**Soil metabolomics - current challenges and future perspectives**

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**Abstract**

Soil is an extremely complex and dynamic matrix, in part, due to the wide diversity of organisms living within it. Soil organic matter (SOM) is the fundamental substrate on which the delivery of ecosystem services depends, providing the metabolic fuel to drive soil function. As such, studying the soil metabolome (the diversity and concentration of low molecular weight metabolites), as a subset of SOM, holds the potential to greatly expand our understanding of the behaviour, fate, interaction and functional significance of small organic molecules in soil. Encompassing a wide range of chemical classes (including amino acids, peptides, lipids and carbohydrates) and a large number of individual molecules (ca. *n* = 105 to 106), the metabolome is a resultant (indirect) output of several layers of a biological hierarchy, namely the metagenome, metatranscriptome and metaproteome. As such, it may also provide support and validation for these “multi-omics” datasets. We present a case for the increased use of untargeted metabolomics in soil biochemistry, particularly for furthering our fundamental understanding of the functions driving SOM composition and biogeochemical cycling. Further, we discuss the scale of the challenge in terms of metabolite extraction, analysis and interpretation in complex plant-soil-microbial systems. Lastly, we highlight key knowledge gaps which currently limit our use of metabolomic approaches to better understand soil processes, including: (i) interpretation of large untargeted metabolomic datasets; (ii) the source, emission and fate of soil-derived volatile organic compounds (VOCs), (iii) assessing temporal fluxes of metabolites, and (iv) monitoring ecological interactions in the rhizosphere. While the application of metabolomics in ecosystem science is still in its relative infancy, its importance in understanding the biochemical system in relation to regulation, management and underpinning the delivery of ecosystem services is key to further elucidating the complex links between organisms, as well as the fundamental ability of the biological community to process and cycle key nutrients.

**Keywords:** dissolved organic carbon, soil organic matter, biochemical profiling, nutrient cycling, soil quality.

**1. Introduction**

Soil is key to the survival of life on Earth and supports a wide range of ecosystem services, for example, nutrient cycling, carbon (C) sequestration, climate regulation and flood prevention (Brown et al., 2021a; Chomel et al., 2016; Jones et al., 2014; Lehmann et al., 2020; Pereira et al., 2018). However, sustaining soil function into the future is a major challenge, due to increasing agricultural intensification, acidification, salinisation, biological invasions, desertification and urbanisation (Pravalie, 2021), in addition to increasingly unpredictable weather patterns (Borrelli et al., 2020). Consequently, a deeper understanding of soil processes and the design of effective management strategies remains key to maintaining soil health and quality (Baveye, 2015). Soil biology is now recognised as the key driver of soil functioning. However, it is generally underrepresented in soil quality assessments, likely due to its immense complexity, both in terms of multispecies interaction, and interpretation, compared to more traditional chemical and physical properties (Bünemann et al., 2018). A combined suite of approaches is therefore needed to fully integrate biological indicators into routine soil monitoring. While major advances have been made in evaluating the diversity of organisms which live in soil (e.g., via metagenomics and community profiling), directly linking this to changes in the amount and chemical properties of soil organic matter (SOM) and soil functioning has proven difficult (Lehmann et al., 2020).

SOM, consisting of any material in the soil that was originally produced by living organisms, at is the universal substrate to which most of life on Earth owes its existence. In addition, the amount and quality of SOM and its predominantly biotically-mediated transformations underpin the delivery of most ecosystem services and nutrient cycles in soil (Jiao et al., 2019; Roth et al., 2019). The cycling of nutrients in soil (i.e., C, nitrogen (N), phosphorus (P) and sulphur (S)), while occurring on a nanoscale, have global implications in terms of C (and nutrient) cycling (net emission or sequestration; Gougoulias et al., 2014). Generally, we have a reasonable understanding of the higher-level processes governing the cycling of these nutrients (e.g., how environmental conditions affect aerobic and anaerobic soil respiration, C use efficiency, or nitrification/denitrification), however, their fluxes are notoriously difficult to quantify *in situ* and their measurement must be interpreted with caution (Hazard et al., 2021). Further, there is little understanding of the small molecule interactions underpinning these processes. This is not surprising given the multitude of compounds, organisms and potential metabolic pathways likely to be involved (Dijkstra et al., 2022). To date, most characterisation of SOM has been associated with techniques that provide a broad view of its chemical composition (e.g., nuclear magnetic resonance spectroscopy (NMR; Sonsri et al., 2022), Fourier-transform infrared spectroscopy (FTIR; Pärnpuu et al., 2022), pyrolysis-gas chromatography-mass spectrometry (Py-GC-MS; Chen et al., 2020)). In addition, the turnover rate of SOM is normally quantified by measuring total soil respiration or heat production (calorimetry; Barros et al., 2007; Dufour et al., 2022; Fraser et al., 2016). While this provides a total integration of biological activity occurring in a soil, it provides no detail about the processes involved. Similarly, research has largely focused on measuring the total dissolved organic C (DOC) pool in soil solution which provides little functional insight (Wang et al., 2021). It is also thought that the composition of this DOC pool largely reflects the high molecular weight end-products of microbial SOM turnover, rather than the compounds that underpin soil respiration, which are cycled very rapidly through the DOC pool (van Hees et al., 2005; Glanville et al., 2012). Therefore, the exo- and endo- microbial metabolome, as a subsection of the DOC pool, is a rich source of information which can provide functional insights into soil processes. Here, we propose that the holistic study of metabolites provide an excellent opportunity to explore soil function as the output of the metabolome on a cellular level is a product of several layers of a biological hierarchy, namely the genome, transcriptome and proteome (see Fig. 1). It should be noted that microbial death may also contribute to the release of metabolites into soil, however, these are expected to be short lived (van Hees et al., 2005).

Metabolomics, the study of small molecules (typically 100–1500 g mol−1; i.e., metabolites, chemical substances produced by organismal metabolism), is a cross-cutting technique and has been widely applied across the life sciences. For example, the application of metabolomic analysis to a single cell, organism or species is widely used to understand; virus-host interactions (Kumar et al., 2020), environmental stresses on shellfish (Viant et al., 2003), or nutritional requirements of single species (Creek et al., 2013). However, in recent years the field of metabolomics in ecosystem science has evolved, enabling the ability to characterise the interactions between a community of organisms and their environment (Bundy et al., 2009), for example assessing functional metabolome change with substrate addition (Brown et al., 2022) or environmental change (e.g., drought; Warren, 2014; Brown et al., 2021a).

