (below 600 Da). Their persistence in soils is not due to any inherent recalcitrance of these molecules, but rather to factors related to the environment, such as absence of degraders or consumers in the immediate environment, sorption onto mineral surfaces, formation of noncovalently bonded aggregates, water availability, pH, and redox state (Schmidt et al. 2011).

2 Exometabolomics for Analysis of Soil Organic Matter

Metabolomics involves the study of the metabolome, defined as the low molecular-weight metabolites (typically less than 2000 Da) present in a cell or living organism under a given set of physiological conditions (Harrigan and Goodacre 2003; Oliver et al. 1998). By contrast, exometabolomics aims to characterize extracellular small metabolites (Silva and Northen 2015). By studying

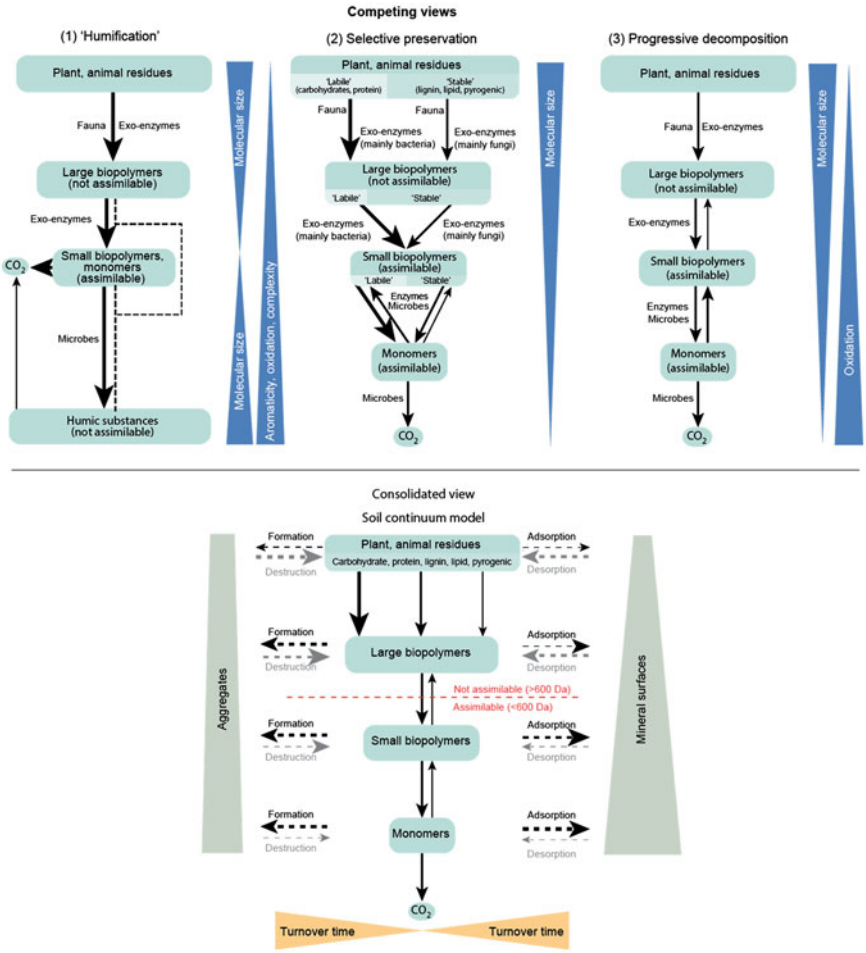

Fig. 1 Schematic representation of three competing models for the fate of organic inputs to soil (*top*), and the recently proposed soil continuum model (*below*). Selective preservation assumes that some organic materials are preferentially mineralized, leaving intrinsically 'stable' decomposition products behind. Progressive decomposition reflects the concept of microbial processing of large plant biopolymers to smaller molecules. In the proposed SCM, a continuum of organic fragments is continuously processed by the decomposer community from large plant and animal residues toward smaller molecular size. At the same time, greater oxidation of the organic materials increases solubility in water as well as the opportunity for protection against further decomposition through greater reactivity toward mineral surfaces and incorporation into aggregates. *Dashed arrow lines* denote mainly abiotic transfer; *solid lines* denote mainly biotic transfer; *thicker lines* indicate more rapid rates; *larger boxes* and *ends of wedges* illustrate greater pool sizes; all differences are illustrative. All *arrows* represent processes that are a function of temperature, moisture, and the biota present. Reprinted from Lehmann and Kleber (2015), with permission

metabolites consumed from or secreted into the extracellular environment, insights can be gained into the metabolic activity of the cell (Kell et al. 2005). This approach (also known as metabolic footprinting) has been applied to characterize yeast mutant metabolism and phenotypes (Allen et al. 2003; Castrillo et al. 2007; Mas et al. 2007). Exometabolomics has also been applied in industrial settings, where analysis of extracellular fermentation media are part of the process of optimizing yeast fermentation conditions (Devantier et al. 2005; Fu et al. 2014), for monitoring various industrially important bacterial and yeast strains in bioreactor cultures (Paczia et al. 2012), and to study the breakdown of polysaccharides by anaerobic bacterial strains (Villas-Boas et al. 2006). Apart from some recent studies (Swenson et al. 2015b; Warren 2014), few examples of the application of exometabolomics to characterize soil microbial communities have been reported, though dissolved organic matter has been characterized in sea and river water with an exometabolomics approach (Kido Soule et al. 2015; Morales-Cid et al. 2009).

