Chapter 6
Soil Microbial Metabolomics

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1 Soil Complexity

The world demand for food is currently expected to increase by two- to fivefold by 2030 (Ewert et al. 2005; Food and Agriculture Organization (FAO) 2002). This projection requires food production to increase from 60 to 70 % (Clair and Lynch 2010), although there is some confusion due to the types of food stuffs the global population will be consuming at that time (Alexandratos and Bruinsma 2013) and the exclusion from earlier studies of important food types (e.g. fruit and vegetables). Agricultural practices over the last century have succeeded in significantly increasing crop yields. For instance, global cereal production doubled between 1960 and 2000 (Tilman et al. 2002). However, the yield increases were driven largely by intensification in the use of non-renewable synthetic fertilisers (Lynch 2007). They were seminal in improving western lifestyles but provided limited relief in many regions of the world such as Africa, Asia and South America. Moreover, the technologies were double edged, with gains in agricultural production coinciding with increased soil erosion (Matson et al. 1997), industrial agricultural pollution (Horrigan et al. 2002), declines in water quality (Foley et al. 2005) and, possibly most importantly, loss of biodiversity (including genetic erosion) (Aguilar et al. 2008; Balestrini et al. 2015). Arguably, a sustainable way forward is 'ecological intensification'. This paradigm expands agricultural intensification that promotes the development of food systems with enhanced nutrient uptake and water use efficiency (Cassman 1999), reductions in pest and disease

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control (Bommarco et al. 2013) and a restoration in soil fertility (Balestrini et al. 2015; Matson et al. 1997; Tittonell 2014). Many of these goals are only achievable today due to the advances in analytical technologies and scientific knowledge that underpins methodologies like metabolomics. As useful as the broad brush of traditional physicochemical analyses has been for agricultural systems (Sinsabaugh et al. 2016) (to understand how to manipulate plant growth), it would be advantageous to determine biogeochemical processes that occur in soil (Rockström et al. 2009; Sardans et al. 2011).

The soil matrix is one of the most diverse ecological systems on the planet (Torsvik and Øvreås 2002). Home to a plethora of organisms, from the largest redwood to the smallest microbe, there are numerous chemical (McBride 1994), physical (Marshall and Holmes 1980) and biological (Barea et al. 2005; Lorenz and Wackernagel 1994) interactions occurring in soil. Until recently, quantifying these biogeochemical processes as a metabolome of "soil" was not considered feasible, with only narrow components of the soil matrix, such as plants and animals, being studied (Maron et al. 2011; Mendes et al. 2013; Mosier et al. 2013). However, a holistic approach to understanding the soil community is something of increasing interest with examples in organic nutrient pools [(Warren 2013, 2014), Table 1], pollution assessment (Jones et al. 2014; Tremaroli et al. 2009), effects of climate change [(Pang et al. 2016), Table 2], plant [(Badri et al. 2013a; van Dam and Bouwmeester 2016), Table 3] and/or microbial metabolism (Ponomarova and Patil 2015; Warren 2015). Recent progress in performing untargeted metabolomics to identify soil organic matter (SOM) metabolites (e.g. lipids and organic acids), which can be linked to nutrient uptake [(Swenson et al. 2015b), Table 3], has shown that "soil metabolomics" is now beyond theory. Still, the ability to study soil holistically is at an early stage of development. The potential for using metabolomics to advance study into how the soil matrix operates will increase as the technologies underpinning the methodology (i.e. gas and liquid chromatography–mass spectrometry (GC–MS and LC–MS), and nuclear magnetic resonance (NMR)) improve (Singh 2006). Likewise, metabolomics can provide a holistic understanding of the impact of the increasing resource demands has on soil (Rockström et al. 2009). Through understanding of the important metabolomic pools and fluxes in soil, so as to understand and monitor soil health as it is managed by farmers, foresters and the community, is likely to improve productivity and the environment while reducing costs (Abhilash et al. 2012; Desai et al. 2010).