The application of metabolomics to soil science is a nascent field, but one with large potential. From the 4 × 105 vascular plant species on the planet (Willis, 2017) it is estimated that there are between 2 × 105 and 1 × 106 individual active metabolites (Rai et al., 2017; Fang et al., 2019), with any single species containing ≥ 5 × 104 compounds (Fernie et al., 2004). These will include a range of molecules from the various subsets of metabolome, for example the central metabolites, sugars and carbohydrates, organic acids and purines, amines and peptides, free fatty acids and lipids as well as volatile compounds (isoprene and monoterpenes) (Brown et al., 2022; Demiwal et al., 2024; Hoyos et al., 2024). This metabolic diversity is key to organismal development, function and maintenance of a diverse community, often occupying a plethora of metabolic and environmental niches. It also indicates that the thousands of metabolites are simultaneously being turned over in soil at any point in time (e.g., in response to changes in often dynamic environmental conditions; plant cell lysis, apoptotic events etc.). The number of compounds in plants and those subsequently detected in the soil also suggests that a large proportion probably remain undetected with conventional analytical approaches. While turnover rates for some metabolites in plants and the bacterium *E. coli* have been shown to be in the order of seconds (Stitt, 1990; Yuan et al., 2006), little work has performed to understand these rates in soils. Intermediates may also be generated during the formation and breakdown of cellular metabolites. This is exemplified by proteolysis which can occur either extracellularly or intracellularly. Given the chemical and structural diversity of proteins in the microbial community, their breakdown may lead to the formation of numerous oligopeptides. For example, *E. coli* contains 4285 individual protein-encoding genes with each cell containing 107 proteins per cell, proteolysis may therefore lead to the generation of > 1015 different oligopeptides (Serres et al., 2001; Shaffer et al., 2022; Soufi et al., 2015).

Metabolomics encompasses a broad range of analytical techniques and metabolic subclasses (Table 1). Therefore, the ability to tailor analysis to answer specific hypotheses and research questions is a strength. However, the complexity of the metabolome is often a hindrance to interpretation (Fig. 2). This has led to most studies being undertaken under controlled laboratory conditions, but rarely validated in the field. Here, we focus on untargeted metabolomics, the analysis of all the measurable analytes in a sample, including chemical unknowns, with a focus on primary and some secondary metabolism (defined in Table 1). This paper aims to identify the challenges in terms of metabolite extraction and interpretation in complex plant-soil-microbial systems, and highlight key knowledge gaps which currently limit the use of metabolomic approaches to better understand soil processes.

**2. The importance of metabolomics in the study of soil biochemistry**

Previously, particular focus has been placed on understanding SOM quality (segregated into water extractable, i.e., accessible compounds, and solvent extractable, i.e., intracellular compounds and as well as more recalcitrant/polar extracellular compounds) (Swenson et al., 2015; Swenson and Northen, 2019). However, little is known about the small molecule composition of soils and their fate. Where this work has been undertaken, it has tended to focus on a specific group of compounds including organic acids (Jones, 1998; Henneron et al., 2020), lipids (Bull et al., 1998; 2000a), amino acids (Czaban et al., 2018; Noll et al., 2019), amino sugars (Amelung et al., 1999), and allelochemicals (Scavo et al., 2019) which provide only small pieces of the metabolic jigsaw puzzle. However, an untargeted approach, examining the whole metabolome, may provide a greater understanding of C cycling, with potentially significant implications for C budgets and fluxes within and from soils (Brown et al., 2022; Overy et al., 2021). When linked to stable isotope labelling of SOM (e.g., 13C/12C, 15N/14N, 34S/32S, 18O/16O) it is also possible to simultaneously trace the flux of individual metabolites in soil. This stable isotope probing approach has been used extensively for phospholipids (e.g., Bull et al., 2000b; Maxfield and Evershed, 2014; Watzinger et al., 2019) and amino acids (e.g., Knowles et al., 2010; Dippold et al., 2014) and extended to amino sugars (e.g., He et al., 2006; Reay et al., 2019), however, it has not yet been widely applied to other compound groups. This approach also has the potential to track the source and fate of metabolites derived from plants by 13CO2 labelling the shoots (Huege et al., 2007).

Equally, ecological metabolomics (ecometabolomics), the exploration of the ecophysiological and ecological function of organisms and ecosystems through metabolomic analysis, is also a field of growing importance (Sardans et al., 2011). In this sense, metabolomics application is not only limited to soil microbial communities, but also to the metabolic health of soil dwelling macrofauna (e.g., earthworms; Rochfort et al., 2008; Tang et al., 2020; Zhu et al., 2020). As the performance of analytical equipment improves, the resolution at which inter- and intra-species biochemical interactions may be observed increases. This is particularly the case with extracellular volatile metabolites (the “volatilome”, a subset of the metabolome; Tholl et al., 2021), which may be profiled in real-time, non-invasively (Seewald et al., 2010).

While only beginning to be elucidated, small molecule interactions are likely to play a central role in the fundamental understanding of environmental interactions and ultimately environmental quality. Examples of its use include pollution monitoring (Jones et al., 2014), soil quality assessment (Brown et al., 2021b; Withers et al., 2020) and quantifying soil stress (Lankadurai et al., 2013) as well as the discovery of new compounds for pesticide and drug discovery (Aliferis et al., 2010; Atanasov et al., 2021; Gupta et al., 2018). While soil biological function is typically estimated from an integrated signal of many processes operating simultaneously (e.g., greenhouse gas emissions), metabolomics may be used to elucidate functional change at much higher resolution and smaller spatial scale. Increasing our understanding of functional metabolomics to that of functional metagenomics (Ungerer et al., 2007; Yan and Xu, 2018; Danczak et al., 2020), may enable metabolites to be ascribed to, or be indicative of, specific processes and further elucidate metabolic pathways adding to the Kyoto Encyclopaedia of Genes and Genomes (KEGG) and equivalent, environmentally focused, databases (Kanehisa and Goto, 2000; Kanehisa et al., 2017). Known examples of this include the role of organic acids in regulating P availability in soil (Menezes-Blackburn et al., 2016; Zhu et al., 2021), amino acids in drought stress tolerance (Kushal et al., 2015; You et al., 2019) and specific peptides in disease regulation (Datta et al., 2015; Khademi et al., 2020). This search for “biomarkers” maybe useful in the assessment of biological soil health categorising or identifying soils at risk, similar to the approach taken in the biological and medical sciences (Chadeau-Hyam et al., 2010). However, these represents the tip of the iceberg, with molecular diversity having been proposed as a key factor in the persistence of soil organic carbon (SOC) (Lehmann et al., 2020). Significant work, including high powered ‘discovery’ metabolomics (Chen et al., 2021), is therefore required to fully elucidate the biochemical complexity of the soil, and its links to the wider environment.