A major reason for the paucity of soil exometabolomics studies is the complexity of soil, and the associated challenges of extraction and sample preparation. The extraction method used in any metabolomics experiment is critical to the quality of the data obtained. The choice of extraction method should allow effective extraction of metabolites from the system under study, without artifact formation or compound degradation. In our current understanding of the nature of SOM (Lehmann and Kleber 2015), the organic compounds that make up SOM exist in different compartments with different degrees of biological accessibility. The soluble component of SOM is the most accessible to processing by soil microbes, and is referred to as dissolved organic matter (DOM). DOM is often defined as dissolved metabolites able to pass through a 0.45 μm filter (Gregorich et al. 2000), to differentiate it from particulate organic matter. In order to characterize DOM in traditional soil science, various methodological approaches involving extraction from soils have been developed (Zsolnay 2003). These often involve extraction of soil under relatively gentle conditions (e.g. aqueous salt solutions) to yield a fraction referred to as water extractable organic matter (WEOM). This fraction conceptually consists of the mobile and available portion of the total DOM pool (Corvasce et al. 2006). An example of such an extraction procedure involves extraction of soil with concentrated salt solutions (e.g. up to 500 mM K_2SO_4) for a few hours, followed by filtration or centrifugation, and analysis for total organic carbon (Jones and Willett 2006). The high salt concentration in the extraction buffer helps extract mineral sorbed metabolites, but can cause issues with downstream sample preparation and metabolite analysis in metabolomics methods used to characterize individual components of DOM (e.g. formation of salt crystals in samples, ion suppression in mass spectrometry, and decrease in sensitivity in NMR) (Annesley 2003; Kelly et al. 2002). Therefore, in recent metabolomics studies, water-based extraction methods were developed to extract organic matter from soils. Warren (2013a, b) extracted field-moist soils in water by shaking for 10 min, followed by centrifugation and filtration. The relatively short extraction time addressed concerns over continued metabolism during extraction, which could give rise to altered metabolite profiles (Rousk and Jones 2010). Swenson et al. (2015b) followed a similar process

but performed aqueous extraction of soils for one h at 4 °C to slow any metabolic activity present. Another concern with aqueous extraction is enrichment of the metabolite profile by intracellular metabolites. Osmotic shock can potentially lyze microbial cells and cause leakage of metabolites (Gregorich et al. 2000). Swenson et al. (2015b) compared aqueous soil extracts to samples extracted in 10 mM K_2SO_4 and 10 mM NH_4HCO_3, and found no significant qualitative differences in metabolites detected. It was concluded that water is a suitable extractant for soil exometabolomics of DOM and that these extracts would be most representative of the types of resources available for soil microbes.

In some cases, an experiment may require analysis of soil intracellular and extracellular metabolites. In this case, cell lysis is an important and desirable step in sample preparation. To access intracellular metabolites, a traditional approach used in soil science involves chloroform fumigation of soil to lyze microbial cells, followed by extraction (Brookes et al. 1985; Vance et al. 1987). Swenson et al. (2015b) compared metabolite profiles of water extracts of fumigated and unfumigated soil samples (Fig. 2). A significant increase in the number and abundance of metabolites was observed, however, the fumigation technique requires long times of exposure to chloroform vapors, which raise concerns about continued metabolic activity or increased enzymatic degradation of metabolites (Warren 2013a, b). The use of organic solvents which are able to lyze microbial cells is another way to obtain soil extracts containing intracellular and extracellular metabolites. This was demonstrated by Swenson et al. (2015b) who used hierarchical cluster analysis to show similarity in metabolite patterns between fumigated soil extracts and organic solvent extracts of unfumigated soil. Soil was also directly extracted with chloroform and K_2SO_4 solution (1:4, v/v) to obtain extracts containing intracellular metabolites (Kakumanu et al. 2013). Rochfort et al. (2015) extracted freeze-dried, finely ground soil with an 8:2 methanol-water solution by sonication for 10 min. Since this method breaks up soil aggregates in addition to using organic solvents, it is not surprising that it provided extracts with a wide coverage of metabolite classes, derived from the intracellular and extracellular soil metabolite pools.

The major analytical methods used in the field of metabolomics are based on Mass Spectrometry (MS) and Nuclear Magnetic Resonance (NMR) (Dettmer et al. 2007; Dunn et al. 2005; Forseth and Schroeder 2011). Each method has advantages and disadvantages related to sensitivity, structural information, ease of quantitation, breadth of metabolite coverage, and availability of structural databases for identification (Lenz and Wilson 2007). Although studies on the soil microbial exometabolome are limited, a few recent examples demonstrate the utility of these methods to this field.

Gas Chromatography coupled with Mass Spectrometry (GC-MS) has previously been applied to targeted analyses of particular chemical classes of small soil metabolites such as sugars or amino acids (Kakumanu et al. 2013). GC-MS is also well suited for measuring a broad range of small metabolite classes, and has been widely used in untargeted metabolomics studies in plants (Jenkins et al. 2004), human biofluids (Garcia and Barbas 2011), and microbial biomass (Koek et al. 2006). Analysis of hydrophilic/polar metabolites by GC-MS requires derivatization