Metabolomics of the soil would identify the largest component of metabolites coming from the most varied and numerous collection of microbes known (Barea et al. 2005; Huang et al. 2014; Mendes et al. 2013), with estimates of between 1 and 40 million species per gram of soil (Burns et al. 2013; Desai et al. 2010; Řezanka and Sigler 2009). Despite the importance of microbes to the agricultural and environmental communities, knowledge of the composition of the microbial biomass is limited. For instance, estimates of fungi range from 700 000 to 1.5 million (Lumbsch and Leavitt 2011; Ponomarova and Patil 2015; Rastogi and Sani 2011). However, only 100,000 have been detailed. A large part of this struggle to understand the composition of the soil microbial community is that <1 % has been

Table 1 Typical metabolites found in soil-related samples using other techniques

Metabolite class	Technique (Column for GC or LC)	Spectroscopic range (min)	Why?	Publication
Amino acids, N containing compounds, dipeptides	CE–MS (bare fused silica capillary)	10.99–35.32	Identification of organic N molecules in soil water	Warren (2013)
FAME	GC-FAME	8.23–11.71	Influence of nanoparticles on soil microbes	Shah and Belozerova (2009), Sasser (2006)
Essential oils	GC-FID (Supelcowax 10)	10.70–25.20	Rhizobacteria inoculation	Cappellari et al. (2013)
PLFA	GC-IRMS (HP 5-MS fused silica capillary column)	22.30–40.79	Microbial PLFA biomarkers using stable isotope methods	Watzinger (2015)
Carbohydrates, amino acids, phenolics	HPLC	8.07–18.62	Effects of VAM fungi on maize	Azaizeh et al. (1995)
Flavonoids, organic acids, resorcinols	RP-HPLC (DAD) and LC–MS (Nucleodur Sphinx RP)	15.34–69.73	Secondary metabolite profiling to identify bacterial–rice associations	Chamam et al. (2013)

able to be cultured (Kirk et al. 2004; van den Berg et al. 2015). Metabolomics, being a microbe-independent technique, could shed light on this rich network via metabolite fluxes and identifying previously unknown metabolites and biogeochemical processes, with genomic studies identifying numerous other microbial clades that have been identified only by their molecular sequences (Cesco et al. 2012; Cheynier et al. 2013; Swenson et al. 2015b). There is potential to extract useful information using metabolomics (Alivisatos et al. 2015); although it is acknowledged that to date, studies have been limited due to the complexity of the soil matrix (Ponomarova and Patil 2015).

This chapter uses the lens of metabolomics to discuss connections between soil microbes and the complex interactions they undergo with plants, animals and human intervention. Although studies of soil microbes using metabolomic techniques are scant, associated studies that can shed light on the complex biogeochemical interactions have been undertaken and provide direction. Due to the drive to feed the planet and increase agricultural productivity, the rhizosphere of plants and how to manipulate plant–microbe interactions has probably been of the greatest research interest in this area (Bertin et al. 2003; De-la-Pena and Loyola-Vergas

Table 2 Typical metabolites found in soil-related samples using 1H-NMR

Sample; treatments	Metabolite class	Techniques used	Metabolites	Spectroscopic range (ppm)	Why?	Publication
(a) Grape skin; soil, weather, cultivar. (b) Soil; bacterial strain, location. (c) Worms; soil type, organic versus chemical fertiliser	Amino acids	1H-NMR (D_2O, TSP), 1H-NMR (D_2O, TSP or $CDCl_3$)	Isoleucine, leucine, valine	0.90–1.10	Biomarkers	(a) Pereira et al. (2006). (b) Liebeke et al. (2009). (c) Rochfort et al. (2009)
(a) Plant cuts; time treatments. (b) Seedlings and plants; NaCl and $NaHCO_3$. (c) Worms; soil type, organic versus chemical fertiliser	Amino acids	1H-NMR TOCSY, NOESY, HSCQ; (D_2O with TSP), 1H-NMR NOESY (D_2O, Na_2HPO_4, NaH_2PO_4, TSP), 1H-NMR (D_2O, TSP or $CDCl_3$)	Threonine	1.30, 1.45	Biomarkers, Salinity	(a) Bertram et al. (2010). (b) Pang et al. (2016). (c) Rochfort et al. (2009)
Worms; soil type, organic vs. chemical fertiliser	α-Hydroxy acids	1H-NMR (D_2O, TSP or $CDCl_3$)	Lactate	1.30	Biomarkers	Rochfort et al. (2009)
Wine; bacterial strain	Alcohols	1H-NMR NOESYPREAST (D_2O, oxalate, DSS)	2,3-Butanediol	1.37	Vintage	Lee et al. (2009)
(a) Grape skin; Soil, weather, cultivar. (b) Plant cuts; time treatments. (c) Soil; bacterial strain, location.	Amino acids	1H-NMR (D_2O, TSP), 1H-NMR TOCSY, NOESY, HSCQ; (D_2O with TSP), 1H-NMR NOESY (D2O,	Alanine	1.46–1.70	Biomarkers, salinity, vintage	(a) Pereira et al. (2006). (b) Bertram et al. (2010). (c) Liebeke et al. (2009). (d) Pang et al. (2016).