**3. Current limitations of metabolome studies**

*3.1. The inherent complexity of soil as a substrate*

Soil is extremely spatially heterogeneous, reflecting the intrinsic large-scale variability in soil mineralogy, organic matter, physical structure, vegetation and land use management regime (Mao et al., 2014; Gravuer et al., 2020), down to the fine-scale variations in chemistry within individual soil aggregates or exudate composition along a root (Zausig et al., 1993; Dong et al., 2019). Rapid temporal changes almost certainly add to a soil’s complexity, with changes in metabolic profiles occurring within minutes of perturbation (Gunina et al., 2017), or diurnally in response to plant rhizodeposition and microbial turnover (Hubbard et al., 2017; Newman et al., 2022).

Such spatial and temporal variability is just the first level of complexity soil poses as a substrate, and differences in extraction methodologies for the soil matrix provide further complication. To date, a range of available extraction solvents have been used to target different SOM fractions. The two most common approaches involve the use of water (i.e., directly biologically available) or organic solvents (examining intracellular, available and soil adsorbed/absorbed depending on choice of solvent), or a combination or both, to recover metabolites from soil. The choice of solvent, however, can greatly influence which compounds are recovered. Consequently, studies using different extraction methodologies should be compared to one another with caution (Tfaily et al., 2015; 2017; Bell et al., 2022). Metabolomic extraction methods are often optimised for specific soil types (Swenson et al., 2015; Jenkins et al., 2017) or environmental conditions (e.g., saline soils are likely to hinder the analysis of metabolites (Annesley, 2003; Xu et al., 2021)), or have been adapted from other research fields and are potentially not optimised for soil at all (Fiehn, 2016; Withers et al., 2020). With potential for low recovery of distinct compound classes (e.g., anionic and cationic compounds) due to sorption to the solid phase (Swenson et al., 2015), this adds complexity to optimising an extraction method, and a key challenge is ensuring differences in studies encompassing a range of soil (and thus sorption capacities) are reflective of the metabolomic profile rather than artefacts of the extraction procedure. It may therefore be desirable to undertake sequential extraction (e.g. water followed by solvent), or parallel extracts with different solvents that are combined to provide a more holistic view of the metabolome. This also applies to fumigation (Table 2), used to release intracellular metabolites for metabolomic analysis. Continued enzymatic processing during fumigation can be influenced by soil type, or the fumigation procedure itself, further complicating intercomparison between datasets (Blankship et al., 2014; Swenson et al., 2015; Bell et al., 2022). Another factor which needs to be considered is potential contamination by plants roots during soil extraction (Oburger and Jones, 2018). It is extremely difficult to remove soil adhering to the surface of roots without damaging root tissues and releasing cellular content. Similarly, the addition of solvents would also inevitably lead to a loss of solutes. One approach to try and estimate contamination from this source is the quantification of root-specific marker compounds. The separation of roots and analysis of their metabolite profile may also help the interpretation of soil metabolomic profiles, allowing differentiation of metabolite source, this may be particularly pertinent in rhizosphere dominated soil systems (e.g., grasslands).

*3.2. Variation in analytical approaches and scope of identification*

The diversity of analysis techniques (Table 2) creates further difficulties for the direct comparison of datasets. No single analytical method can detect all metabolites due to their molecular complexity and diversity (as illustrated in Fig. 2). Using direct infusion (DI), coupled to high resolution accurate mass (HRAM) mass spectrometry (MS), with high resolving power and mass accuracy (e.g., Fourier-transform ion cyclotron resonance (FT-ICR)-MS, Orbitrap MS; Hawkes et al., 2016; Bahureksa et al., 2021), it is possible to simultaneously detect thousands of compounds. However, spectral congestion can be problematic for peak identification and multi-stage mass spectrometry (MSn) analysis, although less so for FT-ICR-MS (Hawkes et al., 2016; Viant and Sommer, 2013; Bahureksa et al., 2021). Furthermore, identification is often restricted to putative assignment of molecular formulae. This limits analysis in terms of the ability to link to soil function and health, and other ‘omics techniques. Additionally, the assignment of molecular formulae is subjective, with varying criteria applied to define these (Koch et al., 2017). Hence, the use of Direct infusion (DI)-HRAM MS for initial screening, and application of multivariate statistics to identify potentially ecologically significant compounds, prior to identification *via* hyphenated MS, is now commonplace in environmental metabolomics (Ohno et al., 2010; Ruff et al., 2015; Hollender et al., 2017; Pemberton et al., 2019). Coupling MS with liquid chromatography (LC), gas chromatography (GC) or capillary electrophoresis (CE) (i.e., hyphenation) can help resolve spectral congestion, and aid identification by extending the analytical window to cover a broader range of metabolites (e.g., Warren et al., 2014; Jenkins et al., 2017). While hyphenated-MS platforms are routinely used in an untargeted way, it should be appreciated that due to the analytical window of each technique and the resultant decrease in the number of compounds that may be analysed (Table 2), the selected analytical approach is tailored to answer specific hypotheses.

A key advantage of hyphenated-MS is the additional time dimension, which, with MS (and potentially MS*n*), aids metabolite identification and thus linkage to soil function and health. There are a few mass-spectral libraries, both commercially available (e.g., NIST, FiehnLab; Babushok et al., 2007; Kind et al., 2009) and open access (e.g., MassBank, mzCloud) which can be used (Vanaixa et al., 2016). However, in studies where thousands of unique compounds may be detected, the number of complete identifications is generally very low. For example, only 5-10 % of the known metabolites reported in compound-centric databases such as the Human Metabolome Database (HMDB) and METLIN have annotated spectral data (Vanaixa et al., 2016). These databases also rarely include breakdown intermediates due to the huge diversity that may exist (e.g., oligopeptides). Similarly, a significant proportion of naturally occurring unknown metabolites may also exist in samples (metabolic ‘dark matter’; Fig. 2). Systematic extension of such libraries using authentic chemical standards (and potentially the application of machine learning techniques) will improve the ability to confidentially identify metabolites in untargeted soil metabolomics. Extension of identification in untargeted approaches will aid the formation of hypotheses, which are subsequently tested in targeted analyses, enabling absolute quantification and annotation alongside largely qualitative outputs from untargeted approaches. Additionally, with appropriate data storage of existing SOM metabolomic datasets, there is potential to further exploit these findings as the resources available for compound identification improve. One area for improvement is the need to develop standards for curating soil metabolomic data and then to make them open access, mirroring the use of soil genomic data and for metabolomic data in other life science fields (Huson et al., 2007; Zhou et al., 2022). Preferably, this should contain soil metadata to facilitate future metanalyses.