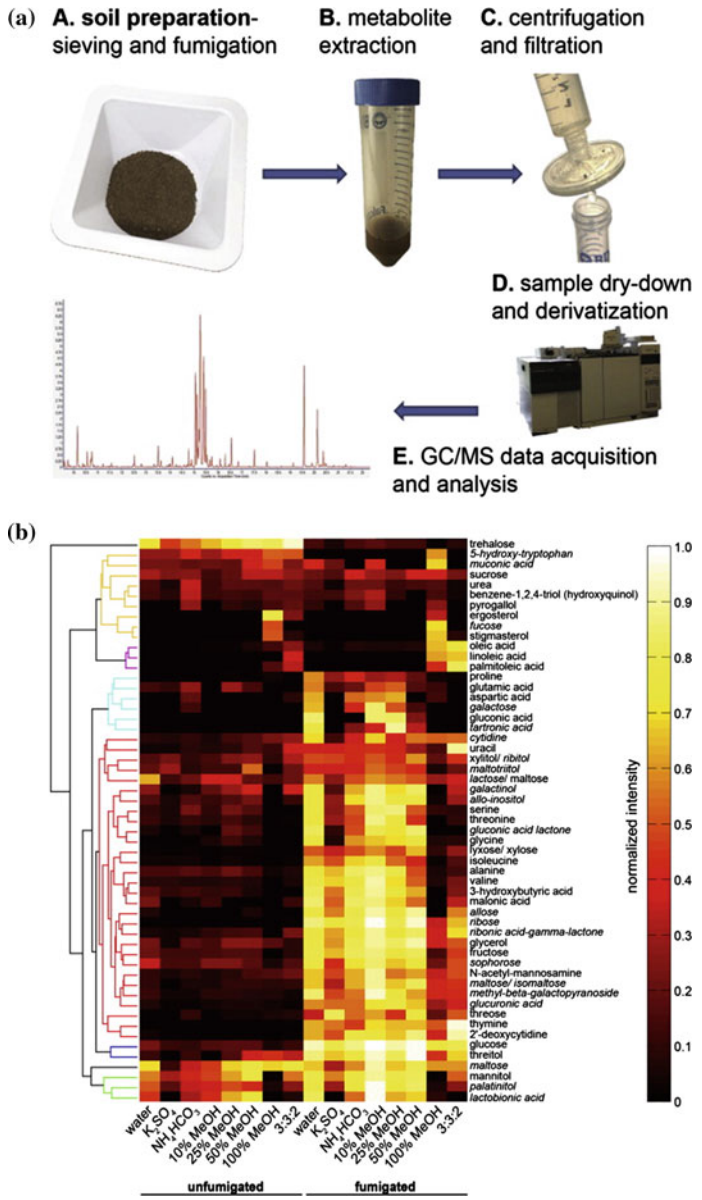

Fig. 2 **a** Workflow for soil DOM extraction: *A* Soil is sieved through 2 mm and fumigated with chloroform for 24 h to access intracellular metabolites or left unfumigated for extracellular metabolites. *B* Soil is extracted with the appropriate solution in a 1:4 ratio (2 g soil: 8 mL extractant) on an orbital shaker for 1 h at 4 °C. *C* Extract is centrifuged to pellet soil and the supernatant filtered through 0.45 μm filter discs. *D* Dried down and derivatized for GC-MS. *E* Data are analyzed for metabolite identification. **b** Relative intensity of metabolites in extracts of unfumigated and fumigated soil prepared with different extractants, and analyzed by GC-MS. Reprinted from (Swenson et al. 2015b), with permission

to increase the volatility of compounds. Swenson et al. (2015) characterized soil extracts using GC-MS. After extraction with different solvents, samples were derivatized by methoxyamination and trimethylsilylation. Hundreds of unique features were detected, of these 55 were confidently annotated using the Fiehn spectral metabolite database (Kind et al. 2009) and comparison with authentic standards. Metabolites detected in all samples included sugars, sugar alcohols, amino acids and amino acid metabolites, nucleobases, carboxylic acids, and sterols (Swenson et al. 2015b).

Liquid Chromatography coupled with Mass Spectrometry (LC-MS) has become an important analytical tool in metabolomics, and has also been applied in studies on many biological systems (Theodoridis et al. 2008; Zhou et al. 2012). Separation of metabolites is achieved by LC using various stationary phases depending on the polarity of the target metabolites. There are various options available for ion sources and mass analyzers in LC-MS systems (reviewed by Zhou et al. 2012). Due to the high structural diversity of metabolites, a particular sample typically needs to be analyzed in positive and negative ionization mode to obtain a good coverage of the metabolome. DOM is by definition composed of small metabolites dissolved in water in situ (Leenheer and Croué 2003). This fraction of SOM is therefore amenable to separation by hydrophilic interaction liquid chromatography (HILIC), a variant of normal phase chromatography (Alpert 1990). Baran et al. (2015) analyzed extracellular soil water, as well as intracellular metabolites of isolates from biological soil crust, with LC-MS using zwitterionic HILIC chromatography and electrospray ionization (ESI). Out of nearly 500 molecular features detected in this study, 79 metabolites were identified based on MS/MS data and comparison with authentic reference standards. A similar method was used by Swenson et al. (2015a) to study sorption of microbially derived metabolites onto mineral surfaces.

Capillary electrophoresis mass spectrometry (CE-MS) is suitable for the analysis of charged metabolites and has found applications in metabolomics studies summarized in a series of reviews (Ramautar et al. 2009, 2011, 2013). In a study focusing on the pool of nitrogen metabolites in soil (dissolved organic nitrogen: DON), Warren (2013a) employed a CE-MS procedure. This method allowed detection of small metabolites ionizable by electrospray that are cationic at low pH. Approximately 100 nitrogen-containing metabolites with a wide range of polarities were detected, of which 57 were identified (Warren 2013a).

Fourier transform-ion cyclotron resonance-mass spectrometry (FT-ICR-MS) is an established method for analyzing natural organic matter, and has been widely used to characterize complex organic mixtures in environmental samples (Kujawinski 2002). This method allows the detection of ions with excellent mass accuracy and resolving power, so that unique empirical formulas can be assigned to most of the thousands of signals detected (Hockaday et al. 2006). Based on atomic ratios (e.g. H:C, O:C) these formulas can be assigned to chemical classes such as carbohydrates, lipids, lignins, tannins, and proteins (Ohno et al. 2014). While individual features are not unambiguously identified using this technique, FT-ICR-MS is very useful for obtaining overviews of patterns in soil DOM dynamics (Hockaday et al. 2006, Kujawinski et al. 2004; Ohno et al. 2014).

NMR is an established analytical platform in the field of metabolomics. It has been applied in analysis of human biofluids (Nicholson and Lindon 2008), plants (Kim et al. 2010), and microbiological samples (Grivet et al. 2003). NMR exometabolomics has been extensively applied to study microbial cell culture systems (Behrends et al. 2014; Resmer and White 2011; Szeto et al. 2010). While solid-state NMR techniques have been employed to analyze macromolecules and structural aspects in soils (Baldock et al. 1992; Kögel-Knabner 1997), there are few examples of NMR used for the characterization of the small metabolite complement (microbial- or plant-derived) of SOM. Jones et al. (2014) analyzed extracts of soils from former mine sites by NMR. The aim was to obtain a survey of the naturally occurring products of soil community metabolism (including intracellular metabolites). NMR spectra were dominated by sugars, and a range of other metabolites such as amino acids and nucleosides were detected. A recent study also characterized soil extracts by NMR for a comparison of native versus agricultural soils (Rochfort et al. 2015). Complex spectra were obtained that were dominated by sugar resonances. Lipophilic compounds (terpenes, lipids) were also detected due to the extraction solvents having a higher organic solvent composition than that used by Jones et al. (2014).