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Table 2 (continued)

Sample; treatments	Metabolite class	Techniques used	Metabolites	Spectroscopic range (ppm)	Why?	Publication
(d) Seedlings and plants; NaCl and $NaHCO_3$. (e) Worms; soil type, organic versus chemical fertiliser. (f) Wine; bacterial strain		Na2HPO4, NaH2PO4, TSP), 1H-NMR (D_2O, TSP or $CDCl_3$), 1H-NMR NOESYPREAST (D_2O, oxalate, DSS)				(e) Rochfort et al. (2009). (f) Lee et al. (2009)
Grape skin; soil, weather, cultivar	Amino acids	1H-NMR (D_2O, TSP)	Arginine	1.70	Biomarkers	Pereira et al. (2006)
(a) Soil; bacterial strain, location, (b) Earthworms; various	Organic acids	1H-NMR (D_2O, TMSP), 1H-NMR (Various)	Acetic acid	1.92	Biomarkers, ecotoxicity	(a) Liebeke et al. (2009). (b) Simpson and McKelvie (2009)
Grape skin; soil, weather, cultivar	Amino acids	1H-NMR (D_2O, TSP)	GABA + proline	1.94	Biomarkers	Pereira et al. (2006)
Grape skin; soil, weather, cultivar	Amino acids	1H-NMR (D_2O, TSP)	Proline	1.98	Biomarkers	Pereira et al. (2006)
Soil; bacterial strain, location	Amino acids	1H-NMR (D_2O, TMSP)	Glutamic acid	2.06	Biomarkers	Liebeke et al. (2009)
Seedlings and plants; NaCl and $NaHCO_3$	Amino acids	1H-NMR NOESY (D_2O, Na_2HPO_4, NaH_2PO_4, TSP)	Glutamate	2.10	Salinity	Pang et al. (2016)
Seedlings and plants; NaCl and $NaHCO_3$	Amino acids	1H-NMR NOESY (D_2O, Na_2HPO_4, NaH_2PO_4, TSP)	Glutamine	2.20	Salinity	Pang et al. (2016)

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Table 2 (continued)

Sample; treatments	Metabolite class	Techniques used	Metabolites	Spectroscopic range (ppm)	Why?	Publication
Grape skin; soil, weather, cultivar	Organic acid	1H-NMR (D_2O, TSP)	Shikimic acid	2.22	Biomarkers	Pereira et al. (2006)
Earthworms; various	Organic acids	1H-NMR (Various)	Acetoacetate	2.23	Ecotoxicity	Simpson and McKelvie (2009)
Soil; bacterial strain, location	Organic acids	1H-NMR (D_2O, TMSP)	Pyruvic acid	2.27	Biomarkers	Liebeke et al. (2009)
Wine; bacterial strain	Organic acids	1H-NMR NOESYPREAST (D_2O, oxalate, DSS)	Acetate	2.28	Vintage	Lee et al. (2009)
(a) Seedlings and plants; NaCl and $NaHCO_3$. (b) Soil; bacterial strain, location. (c) Earthworms; various. (d) Wine; bacterial strain	Organic acid	1H-NMR NOESY (D_2O, Na_2HPO_4, NaH_2PO_4, TSP), 1H-NMR (D_2O, TMSP), 1H-NMR NOESYPREAST (D_2O, oxalate, DSS)	Succinate	2.40, 2.42, 2.82	Salinity, biomarkers, ecotoxicity, vintage	(a) Pang et al. (2016), (b) Liebeke et al. (2009). (c) Simpson and McKelvie (2009). (d) Lee et al. (2009)
Grape skin; soil, weather, cultivar	Amino acids	1H-NMR (D_2O, TSP)	GABA + glutamine	2.46	Biomarkers	Pereira et al. (2006)
(a) Earthworms; various. (b) Seedlings and plants; NaCl and $NaHCO_3$	Amine	1H-NMR (Various), 1H-NMR NOESY (D_2O, Na_2HPO_4, NaH_2PO_4, TSP)	Dimethylamine	2.75, 2.80	Ecotoxicity, salinity	(a) Simpson and McKelvie (2009). (b) Pang et al. (2016)
Soil; bacterial strain, location	Amino acids	1H-NMR (D_2O, TMSP)	Aspartic acid	2.79	Biomarkers	Liebeke et al. (2009)

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Table 2 (continued)