*3.3. Phylogenetic and associated biochemical diversity*

The sheer size and scale of the metabolome is an additional hinderance to analysis, particularly considering the substantial chemical diversity of metabolites and their dynamic range (Fernie et al., 2004; D’Auria and Gershenzon, 2005), which is influenced by the extraction and analysis method. As discussed above, organismal metabolism is extremely complex and diverse (Fernie et al., 2004; Rai et al., 2017; Fang et al., 2019). Furthermore, while primary metabolism is generally conserved, the synthesis of secondary (specialised) metabolites, which can account for a large proportion of the metabolome, can exhibit a large amount of diversity between species (Alseekh and Fernie, 2018). This diversity of metabolism is replicated across the fungal kingdom, and while bacteria are generally considered to be less metabolically diverse, they still exhibit substantial variation, depending on their energy requirements and metabolic mechanisms (e.g., phototrophs vs. chemotrophs; Gomez, 2011).

*3.4. Functional synergy between ‘omics techniques*

Linking metabolite analysis to the underlying genomic, transcriptomic, and proteomic studies remains the (often illusive) gold standard in environmental ‘omic research, as this can provide key insights into microbial function and biochemical interaction in unprecedented detail. The relationship to enzymatic rates may also possible, although this typically only provides an indicator of potential rates rather than the actual rates in soil (Greenfield et al., 2020; Hazard et al., 2021)) Correlation of the presence of metabolites to specific members of the biological community is possible (Taylor et al., 2018); e.g., glutaric acid, 2-ketobutyric acid and α-santonin to PAH degraders in the rhizosphere in biochar treated soil (Li et al., 2020). Furthermore, metabolites have also been linked to specific genes, e.g., core stress and C, N, P and S cycling (Finn et al., 2020; Van Der Hooft et al., 2020), and enable the identification of the biochemical diversity of a genus (Kjærbølling et al., 2018). This translation of multi-omics metrics into a functional understanding of ecosystem processing, and function and service provision, will be key to cementing their relevance in the study of environmental biology in the future (Bahamonde et al., 2016; Biswas and Sarkar, 2018). However, this requires the systematic construction of high-resolution metabolome libraries, including the continual characterisation of yet unidentified metabolites (or ‘metabolic dark matter’, Fig. 2), which it has been suggested may contribute up to 98% of detected peaks (Schymnaski et al., 2014; Mahieu and Patty, 2017; Peisl et al., 2018). Further, the use of metabolic pathway maps (e.g., KEGG) may not capture the diversity of metabolic pathways (particularly of secondary metabolism) which may be active simultaneously within the soil microbiome (Zhou et al., 2022). New approaches are required to evaluate which metabolic pathways are active/operational within mixed microbial communities. Ultimately, this will also aid in the efficient integration with other omics techniques currently utilised in soil research and therefore allow much greater systems understanding of soil biological function.

**4. Perspectives on the study of soil metabolomics**

*4.1. The importance of untargeted analysis*

As highlighted in section 3, targeted metabolomic analysis is often viewed as more powerful compared to untargeted analysis (although every approach does introduce some bias with extraction and/or analysis), due to its quantitative, validated nature. However, untargeted analysis should not be overlooked, as it may allow the detection of unexpected changes in the biochemical profile of the soil. Additionally, many metabolic compounds are yet to be characterised. This so called ‘metabolic dark matter’ (Fig. 2) represents an exciting and yet untapped source of new analogues. A commonly used practical example of this being the discovery of antibiotic metabolites capable of circumventing clinically important resistance mechanisms (Peek et al., 2018; Sharrar et al., 2020). It is only by performing untargeted, ‘discovery’ analysis that the elucidation and association of molecular biomarkers with characteristic conditions may be performed, e.g., typical osmolytes associated with drought such as trehalose, mannitol and glycine betaine (Nawaz and Wang, 2020; Warren, 2020). In addition, this will aid in our understanding of the chemical ecology of soil (Kellogg and Kang, 2020), and fundamental fluxes of small organic molecules through soil, and the inter- and intra-species interactions between soil organisms. However, in both cases (targeted and untargeted), metabolite identification remains a challenge. This is exemplified in protein hydrolysis in soil which is likely to generate many thousands of unique oligopeptides. Nevertheless, computational methods are improving to compensate for the lack of reference standards for soil, as non-model and heterogeneous mediums are difficult to standardise, while LC-based methods also improve the ability to disentangle fragmentation and adducts, aiding in biological interpretation (Chang et al., 2021). We have only scratched the surface in terms of our comprehension of high-resolution C cycling in soils with analytical advances in resolution, annotation and quantification likely to enhance this into the future, potentially to beyond the current level of understanding of the more constrained plant system (Yang et al., 2017).

*4.2. Emission and fate of volatile organic compounds (VOCs)*

The emission, capture and quantification of non-methane VOCs from soil is also an area of interest for future research. VOCs may be sampled using a number of methods, e.g., in real time using proton-transfer-reaction mass spectrometry (PTR-MS; Potard et al., 2017) or through sorption and desorption using GC-MS using tubes or fibres over time (Brown et al., 2021c; Perrault et al., 2014; Table 2). Current soil models focus overwhelmingly on the three most potent greenhouse gases emitted from soil, namely CO2, methane (CH4) and nitrous oxide (N2O). While the effect of these gases is well known in terms of climate change, the emission of VOCs from soil has been little explored. For example, biogenic secondary metabolite emission from soil (e.g., isoprene and monoterpenes) may also contribute to tropospheric ozone and secondary organic aerosol formation (Sindelarova et al., 2014; Ahlberg et al., 2017; Fitzky et al., 2019), and VOCs are broadly seen to have a negative effect on air quality and human health (Soni et al., 2019). With soil VOC emissions having been shown to make up to 5.0 ± 2.0% of C emissions (molar CO2-C equivalents) on rewetting (Rossabi et al., 2018), this may be a significant, previously unaccounted for, loss of C from soil. A range of studies have shown that soils simultaneously emit a diverse range of VOCs, which can be related to the health of the soil (Brown et al., 2021b; Monard et al., 2021; Romero-Olivares et al., 2022). Many of the compounds remain unidentified, however, the dominant groups include alcohols, aldehydes, ketones, terpenoids (mono- and sesqui-) and organosulfurs (Abis et al., 2019; Rossabi et al., 2018; Tang et al., 2019).

While VOCs have been incorporated into some atmospheric models, they do not usually focus on total VOC emission, rather they focus on a small number of the most abundant VOCs, often including compounds not produced from the soil itself, but from the overlying vegetation (Navarro et al., 2014). Although modelling has estimated the annual global atmospheric emission of VOCs from vegetation to be 6.34 × 108 t C yr−1 (Henrot et al., 2017), no accurate estimates for VOC emission from soil exist. To contextualise this, annual global CO2 emission from soils is estimated to be between 6.8 and 9.8 × 1010 t C yr−1 (Bahn et al., 2010; Raich and Schlesinger et al., 2017).