3 Exometabolomics for Analysis of Whole Microbial Communities

The exometabolome of a complex soil microbial community comprises the sum of small metabolites being produced or released, and consumed by all the metabolic activity in the soil. The exometabolome is thus a reflection of the net metabolic state of the community. Studying differences in the microbial community exometabolome under different conditions can lead to insights into the response of communities as a whole. In one of the first exometabolomics studies on complex microbial communities, Henriques et al. (2007) applied an LC-MS based approach to analyze soluble metabolites in wastewater treatment plant communities. Activated sludge cultures from four different wastewater treatment plants were exposed to four different chemical stressors known to affect the processing ability of such communities. Comparisons of metabolite profiles between untreated and treated samples using multivariate statistical methods revealed clear patterns between the different toxin-stressed cultures. A limited number of variables were able to discriminate samples based on chemical treatment, which was community-independent. It was concluded that the discriminant metabolites may be universal biomarkers for these stress conditions, and that these may be used in developing early warning tools for toxins in these systems (Henriques et al. 2007). Exometabolomics has also been applied to analyze uptake and release of extracellular metabolites from microbial biofilm consortia occurring in water pipes (Beale et al. 2010). Small metabolite profiles, obtained by GC-MS, of water flowing through copper pipe systems

differentiated samples exposed to copper corroding microbial biofilms from those that were not (Beale et al. 2012). In a pilot study this approach was applied to a water supply network, where it provided information on biofilm activity in the system. This approach showed potential for elucidating the relationship between specific metabolites in water supply networks and issues related to water quality, caused by microbial biofilms (Beale et al. 2013).

The effect of human activities on soil systems have been the topic of metabolomics field studies. In a report on soils from former mine sites in the UK, Jones et al. (2014) employed NMR and principal component analysis (PCA) to compare metabolite profiles of soil extracts. Soil sites under study were geographically dispersed and had a range of physicochemical properties. The PCA grouped some sites together based on similarity of their overall profiles. The authors concluded that the observed patterns are likely due to the similar pollution patterns at the sites, but did not do further in-depth analysis of the factors potentially underlying the observed patterns. Another NMR-based study compared soil extracts (intra- and extracellular metabolites) of geochemically matched remnant (native) and agricultural (managed) soils (Rochfort et al. 2015). When subjected to multivariate data analysis, samples were grouped together based on land use. NMR resonances responsible for the observed groupings were assigned to characteristic terpene and aromatic compound signals in the remnant soils, and sugar and lipid signals in the agricultural soils. Soil samples were analyzed in parallel by mid-infrared (MIR) spectroscopy, a technique that employs absorption and transmission of photons in the infrared energy range (about 2500–25,000 nm in the electromagnetic spectrum), to characterize molecules based on their constituent bonds. (Bellon-Maurel and McBratney 2011). Multivariate data analysis of these data resulted in samples clustering together based on location, irrespective of land use. Soil extracts were also tested for antibacterial activity, and the most active extracts were from native soil samples that clustered together by PCA analysis. This study established that the two analytical methods captured different aspects of the soil, namely soil biochemistry (NMR) and soil physicochemistry (MIR). It also demonstrated how biochemical characteristics as measured in this metabolomics study can be related to functional aspects of soil communities as a whole.

The above studies followed an untargeted metabolomics approach, where metabolite profiles were measured and compared between samples without identifying the compounds responsible for discriminating groups. Rochfort et al. (2015) were able to assign important discriminating NMR signals to compound classes (e.g. terpenes and aromatics), but noted that further characterization would be needed to confidently identify individual metabolites. This untargeted approach is widely used in other fields employing metabolomics (Sévin et al. 2015), but also points to a larger issue in soil exometabolomics, i.e., very little data on the composition of soil metabolites. The studies mentioned in the analytical methods section comprise the few that have contributed to the broad qualitative profiling of multiple compound classes in soil (as opposed to targeted methods for one compound class at a time). Even fewer have attempted to characterize soil metabolites in a quantitative manner. One exception is Warren (2013b) who performed a broad

analysis of small nitrogen-containing metabolites in different soil types dominated by different vegetation. The relative proportions of the different compound classes in this pool of small metabolites were determined. The relative abundance of the top ten small nitrogen-containing metabolites in each soil type was also analyzed. Even though the study of Warren (2013b) focused on a particular subset of metabolites, such a detailed quantitative analysis lays an important foundation for understanding what is in the soil exometabolome. Similar characterizations are needed that include a broader range of metabolite classes, and relate these to differences in factors such as vegetation type and physicochemical factors.

Warren (2013b) pointed out that soil water extracts may not accurately represent what is biologically available, since differential adsorption to the soil stationary phase may occur. The mineral content and surface area of soils are known to affect the solid-state partitioning of and thereby the accessibility of DOM components to microorganisms (Kalbitz et al. 2000). However, these processes are not understood down to a metabolite-specific level. Recently, Swenson et al. (2015a) investigated the sorption of small metabolites from a soil bacterial lysate on an iron oxide mineral, ferrihydrite. Different metabolite classes were adsorbed to different degrees, with phosphate-containing metabolites, for example, showing the highest sorption (Fig. 3), while other metabolites were not adsorbed, suggesting their higher degree of bioavailability in iron-rich soils. Since high-sorbing metabolites were able to displace sorbed phosphate from the ferrihydrite, the authors hypothesized that the release of such metabolites by soil microbes may be a strategy to access phosphate in soils where it is limiting. More studies of the effects of minerals on the bioavailability of small metabolites will help elucidate the role abiotic factors play in SOM dynamics of different environments.

4 Who Does What in Soil Community: Characterizing Metabolism of Individual Members

One approach to understanding the dynamics of a microbial community is to characterize the individual members of the community in isolation. Studying the uptake and release of metabolites through a particular microbial isolate in the laboratory, insights can be gained into its metabolic interactions with the environment. Baran et al. (2011) used an untargeted metabolite footprinting approach to characterize the marine cyanobacterium *Synechococcus* sp. PCC 7002 cultured in different growth media (Fig. 4). A wide variety of metabolites were found to be taken up by this strain, and analysis of intracellular metabolites also provided insights into which metabolites were actively turned over and which were maintained in cells in their native states. A study on acid mine drainage used an exometabolomics approach to study the role of the primary producer *Euglena mutabilis* in these oligotrophic environments (Halter et al. 2012). The exo- and endo-metabolome of *E. mutabilis* was profiled in situ and also for laboratory grown cultures. A number of metabolites