Sample; treatments	Metabolite class	Techniques used	Metabolites	Spectroscopic range (ppm)	Why?	Publication
Seedlings and plants; NaCl and $NaHCO_3$	Organic acid	1H-NMR NOESY (D_2O, Na_2HPO_4, NaH_2PO_4, TSP)	2-Oxoglutarate	3.00	Salinity	Pang et al. (2016)
(a) Grape skin; soil, weather, cultivar. (b) Plant cuts; time treatments. (c) Plant cuts; time treatments	Neurotransmitter (Amino acid)	1H-NMR (D_2O, TSP)	GABA	1.85, 2.17, 2.25, 2.84, 3.02, 3.06, 3.10, 3.28	Biomarkers, vintage	(a) Pereira et al. (2006). (b) Bertram et al. (2010). (c) Lee et al. (2009)
(a) Seedlings and plants; NaCl and $NaHCO_3$. (b) Plant cuts; time treatments	Amino acids	1H-NMR NOESY (D_2O, Na_2HPO_4, NaH_2PO_4, TSP), 1H-NMR TOCSY, NOESY, HSCQ; (D_2O with TSP)	Choline	3.20, 3.27	Salinity, biomarker	(a) Pang et al. (2016). (b) Bertram et al. (2010)
in situ; Cytochrome P450 inhibitor; xenobiotic	Xenobiotics	1H-NMR (D_2O + TSP), ISP-MS	Thiodiglycolic acid	3.25	Degradation of xenobiotics	Delort and Combourieu (2001)
Seedlings and plants; NaCl and $NaHCO_3$	Amino acids	1H-NMR NOESY (D_2O, Na_2HPO_4, NaH_2PO_4, TSP)	Betaine	3.30	Salinity	Pang et al. (2016)
(a) Grape skin; soil, weather, cultivar. (b) Plant cuts; time treatments	Sugars	1H-NMR (D_2O, TSP), 1H-NMR TOCSY, NOESY, HSCQ; (D_2O with TSP)	Glucose, fructose, sucrose	3.42–4.22	Biomarkers	(a) Pereira et al. (2006). (b) Bertram et al. (2010)

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Table 2 (continued)

Sample; treatments	Metabolite class	Techniques used	Metabolites	Spectroscopic range (ppm)	Why?	Publication
(a) Soil; bacterial strain, location. (b) Earthworms; various. (c) Seedlings and plants; NaCl and $NaHCO_3$	Amino acids	1H-NMR (D_2O, TMSP), 1H-NMR (Various), 1H-NMR NOESY (D_2O, Na_2HPO_4, NaH_2PO_4, TSP)	Glycine	3.56–3.60	Biomarkers, ecotoxicity, salinity	(a) Liebeke et al. (2009). (b) Simpson and McKelvie (2009). (c) Pang et al. (2016)
in situ; Cytochrome P450 inhibitor; xenobiotic	Xenobiotics	1H-NMR (D_2O + TSP), ISP-MS	Glycolate	3.95	Degradation of xenobiotics	Delort and Combourieu (2001)
Seedlings and plants; NaCl and $NaHCO_3$	Amino acids	1H-NMR NOESY (D_2O, Na_2HPO_4, NaH_2PO_4, TSP)	Malate	4.40	Salinity	Pang et al. (2016)
(a) Grape skin; soil, weather, cultivar. (b) Soil; bacterial strain, location. (c) Plant cuts; time treatments. (d) Worms; soil type, organic versus chemical fertiliser	Sugars	1H-NMR (D_2O, TSP), 1H-NMR (D_2O, TMSP), 1H-NMR TOCSY, NOESY, HSCQ; (D_2O with TSP), 1H-NMR (D_2O, TSP or $CDCl_3$)	Glucose, maltose, sucrose	4.62–5.42	Biomarkers	(a) Pereira et al. (2006). (b) Liebeke et al. (2009). (c) Bertram et al. (2010). (d) Rochfort et al. (2009)
Wine; bacterial strain	Organic acids	1H-NMR NOESYPREAST (D_2O, oxalate, DSS)	Tartrate	4.63	Biomarkers	Lee et al. (2009)
Soil; bacterial strain, location	Sugars	1H-NMR (D_2O, TMSP)	Trehalose	5.19	Biomarkers	Liebeke et al. (2009)

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Table 2 (continued)