Plants are also major emitters of VOCs to soil through their roots (Dudareva et al., 2007), of which many compounds are released in response to abiotic or biotic stresses. Therefore, monitoring their emission and diffusion in a non-invasive and non-destructive way may provide further insight into plant health, for example organismal interaction (attraction of beneficial organisms e.g. mycorrhiza (Dearth et al., 2018; Kaling et al., 2018) and bacteria (Schulz-Bohm et al., 2018)) and defence from hostile organisms (Ali et al., 2010; Erb and Kliebenstein, 2020), allelopathy (Effah et al., 2019; Santonja et al., 2019), and stress and disease (Velikova et al., 2011). Equally, VOCs are a labile substrate and may be consumed by the soil microbial community (Owen et al., 2007). Therefore, methods of measurement through the whole soil profile, not just at the surface, are key to understanding the mechanisms of anabolism and catabolism throughout the soil. Future work is required to quantify this loss of volatile C from soil. Furthermore, it is not only the emission of these compounds, but their interaction with the environment and indigenous biology, as well as the degradation pathways and ultimate fate of this C that is largely unknown and warrants further investigation.

*4.3. The rhizosphere – a key hotspot for biochemical and organismal interaction*

The rhizosphere has long been recognised as a ‘hotspot’ for nutrient transformation, biochemical and organismal interaction (Kuzyakov and Blagodatskaya, 2015). Plants release substantial amounts, of their photosynthetically fixed C and N through rhizodeposition (up to 20% and 15% as root exudates and root turnover, respectively (Mohanram and Kumar, 2019; Venturi and Keel, 2016). It is evident that root exudates are key to nutrient acquisition strategies (Wen et al., 2022), as well as shaping the rhizosphere microbial community (Sasse et al., 2018; Zhalnina et al., 2018), and signalling to other plants (Jones et al., 2009; Wang et al., 2021). Root exudate composition (and its associated ‘rhizosphere effect’) is largely defined by the genotype of the plant (Mönchgesang et al., 2016), its developmental stage (Chaparro et al., 2013) and level of abiotic stress (Carvalhais et al., 2013), all of which have been shown to influence and self-select the plant’s rhizosphere microbiome (Sasse et al., 2018) and subsequent inter-kingdom (Durán et al., 2018) and inter-species interactions (Badri et al., 2009; Foster and Bell, 2012). These interactions are of significance to plants, aiding in their resilience to disease and tolerance to stress (Mohanram and Kumar, 2019). There is great interest in manipulating the plant microbiome to improve agricultural productivity and reduce potential N pollutants (N2O and ammonia (NH3) emissions, nitrate (NO3-) leaching) by selecting for plant genes and metabolites that promote greater N uptake or inhibition of key processes which drive N loss (e.g., nitrification, denitrification; Arif et al., 2020; Subbarao et al., 2021). To achieve this, however, further research is also required to understand the fundamental relationship between the soil metabolite profile and the microbial community, as well as the relationship to the regulation of plant rhizosphere processes and subsequent nutrient transformations and processing. Particularly, investigation of the rhizosphere metabolome and microbiome under different plant growth stage and abiotic stresses, including those that are likely to become more prevalent with climate change (e.g., drought, flood, salinisation; Jansson and Hofmockel, 2019), anthropogenic perturbation (e.g., plastic and microplastic loading and other pollution events; Rillig et al., 2017; de Souza Machado et al., 2019), and agriculture (e.g., nutrient loading and multi-species planting; Overy et al., 2021).

One major challenge is how to extract compounds from the rhizosphere without disturbing the root itself (which may lead to a stress response or cell lysis). Therefore, the practical and analytical challenge of disentangling the complex relationship between plant-microbe interactions in the rhizosphere should not be underestimated (Oburger and Jones, 2018). Isotomics, the combination of stable isotope and metabolomic techniques, has been suggested as a potential method of exploring the rhizosphere and the source and flux or individual metabolites (Giavalisco et al., 2008; Kluger et al., 2014). Providing selective, untargeted screening of metabolites derived from root exudates, while minimising the influence of disturbance, could also be used to differentiate between soil and root biochemistry and will further guide our ability to engineer the rhizosphere to promote resistance, resilience, productivity and sustainability (Lohse et al., 2021).

*4.4. Understanding temporal metabolite fluxes*

During SOM turnover, it is often the temporal flux of metabolites through the soil system rather than the absolute size of the metabolite pool, that determines the functional significance (van Hees et al., 2005). The destructive nature of metabolite sampling and high cost of analysis often limits the temporal datasets created in large-scale multi-factorial experiments. While there is currently little analytical scope for real-time monitoring, the focus of most studies is on metabolic pool sizes (Warren, 2013) or temporal “snapshots”. Equally, stable- and radio-isotope analysis of the whole metabolomic profile has been explored, with most studies still confined to a limited number of compounds (e.g., amino acids, lipids). However, a more holistic approach is likely to be powerful in enhancing our understanding of metabolic pathways and networks, as well as overall metabolome (including its numerous subsets) change within the soil environment (Dijkstra et al., 2011; Watzinger, 2015; Bore et al., 2017; Tian et al., 2018; Nakabayashi and Saito, 2020). However, it should be noted that targeted analysis must be performed after compounds of interest have been identified, to fully quantify fluxes. Ultimately, understanding the metabolic dynamics of the soil system is key to understanding the associated functional implications e.g., rates of C and nutrient cycling (Canarini et al., 2019).

In comparison to non-volatile metabolites, the nature of VOCs means that they can be easily measured in soil in a non-destructive way (Gil-Loaiza et al., 2022). This makes them more suited to real-time measurement. One approach that has been used to measure the temporal flux of VOC emissions from topsoil is PTR-MS (Gray et al., 2010; Aaltonen et al., 2013; Potard et al., 2017). However, there are drawbacks of this approach, for example the range of compounds detected is limited and identification is putative and qualitative, unless combined with other analytical techniques (e.g., TOF-MS) (Mancuso et al., 2015). Additionally, significant advances in calibrated sensor technology (e.g., photoionization detectors, PIDs) may allow the non-selective measurement of total VOC fluxes from the soil (Bocos-Bintintan et al., 2019), potentially quantitatively aiding the understanding of VOC emission and secondary metabolic changes at low cost and high (ppb) sensitivity.

*4.5. Disentanglement of the metabolome using modelling*

Mathematical modelling is another approach which has been frequently applied to soil systems to untangle and quantify the myriad of abiotic and biotic processes and interactions that characterise these systems. In this approach, physical, chemical and biological processes are formalised in mathematical equations and coupled together in reactive transport models (RTM) which then can be used to simulate the temporal evolution of these systems (Steefel et al., 2005). By fitting models to measured metabolite data, quantitative predictions can be obtained which are, for example, utilised in bioremediation applications (e.g., Alvarez-Zaldívar et al., 2016). While microbial activity has been traditionally described in a very generalised manner in these models, novel approaches have recently been discussed which also draw on genomic data (Kreft et al., 2017).