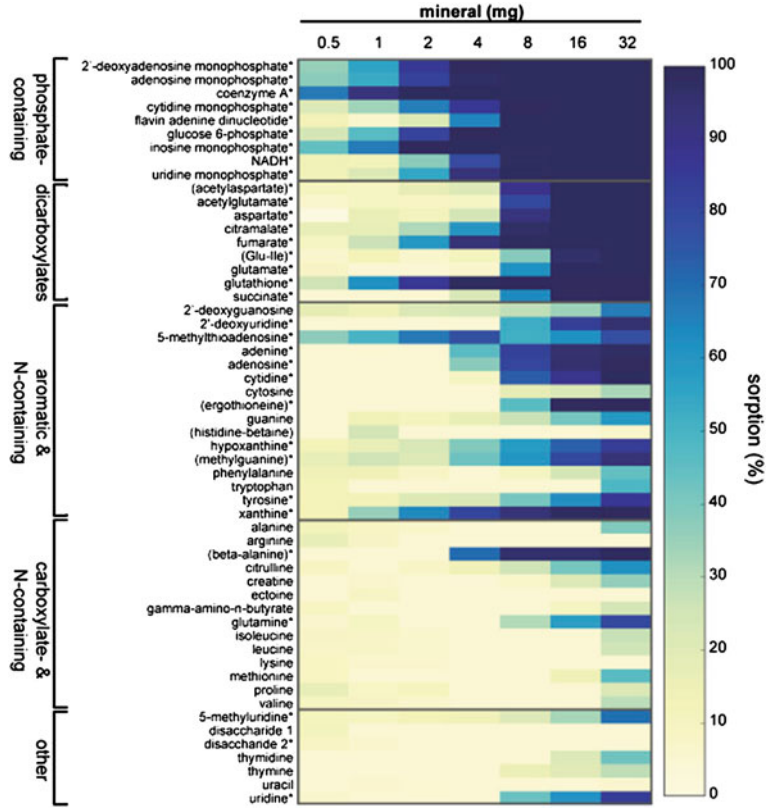

Fig. 3 Sorption of small metabolites from a soil bacterial lysate on an iron oxide mineral, ferrihydrite. For each metabolite, the percent sorption (relative to the non-mineral control) is displayed as mineral concentration increased from 0.5–32 mg. Metabolites were analyzed by LC-MS. Putatively identified metabolites are indicated by parentheses. Reprinted from Swenson et al. (2015a), with permission

in the acid mine drainage exometabolome were found to be secreted by the cells in laboratory cultures. This suggested an important role in organic matter production by *E. mutabilis* for consumption by other microbial strains in this ecosystem.

Baran et al. (2015) extended this approach by characterizing multiple isolates from a biological soil crust (biocrust) community. The primary producer in this community, the filamentous cyanobacterium *Microcoleus vaginatus*, was cultured in the laboratory. Exo- and endo-metabolite profiling revealed that many metabolites were released into the culture medium by this strain. Seven bacterial isolates, representing diverse phyla from the biocrust environment, were cultured individually in different-rich media to characterize their substrate preferences. Only a small proportion of metabolites detected in the media were taken up by any given strain, and there was little overlap between the strains' preferred substrates. Metabolite

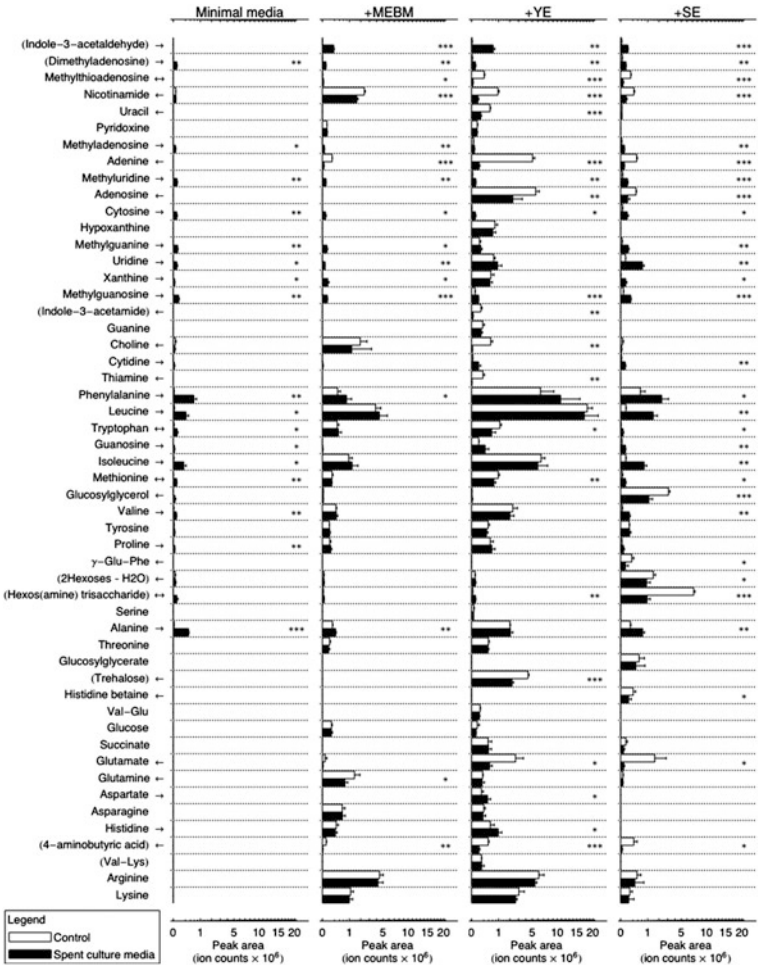

Fig. 4 Comparison of levels of selected metabolites in the growth media following growth of *Synechococcus* (*full bars*) against their levels in control media (*open bars*, $n = 4$), as determined by LC-MS. The peak areas axis was scaled with a square root to improve the visualization of smaller peaks. Statistically significant differences are indicated as "*" ($p < 0.05$), "**" ($p < 0.01$), or "***" ($p < 0.001$). An *arrow* is shown next to the name of a metabolite if it was found to be significantly consumed (←), released (→), or both consumed and released (↔). Reprinted from Baran et al. (2011), with permission

profiling of the biocrust soil water was also performed to link the observed patterns from the isolates to the intact microbial community. Changes in metabolite profiles at different times following wet up of desiccated biocrust showed patterns similar to those observed in the individual isolate experiments. This study revealed the particular substrate preferences of sympatric isolates from a soil community, which suggest that exometabolite niche partitioning may be an important driver in

maintaining soil microbial diversity. Conversely, if different microbial phyla have different roles in processing soil organic matter, it follows that changes in soil microbial diversity may affect carbon cycling in soils.