Sample; treatments	Metabolite class	Techniques used	Metabolites	Spectroscopic range (ppm)	Why?	Publication
Soil; bacterial strain, location	Organic acids	1H-NMR (D_2O, TMSP)	Malic acid	6.03	Biomarkers	Liebeke et al. (2009)
(a) Worms; soil type, organic versus chemical fertiliser. (b) Seedlings and plants; NaCl and $NaHCO_3$. (c) Soil; bacterial strain, location. (d) Earthworms; various	Organic acids	1H-NMR (D_2O, TSP or $CDCl_3$), 1H-NMR NOESY (D_2O, Na_2HPO_4, NaH_2PO_4, TSP), 1H-NMR (D_2O, TMSP), 1H-NMR (Various)	Fumarate	6.20–6.52	Biomarkers, ecotoxicity	(a) Rochfort et al. (2009). (b) Pang et al. (2016). (c) Liebeke et al. (2009). (d) Simpson and McKelvie (2009)
Earthworms; various	Organic Acids	1H-NMR (Various)	Orotic acid	6.20	Ecotoxicity	Simpson and McKelvie (2009
(a) Soil; bacterial strain, location. (b) Worms; soil type, organic versus chemical fertiliser	Amino acids	1H-NMR (D_2O, TMSP), 1H-NMR (D_2O, TSP or $CDCl_3$)	Tyrosine	6.92–7.00	Biomarkers	(a) Liebeke et al. (2009). (b) Rochfort et al. (2009)
Worms; soil type, organic versus chemical fertiliser	Amino acids, Sulfonate, Lipids, Triacylglycerides (TAG)	1H-NMR (D_2O, TSP or $CDCl_3$)	Phenylalanine	7.40	Biomarkers	Rochfort et al. (2009)
(a) Worms; soil type, organic versus chemical fertiliser. (b) Soil; bacterial strain, location. (c) Earthworms; various	Organic acids	1H-NMR (D_2O, TSP or $CDCl_3$), 1H-NMR (D_2O, TMSP), 1H-NMR (Various)	Formate	8.35	Biomarkers, ecotoxicity	(a) Rochfort et al. (2009). (b) Liebeke et al. (2009). (c) Simpson and McKelvie (2009)

Table 3 Details of selected GC–MS analyses reported in this review

Sample; treatments	Metabolite class	Technique (Column)	Spectroscopic range (min)	Why?	Publication
(a) Soil: time, fumigation, ^{13}C incorporation. (b) Soil columns on farm; time	PLFA	(a) GC–MS (DB1-MS column). (b) GC–MS (HP-1 methylpolysiloxane)	1.50–48.83	(a) Transformations of soil monosaccharides (b) Incorporation of organic compounds into microbes	(a) Apostel et al. (2015). (b) Gunina et al. (2014)
Root exudates; soil; solvent extraction fraction	Sugars, Phenolics, Amino acids, Sugar alcohols	GC–MS (rtx5Sil-MS column)	3.57–17.14	Identifying root exudates that affect soil microbiome	Badri et al. (2013a)
Roots; microbe inoculation, humic acids, time	Nitrogenous compounds, Carbohydrates, Fatty acids, Organic acids, Aromatic and phenol derivatives, Terpenoids and steroids, Alcohols	GC–MS (Rtx-5MS WCOT capillary column)	3.98–45.54	Root exudates after inoculation with microbes and humic acids	Lima et al. (2014)
Plant rosettes; NaCl loading, plant species, water concentration	Amino acids, Organic acids, Alcohols, Carbohydrates, Amines	GC-QQQ-MS (Rtx-5Sil MS capillary column) and GC-FID (J&W DB5)	10.68–47.99	How *Arabidopsis* and *Thellungiella* react to salt and/or water stress	Lugan et al. (2010)
Untargeted soil profiling, fumigation, labelled (^{13}C)	Amino acids, Organic acids, Sugar alcohols, Carbohydrate, Lipids, Nucleosides, Nucleobases, Sugars, Sterols and others	GC–MS (Rtx5Sil-MS column)	8.42–24.32	Development of a simple soil metabolomics workflow and a novel spike recovery approach using ^{13}C bacterial lysates to assess the types of metabolites remaining in the water extractable organic fraction	Swenson et al. (2015b)

2014; van Dam and Bouwmeester 2016; Zahir et al. 2004). Other areas of discussion beyond the plant–microbe interactions include specific foci on how soil microbes are affected by pollution, diseases, pests and potential climate change. A theme of the chapter will be how metabolomics may be used to improve soil management (Rochfort et al. 2015; Zhang et al. 2015), both for increasing productivity of the soil and mitigating environmental effects.