Of particular interest are so-called constraint-based techniques which operate on metabolic networks (Lewis et al., 2012). Such enzymatically driven reaction networks can be derived for whole communities, based on metagenomic data, or for individual organisms if their genome has been sequenced, or at least a metagenome-assembled-genome (MAG) could be inferred (Bickhart et al., 2022). Metabolic network models up to the genome scale can be constructed by collecting all reactions which are known to be catalysed by all enzymes encoded on a species’ genome. By assuming that intracellular metabolites are at steady state, feasible flux distributions can then be inferred.

Flux-Balance-Analysis (FBA) is one of the most popular approaches applicable to single species. This works on the assumption that microbial cells try to maximise growth, enabling prediction of specific growth rate and a specific flux distribution. They also incorporate metabolite uptake from the external medium and secretion fluxes. Such predictions are useful for mapping metabolites to those species that affect their fate. This approach has also been extended to consider dynamics of microbial communities, requiring one FBA model for each member species and providing quantitative predictions for metabolite exchange (Harcombe et al., 2014; Bauer et al., 2017; Popp and Centler, 2020). While FBA modelling has been coupled to RTMs (Scheibe et al., 2009; Rubinstein et al., 2021), the construction of genome-scale metabolic models remains a challenge, although (semi-) automatic pipelines now exist for their creation (reviewed in Mendoza et al. (2019); with recent additions by Zorilla et al. (2021) and Zimmermann et al.(2021)).

In contrast, microbial community wide metabolic networks circumvent this problem. In this approach, a metagenome is treated as if originating from a single organism. Hence, species boundaries are resolved, and metabolites are assumed to be accessible to any enzyme found to be encoded on the metagenome, irrespective of its organismic origin. Despite this stark simplification, such an approach was able to detect seasonal changes in overall metabolic pathway activity for a marine environment (Larsen et al., 2011). While relying on genomic data for generating the metabolic network, the discussed modelling approaches can also take advantage of meta-transcriptomics and meta-proteomics data as experimental evidence for pathway activity, which can be incorporated into the models as additional constraints and improving predictive power (Tian and Reed, 2018). By drawing on sequence data on the one hand and providing predictions on uptake and secretion of individual metabolites on the other hand, constraint-based metabolic network modelling is a valuable avenue for connecting genomic and metabolomic data, linking individual microbial species to the fate of individual metabolites. Further, the production of spatially-explicit metabolic models which link microbial function to soil physical structure (microniches) is now also possible (Borer et al., 2019).

Despite this potential, constraint-based approaches have their limitations. For example, they rely heavily on the current knowledge regarding the mapping from gene sequences to enzymes and their metabolic activity. While conserved enzymes and pathways will reliably be detected and incorporated in such models, metabolic activities at the fringe of our current metabolic knowledge will be absent until it may be formalised and incorporated into appropriate databases. This is especially relevant when dealing with uncultured and/or unculturable organisms (which dominate soil; Bodor et al., 2020), relying on MAGs for example, as these might harbour not yet described metabolic potential which will not be reflected in the corresponding models. In this context, another limitation of FBA is the need to specify the molecular composition of a species’ biomass and the characterisation of uptake kinetics for dynamic simulations. Such data are hard to obtain for uncultured organisms and can only be estimated. Further, uptake kinetics in liquid culture may not reflect those in soil (Jones et al., 1996). Gene regulation is another aspect which is not addressed in classical FBA but might be important in nutrient-deprived and dynamic environments such as soils. The assumption, that an organism is trying to maximise its growth might also not always hold true under such conditions. These two aspects, however, can be addressed by directly incorporating meta-transcriptomic and meta-proteomic data into modelling. Further research is required to address these gaps in the knowledge regarding metabolic modelling, particularly in soils, where most organisms are unculturable, and community dynamics and biotic and abiotic stress are likely to impact growth rates and cell sizes.

*4.6. Towards functional environmental and ecological ‘omics*

Functional metabolomics is a concept originating from the biomedical sciences with the aim of overcoming the descriptive nature of interpretation, largely limited to speculating on metabolite function based on previous literature (Yan and Xu, 2018). To truly understand function, an integrative approach drawing on genomic, transcriptomic and proteomic approaches is required. Though, the functions of most genes are yet to be elucidated, particularly in the case of the gene-protein-metabolite regulation network. Additionally, biochemical characterisation of enzyme activity and substrate utilisation is important too. This integration of multi-omics data can be utilised to tentatively reconstruct the multi-layer regulation network, potentially providing a more comprehensive and informative understanding of the regulation of genes to transcripts, transcripts to proteins and proteins to metabolites. What is more, different levels of ‘omics data can be used to validate each other and used to improve model accuracy (Zhou et al., 2022). While likely to be resource intensive work and require significant collaboration between disciplines (microbiologists, biochemists, chemical ecologists, environmental scientists, bioinformaticians and modellers), future research towards functional environmental ‘omics must aim to integrate and draw on multiple levels of omics data (Shaffer et al., 2022). This integration also requires minimum reporting standards for metabolomic data and metadata and publicly accessible data repositories like that of genomic sequencing data, to allow future comparison in meta-analyses and modelling to maximise use and compatibility of datasets. Previously minimum reporting standards have been suggested (e.g., Fiehn et al., 2007; Sumner et al., 2007; Viant et al., 2019), however not widely adopted, particularly in the environmental sciences.

**5. Conclusions**

Soil metabolomics has great potential to provide deep insights into soil biochemistry and chemical ecology, aiding our understanding of the complex small molecule interactions which take place within soils. Due to its relative infancy as a technique, there are many possibilities for its application to unravel the inherent biological complexity and interplay that exists between organisms that interact with soil ecosystems (i.e., plants, microorganisms, mesofauna, livestock). The use of untargeted ‘discovery’ metabolomics should not be undervalued, particularly in a complex system such as soil. Equally, both the rhizosphere and volatile products of metabolism are relatively unexplored research areas using metabolomic and volatilomic techniques. Looking to the future, there is a need to develop robust and standardised methods for metabolite extraction and analysis from soil alongside recognised data curation approaches to facilitate downstream analysis and cross-comparison of studies. The goal should be the integration of metabolomics with other ‘omics approaches, with an emphasis on providing a functional understanding to key soil processes e.g., C and nutrient cycling, which are essential to ecosystem service provision, as well as providing novel metrics for evaluating soil health.