Integrating exometabolomics data from various soil isolates would be a useful way to form hypotheses about the relationships between different strains in a particular environment. An online data repository, Web of Microbes, has been developed for such exometabolomics data (webofmicrobes.org). This tool allows rapid visualizations of large exometabolomics datasets of individual isolates that enables predictions to be made about how they behave in a community. This includes interactions such as potential resource competition and cross-feeding between strains, and how these relationships would be affected by changes in the chemical environment. Characterizing individual isolates from a soil community can shed light on how they behave in relation to other soil community members. However, this approach is limited to strains that can be cultured outside of their native habitat, thereby excluding the vast majority of the soil microbial diversity (Schloss and Handelsman 2003). Hence, there is a need for methods that enable the study of soil microbial communities in situ, to link specific functions to particular community members, and to elucidate the metabolic interactions between them.

5 Stable Isotope Probing: Tracking Flow of Substrates Through Communities

Stable isotope probing (SIP) techniques involves addition of a stable isotope enriched substrate, and tracking its fate as it is transformed by the metabolism of community members into labeled molecules/biomarkers (Dumont and Murrell 2005). Variations of SIP target different biomarkers that become labeled as a result of growth on the labeled substrate. One approach targets microbial phospholipid fatty acids (PLFAs). Since different microbial classes possess characteristic fatty acids as part of their cell membranes, selective extraction and analysis of PLFA patterns is an established approach for determining the composition of microbial communities (Zelles 1999). In PLFA-SIP, tracking which is the characteristic phospholipid fatty acids become labeled with the stable isotope yields information about which groups of microbes were responsible for metabolizing the labeled substrate. This approach has been used to identify groups of microorganisms performing particular functions in soils based on labeling with substrates such as $^{13}CH_4$ (Bull et al. 2000), ^{13}C-acetate (Boschker et al. 1998), ^{13}C- glucose, and -ribose (Apostel et al. 2015). Uniformly labeled ^{13}C-cellulose and ^{13}C-lignin were substrates in a PLFA-SIP study on the role of diverse microbial groups in plant polymer degradation (Torres et al. 2014). Treonis et al. (2004) combined a $^{13}CO_2$-labeling experiment with PLFA analysis to identify microbes assimilating plant root

exudates. The disadvantage of this approach is based on PLFA biomarkers determined from cultivated microbes limiting application to uncultivable microorganisms.

Another approach relies on combining SIP with nucleic acid analysis. This relies on the incorporation of the isotopic label into DNA or RNA, so that subsequent separation and sequencing of the labeled fraction identifies community members actively incorporating the labeled substrate (Dumont and Murrell 2005). DNA-SIP has mostly been used with ^{13}C-labeled substrates, such as $^{13}CH_3OH$ to study soil methylotrophs (Radajewski et al. 2000), and ^{13}C-naphthalene and other organic compounds to characterize pollutant biodegraders (Padmanabhan et al. 2003). Using ^{13}C-cellulose as a substrate, El Zahar Haicher et al. (2007) identified cellulose degraders in a soil community using a DNA-SIP approach. These included bacteria not previously known to have this ability, as well as a number of uncultured strains. A disadvantage of DNA-SIP is that a relatively large amount of labeled substrate is needed, together with long incubation times to allow production of highly labeled ^{13}C genomic DNA. Artificially high substrate concentrations are thus often applied to soils, which can cause biases in how the community behaves (Dumont and Murrell 2005). The incorporation of ^{13}C into RNA occurs earlier than DNA (Manefield et al. 2002), therefore targeting RNA as the labeled biomarker molecules in RNA-SIP allows the use of shorter incubation times. RNA-SIP also allows detection of cells which are metabolically active, even though they are not dividing or growing (El Zahar Haichar et al. 2007). A combination of DNA- and RNA-SIP can potentially be a very powerful approach. Recently, $H_2^{18}O$ was applied to a soil bacterial community as a universal substrate, and was found to be an effective label for DNA- and RNA-based SIP approaches for studying active members of the community (Rettedal and Brözel 2015).

SIP approaches monitoring the changes in labeling patterns over time can yield valuable information on how nutrients flow through a microbial community. Labeled substrates will become incorporated into cells (e.g. as part of PLFA, DNA, RNA) but a proportion will be transformed and transported out of the cell, where it may be consumed by other members of the community food web (DeRito et al. 2005). Extending the analysis of labeled biomolecules to the community exometabolite pool is thus a potentially powerful approach for elucidating how these trophic interactions occur. Date et al. (2010) combined DNA-SIP with NMR exometabolomics to study microbial variability and metabolic dynamics in a mouse gut microbial community. Using labeled glucose as a sole carbon source, metabolic-microbial correlation analysis was performed, allowing identification of glucose-utilizing gut microbes and their extracellular metabolites. Microbial strains consuming the metabolites produced by the glucose utilizers were also identified, together with their extracellular metabolites. The study demonstrated that the feasibility of this approach for tracking carbon flux within a microbial community by identifying members of the community involved at different steps, as well as the metabolites that mediate their interactions. This approach clearly has great potential to address questions about carbon flux in the context of soil microbial communities.

Labeling experiments with stable isotopes can also aid analysis of the highly complex soil exometabolome. For example, in NMR-based studies, the low natural abundance of the magnetic isotope of carbon (^{13}C) results in low sensitivity of detection in unlabeled systems. Signals are dramatically enhanced as metabolites become labeled with ^{13}C isotopes, thereby facilitating identification of metabolites downstream of the labeled substrate (Schneider et al. 2003). In mass spectrometry based methods coupled to chromatographic separations, labeled metabolites can be detected at the same retention time as their unlabeled counterparts, with characteristic shifts in mass spectral features corresponding to the number of incorporated labeled isotopes (Rodgers et al. 2000). Computational methods have been developed for the quantitative detection of features derived from a particular labeled compound, even when not all metabolites are identified (Hiller et al. 2010). However, stable isotope labeling can greatly facilitate unambiguous assignment of chemical formulas, and thereby identification of unknown features (Baran et al. 2010). Thus, SIP methods show great promise for reducing noise by highlighting relevant metabolites and pathways, and identifying unknown metabolites in complex datasets such as those generated in soil exometabolomics experiments.