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**Declaration of Competing Interest**

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

**CRediT author statement**

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**Figure and Table Captions**

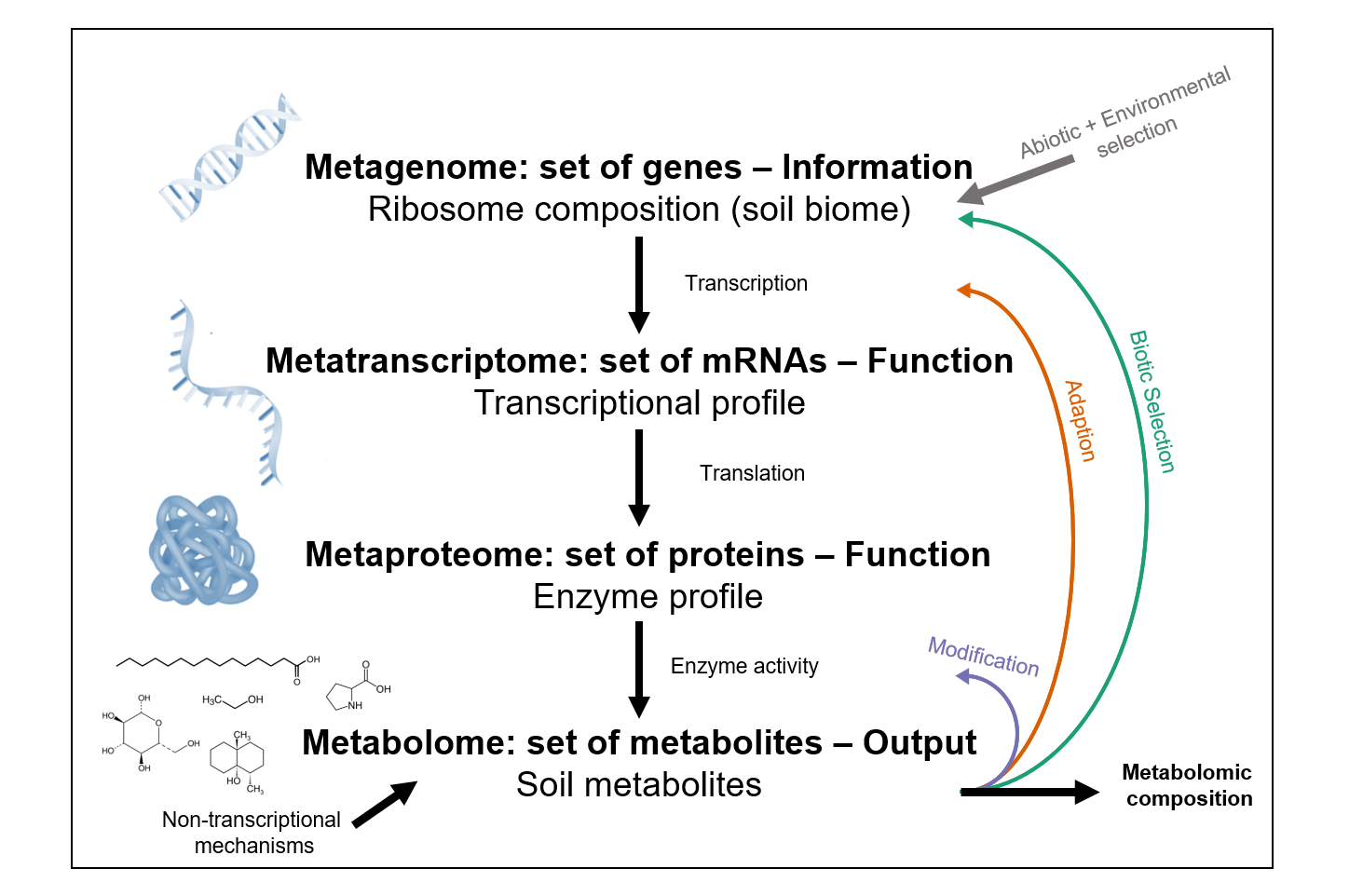
**Figure 1.** A conceptual representation of the biological hierarchy, starting with the genome and culminating with the metabolome, which is sensitive to environmental and organismal change. Adapted from Takahashi et al. (2012).

**Figure 2.** The Kyoto Encyclopedia of Genes and Genomes (KEGG) schematic representation of known metabolomic pathways (Kanehisa et al., 2016). However, the database is not extensive with the potential for many other yet to be identified metabolic pathways, or ‘metabolic dark matter’. Equally, while the focus of most studies to date is the emission of the three most prolific greenhouse gases from soil (CO2, CH4 and N2O), volatile organic compounds (VOCs) may potentially be emitted as metabolic intermediaries from the anabolism and catabolism of various other primary and secondary metabolites (Insam and Seewald, 2010), potentially representing a significant flux of carbon from the micro- and phyto-biome.

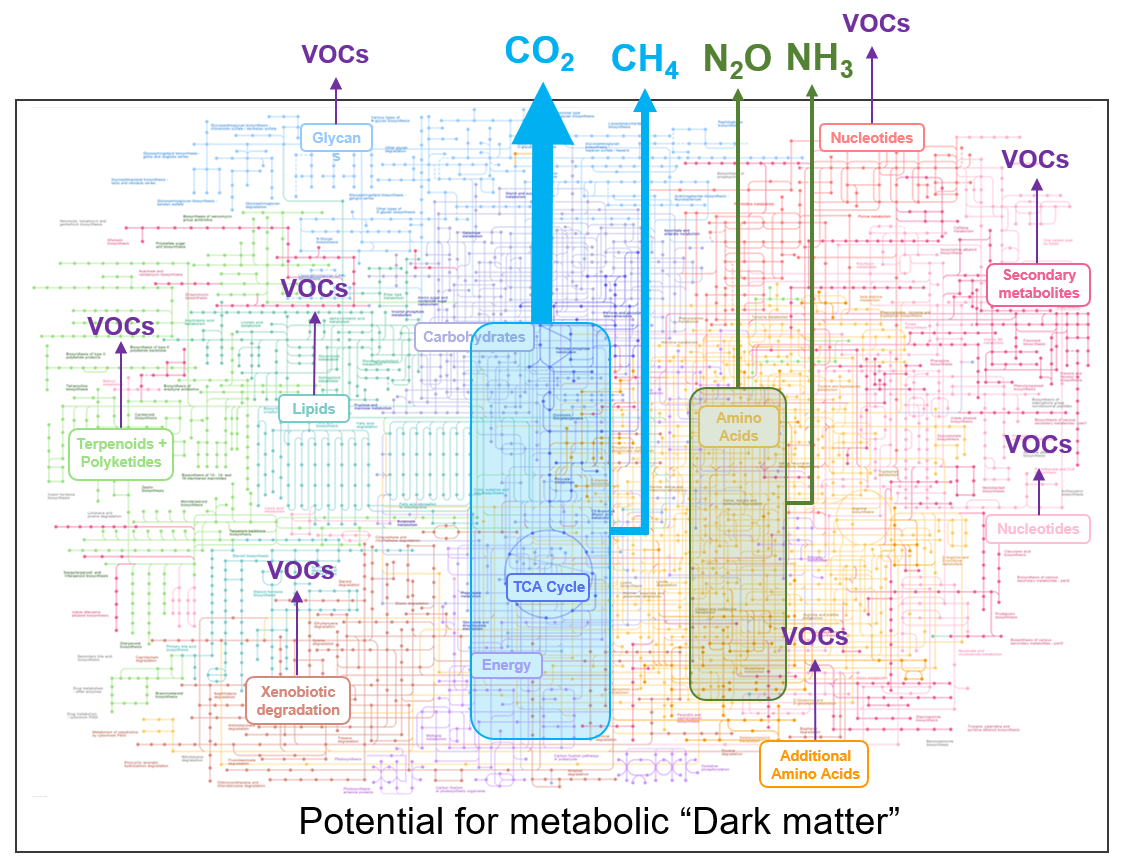
**Table 1.** Brief description of the subtypes of metabolomics.

**Table 2.** Summary of common extraction techniques and analysis platforms

**Figure 1.**



**Figure 2.**

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| **Sub-types of metabolomic analysis** | **Brief description** | **References** |
| Targeted | Analysis of defined groups of chemically characterized and biochemically annotated metabolites. | Roberts et al. (2012) |
| Untargeted | Comprehensive analysis of all the measurable analytes including chemical unknowns, limited by analysis and extraction technique. | Want et al. (2018) |
| Primary metabolites | Compounds involved directly in the normal growth, development and reproduction of an organism or community. Generally, endogenic, often produced in the log phase of growth. | Crueger and Crueger, (1990)  Sanchez et al. (2008) |
| Secondary metabolites | Compounds that are not required for the growth or reproduction of an organism or community but are usually produced to gain a selective advantage. Usually, exogenic, often produced in the stationary phase of growth. | Karlovsky, (2008)  Chomel et al. (2016)  Erb and Kliebenstein, (2020)  Ruiz et al. (2010)  Isah, (2019) |
| Lipidomics | Lipid compounds generally transcend the categories of primary and secondary metabolites. For example, some groups are crucial for cell function e.g. PLFAs as membrane lipids and TAGs as storage lipids whereas others are used as signalling compounds. | Zhao et al. (2018)  Soto et al. (2019)  Mahfouz et al. (2020)  Frostegard et al. (2015) |
| Volatilomics | A subset of secondary metabolomics concerning all volatile metabolites, often referred to as volatile organic compounds (VOCs). | Leff and Fierer, (2008)  Insam and Seewald, (2010)  Kesselmeirer and Staudt, (1999)  Brown et al. (2021)  Brilli et al. (2019) |

Table 1.

Table 2.

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| --- | --- | --- |
| **Extraction/sampling techniques** | **Brief description** | **References** |
| Fumigation-extraction | Fumigation of a soil sample with chloroform has been shown to lyse cells and release intracellular metabolites prior to extraction. This may be combined with a H2O- or solvent-based extraction. | Vance et al. (1987)  Swenson et al. (2015) |
| H2O extract | This method is used for the extraction of water extractable organic matter (WEOM) or soil exometabolites. Effective at extracting a broad range of polar compounds, however, may significantly underrepresent fatty acids and sterol compounds. Low concentrations of salt may be included to reduce the effect of cell lysis and osmotic shock, however, salt must typically be removed before analysis. | Swenson et al. (2015)  Gregorich et al. (2000) |
| Solvent based extraction | Solvents may be used either individually or in combination. Common extraction solutions include, methanol as well as combinations of isopropanol, methanol and water or isopropanol, acetonitrile and water. Generally, combining solvents allows for the extraction of a broader range of metabolite compound classes including a greater number of non-polar molecules compared to H2O only. | Swenson et al. (2015)  Ser et al. (2015)  Capriel et al. (1986)  Withers et al. (2020)  Roberts et al. (2012)  Tfaily et al. (2015)  Tfaily et al. (2017) |
| Sorption | Volatile organic compounds can be sorbed onto the extraction (stationary) phase. A number of different phase materials exist to capture a range of molecules, e.g., polydimethylsiloxane (PMDS) for semi-polar molecules or Divinylbenzene/Carboxen/Polydimethylsiloxane (DVB/CAR/PDMS) for a range of polar and semi-polar molecules.  Sampling may be through tubes (with can be deployed in the laboratory or field, or various fibres and probes e.g., solid phase microextraction (SPME) fibres (which are more applicable to laboratory-based experiments).  The phase can then be thermally desorbed into a GC-MS for analysis. | Brown et al., (2021c)  Perrault et al., (2014) |
| **Analysis platforms** |  |  |
| Nuclear Magnetic Resonance (NMR) spectroscopy | Highly repeatable, easy metabolite identification and non-destructive analysis. However, generally low resolution and sensitivity hinders the determination of metabolites to ≤ 50 per sample. | Emwas et al. (2019) |
| Direct Infusion-Mass Spectrometry (DI-MS) | Complex mixtures injected directly into high resolution accurate mass (HRAM) MSxMS allowing simultaneous detection of thousands of compounds. Compounds assigned molecular formulae which can be compared to infer compositional differences.  Fourier-transform ion cyclotron resonance (FT-ICR) – the most advanced mass analysers in terms of high accuracy and resolving power and sensitivity, with sub-parts-per-million mass accuracy. Potentially greater information about heteroatom-rich samples.  Orbitrap – lower cost HRAM MS increases accessibility but marginally lower resolving power than FT-ICR.  FT-ICR and Orbitrap can also be used in hyphenated approaches. | Kirwan et al. (2014)  Ghaste et al. (2016)  Simon et al. (2018)  Hawkes et al. (2016) |
| Gas chromatography (GC)-MS | Flexible with sensitivity to volatile-semi volatile compounds, including volatile organic compounds, lipids and derivatizable molecules, allowing for targeted or untargeted analysis. Often identifying > 100 compounds per sample the analytical window is smaller than for LC based techniques. The technology is easily combined with mass spectrometers and accurate mass measurements. This is complimented by large supporting spectral libraries and standardised methodologies. | Schauer and Fernie (2006)  Fiehn et al. (2016) |
| Liquid chromatography (LC)-MS | Flexible, high resolution and sensitivity to a broad range of compounds Often identifying > 400 compounds per sample. Often provides increased resolution for semi-polar metabolites. | Zeki et al. (2020) |
| Capillary electrophoresis (CE)-MS | Separation for polar and charged metabolites with  Less reproducible than GC and LC techniques, but developing area of metabolomics to improve hyphenation with HRAM MS. | Zhang and Ramautar (2021) |
| Proton Transfer Reaction (PTR)-MS | High sensitivity, real-time measurement of VOCs, requiring no sample pre-preparation. Although, a limited range of molecules are detectable (only molecules with a proton affinity higher than water) and isomers cannot be easily separated. | Mancuso et al. (2015)  Hewitt et al. (2003)  Han et al. (2008) |