6 Metabolite Imaging: Microbial Interactions Through Space

Soil is a very heterogeneous matrix, in which biotic and abiotic factors combine to create diverse microclimates. Studies on soil microbial communities are often conducted on homogenized bulk soil samples, in which the spatial structure of soil and soil microorganisms are disrupted (Becker et al. 2006). Yet, observations of microbial communities at the micron scale have revealed defined spatial organization. For example, in dental plaque, a nine taxon microbial consortium was observed to be radially arranged around cells of filamentous bacteria (Welch et al. 2016). Obligate aerobes were arranged around the periphery of the consortium, anaerobes were found in the interior, and others were localized in ways suggestive of their functional roles in the consortium. Such structured assemblages have been observed in biofilms and consortia occurring in aquatic systems, on leaf and root surfaces, and in pathogenic or commensal associations with humans (Almstrand et al. 2013; Wessel et al. 2013). It is believed that the spatial arrangement of soil microbial communities is a very important driver of microbial diversity in soil, thereby also of microbial community functioning (Ettema and Wardle 2002). Therefore, the next level of detail required to understand microbial soil communities is characterizing their functioning in space. To achieve this, experimental methods are needed to observe specific community members and their activities in relation to other community members in their native spatial arrangement.

Experimental approaches utilizing labeled substrates have been successfully used to visualize and identify labeled microbial cells taking up a specific compound. FISH-microautoradiography or fluorescence are approaches for detecting bacterial cells that have consumed and metabolized a specific radioactive substrate, and identification of these cells using an oligonucleotide probe (Adamczyk et al. 2003; Lee et al. 1999). The use of radioactive labels are less desirable, and recent technological advances have yielded promising alternative approaches for analyzing interactions between microorganisms and their chemical environment (Wessel et al. 2013). Orphan et al. (2001) used FISH in combination with Secondary Ion Mass Spectrometry (SIMS) to detect and visualize ^{13}C profiles in microbial consortia composed of archaea and sulfate-reducing bacteria. Lower $^{13}C/^{12}C$ ratios in both the archaea and associated bacteria provided evidence that methane was consumed by the former, and that methane-derived carbon was transferred to the latter consortium members. SIMS is ideally suited to detect isotopes at very fine resolution, for example, nanoSIMS can image with some 50 nm resolution. Therefore, the combination of SIMS with stable isotope probing (SIP) shows great promise for spatially resolved analysis of single microbial cells and their utilization of particular substrates (Behrens et al. 2008; Chandra et al. 2008; Cliff et al. 2002).

The above-mentioned methods allowed tracking of substrates into identifiable microbial cells. Ideally, the metabolites released into the environment and exchanged between community members should also be characterized. Besides SIMS, other Mass Spectrometry Imaging techniques are potentially well suited to do this, since a broad range of metabolites can be detected without the need for labeling with a radioactive or stable isotope (reviewed by Watrous and Dorrestein 2011). In Mass Spectrometry Imaging techniques, ionization probes generate ions on the sample surface, and the sample stage is moved in the *x*–*y* plane so that this is done across a defined sample area. Mass analyzers detect the generated ions, resulting in a grid of data points each with its own mass spectrum. An ion map can be made from these data showing the location and intensity of detected ions across the measured sample surface.

In specific Mass Spectrometry Imaging techniques (e.g. nanoDESI-IMS), samples are detected at atmospheric pressure, and samples can be wet (e.g. fresh samples of bacteria on an agar plate can be analyzed directly), while for others (e.g. MALDI-IMS), dry samples are covered in matrix and ionization and detection occurs under high vacuum (Wessel et al. 2013). In nanostructure-initiator mass spectrometry (NIMS), microbial agar cultures cannot be analyzed directly since desorption/ionization occurs at the bottom of the sample (Woo et al. 2008). For this technique, "replica extraction transfer" is used to transfer metabolites from the culture surface to the NIMS surface, allowing spatial arrangement of metabolites to be retained (Louie et al. 2013). These approaches have been used to characterize metabolites produced and released by microorganisms on solid culture media (Fig. 5). For example, Traxler et al. (2013) detected a suite of secondary metabolites released by *Streptomyces coelicolor* in response to interactions with various Actinomycetes. Watrous et al. (2013) used nanoDESI to profile metabolites in single colonies of *Schewanella oneidensis* MR-1 and *Bacillus subtilis* 3610, as well

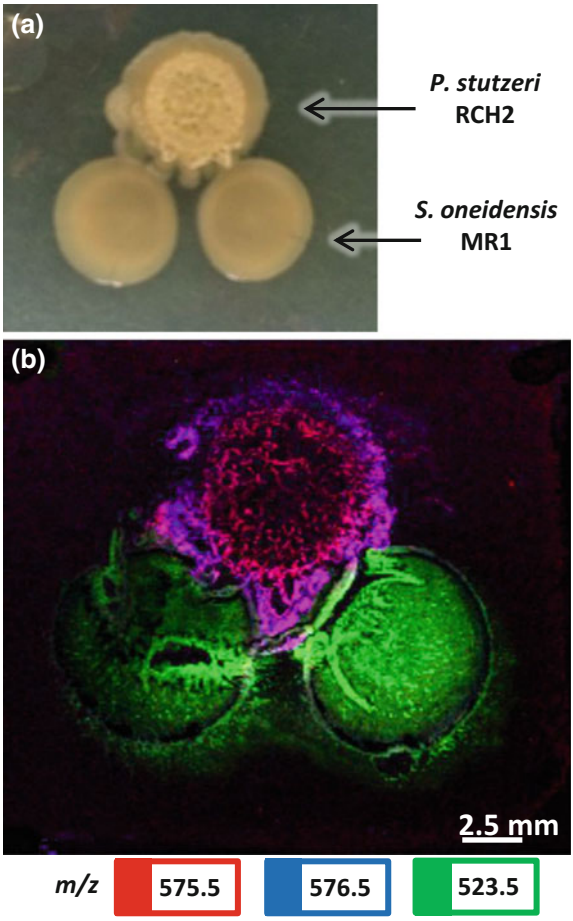

Fig. 5 Mass spectrometry imaging of *P. stutzeri* RCH2 and *S. oneidensis* MR1 coculture. **a** Optical image of coculture on solid medium. **b** Tricolor mass spectrometry image of coculture with *m/z* corresponding to species-specific lipids (Katherine Louie, unpublished)

as a mixed biofilm of these strains. A range of peptides, lipids, and small molecules were detected (Watrous et al. 2013). A REX-NIMS approach was used by Louie et al. (2013) to identify ions localized to regions within and between bacterial colonies cultured individually and in coculture on agar media. In a study on methanotrophic microbial mats from sea shelf methane seeps, Time-of-Flight Secondary Ion Mass Spectrometry (TOF-SIMS) was used to characterize microbial lipid biomarkers (Thiel et al. 2007). Characteristic lipid classes were detected in distinguished areas, which matched the presence of different microbial colonies as determined by conventional microscopic techniques.

Mass Spectrometry Imaging techniques show great promise for characterization of microbial communities in space, however, technical challenges limit their application to complex microbial communities in soil environments. Benefits and challenges of different imaging mass spectrometry techniques were comprehensively reviewed by Watrous and Dorrestein (2011). While able to detect metabolites with a wide mass range, the resolution of methods such as MALDI-IMS and nanoDESI currently does not allow imaging down to the single-cell level. Dynamic SIMS offers the best spatial resolution (sub-μm scale) but does not provide molecular information beyond elements and small atomic clusters. Another challenge of imaging experiments targeting small metabolites lies in the sample preparation. Methods for preparing thin soil sections as used in soil sciences typically involve fixing in formaldehyde, washing and impregnation in resin (Nunan et al. 2001), which would not be suitable for IMS. Tissue samples (e.g. mammalian, plant) are usually embedded in a substance such as OTC polymer, gelatin, or agarose gel for stabilization during cryosectioning (Cornett et al. 2007; Lee et al. 2012). Even if such treatment would not cause delocalization of metabolites in soil samples, the heterogeneous physical structure of soil may hamper cutting thin sections for imaging. Most IMS experiments to date involve laboratory cultured agar samples which can be analyzed fresh or mounted and dehydrated in preparation for imaging experiments (Traxler et al. 2013; Yang et al. 2012). Studies where microbial consortia from a natural environment were used either involved smears of soil on the sample target (Orphan et al. 2001), or cryosectioning of well-structured communities such as microbial mats (Thiel et al. 2007) or symbionts associated with other organisms (Lechene et al. 2007; Schoenian et al. 2011). Given the great potential of these approaches, there is an urgent need for improved sample preparation methods that will enable small metabolite imaging of soil microbial communities in their natural spatial arrangement.

7 Conclusions and Future Outlook

Recent technological and methodological advances have led to great progress in understanding the linkages between microbial diversity and ecosystem functioning (Bardgett et al. 2008). Metagenomics approaches have enabled characterization of the members of the soil community, including uncultivable microorganisms. Other

molecular methods and SIP approaches have improved understanding of the particular members of the soil community's metabolic capabilities. What members of a particular soil microbial community actually do, will depend on the substrates that are available in their environment. Therefore, exometabolomics is a very promising approach in that it provides direct evidence of the soil metabolites available to soil microorganisms and how the available substrates are transformed by microbial community metabolism.

This chapter reviewed the handful of reports where an exometabolomics approach was applied to the study of intact soil microbial communities, or to laboratory experiments focusing on a particular aspect of such complex systems. Soil microbial communities are very complex, and soils are extremely heterogeneous matrices, so it is not surprising that there are many technical challenges that remain to be resolved in this field. Care should be taken to use sample preparation methods appropriate for the specific question being asked and analytical method being used. There is no single analytical method that can detect the massive diversity of metabolites across large dynamic ranges in an unbiased way. The choice of analytical method will depend on the focus and needs of the study, and combinations of complementary techniques may offer a more comprehensive coverage of diverse chemical classes (Simpson et al. 2004; Werf et al. 2007). It is important to keep in mind that the mineral composition and other factors may confound analysis by preferentially sorbing certain metabolites (e.g. ferrihydrite sorbing phosphate containing metabolites) making it challenging to compare soil types.

As with any metabolomics workflow, soil community exometabolomics experiments generate large datasets. Untargeted metabolomics results usually include many detected features that remain unidentified. There are several well-established mass spectrometry and NMR databases that can aid in identification of such unknowns (Kind et al. 2009; Smith et al. 2005; Wishart 2007). Many of these target intracellular metabolism of organisms such as yeast or plants (Bais et al. 2010; Hummel et al. 2007; Jewison et al. 2011). Since much soil organic matter is derived from plant and microbial biomass, these are useful to soil organic matter characterizations. There is currently great interest in secondary metabolites from soil microorganisms, and increasing the number of entries in databases of such compounds will also be very helpful in the context of soil exometabolomics (Hadjithomas et al. 2015). Many workflows have been developed for the analysis of large metabolomics datasets, which are also applicable to exometabolomics data analysis (Bowen and Northen 2010; Rübel et al. 2013; Tautenhahn et al. 2012; Xia et al. 2012). Data analysis tools for interpreting data in SIP experiments will be of particular value (Hiller et al. 2010; Huang et al. 2014). Any experimental setup and data reported should meet the quality and reporting standards as set by the larger metabolomics community (Goodacre et al. 2007).

The full potential of soil microbial community exometabolomics will be realized when it can be integrated with other approaches such as metagenomics, metatranscriptomics, and metaproteomics. A recent review describes examples where such multiomics approaches were applied to understanding microbial communities

(Franzosa et al. 2015). Careful planning of experimental design and data integration strategies are needed to derive the most value out of such combined approaches (Muller et al. 2013). Such data integration should result in improved mechanistic models of the structure and functioning of soil microbial communities that can be tested in combinations of laboratory and field experiments (Franzosa et al. 2015). This will enable better predictions of the effects of environmental perturbations on soil carbon cycling by soil microorganisms.

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