2 Soil Metabolomics Is a Nascent Field

Soil is often a secondary topic for metabolomics analyses. The focus of the research is often on how microbes interact with plants such as grasses (e.g. *Trifolium*: clover) or legumes [(Bertram et al. 2010), Table 2], weeds such as *Arabidopsis* [(Gamir et al. 2014), Table 4], trees such as *Aspen* (Wallenstein et al. 2010) or invertebrates that inhabit the soil such as earthworms *Aporrectodea caliginosa* [(Rochfort et al. 2009), Table 2] or *Eisenia fetida* (Simpson and McKelvie 2009). Recent reviews have discussed soil microbial metabolites within the concept of environmental metabolomics. For instance, a review on environmental metabolomics suggests the field is fragmented, as this new "holistic" methodology was mainly being used to study single species of the researchers' interest, including those involving soil microbes (Bundy et al. 2009). The authors reiterated that this misses one of environmental metabolomics assets in gathering an understanding of the interactions between species and their environment. Another review focused on using metabolomics to assess soil contamination (Hernandez-Soriano and Jimenez-Lopez 2014). Earthworms, usually of the genus *Eisenia*, were found to be typical subjects for metabolomic analyses. Studies related to microbial metabolomics were small, with the only reference to work on the response to tellurite by *Pseudomonas pseudoalcaligenes* (Tremaroli et al. 2009). Earthworms were also considered the main target for soil metabolomics of another review on environmental sciences and metabolomics (Lin et al. 2006). This review detailed the various effects of contaminants on the metabolic profiles of a variety of earthworms, particularly halogenated compounds and metal-contaminated soils. Another review discussed 'eco-metabolomics', the use of metabolomics and how it relates to ecology, with regard to interactions between organisms, including those in the soil (Sardans et al. 2011). While mainly related to how plants and worms react to changes in environment, some discussion was made on how fungi *Magnaporthe grisea* and *Sclerontinia sclerotiorum* release metabolites to suppress plant defences.

Various studies that have examined microbes and/or the metabolites associated with them. However, only in 2015 has there been research that specifically mentions the use of "soil metabolomics" to identify biogeochemical processes occurring in soils [(Swenson et al. 2015a; Swenson et al. 2015b), Table 4]. The research sought to understand the fluxes of microbial metabolites in SOM that might occur due to climate change. Comparing fumigated and unfumigated soil samples using

Table 4 Details of selected LC–MS analyses reported in this review

Sample; treatments	Metabolite class	Technique (Column)	Spectroscopic range (min)	Why?	Publication
Plant roots; arbuscular mycorrhizal fungi (AMF) strains, time	Amino acids, Organic acids, Phosphate compounds, Acetates, Nucleosides, Vitamins, Indoles, Purines	LC-ESI Q-TOF-MS (SunFire C18 analytical column)	0.50–13.40	Mycorrhizal association of AMF with tomato plants	Rivero et al. (2015)
Roots; AMF and/or plant growth-promoting rhizobacteria (PGPR)	Lipids (PE, PC, LPE, Carnitine)	LC-HILIC-Q-TOF-MS (Acuity 1.7 µm BEH HILIC) or GC-TOF-MS (Rtx-5SilMS column)	2.60–8.82	AMF effects on wheat in N limited, P-rich environment	Saia et al. (2015b)
Arabidopsis cells; Nutrients	Phytochelatin (PC)	LC–MS (X-Terra MS C18 column)	9.70–25.00	Plant response to cadmium stress	Sarry et al. (2006)
Ferrihydrite: Organic Carbon, temperature	Phosphate, Dicarboxylate, Aromatic, N-carboxylate, other N organic compounds	LC–MS (ZIC-pHILIC)	1.70–23.6	Microbial metabolites on ferrihydrite	Swenson et al. (2015a)
Leaves; pre- and post-fungal treatments	Amino acids, Organic acids, Aldehydes, Indoles	LC-QQQ-MS (Pack ODS-A reversed-phase C18)	0.53–13.40	Understanding priming of plants against microbes	Gamir et al. (2014)

water extractable SOM, it was possible to identify metabolites associated with microbial species. Initial studies using GC–MS identified 55 metabolites via comparison with accurate standards or carbon-labelled samples (Swenson et al. 2015b). Up to 300 molecular features (after extensive sample preparation using GC-friendly water soluble solvents) were identified in soil samples following optimisation of the extraction media water, aqueous potassium sulphate or ammonium carbonate, isopropanol and methanol. Stable isotope labelling studies, using ^{13}C-acetate as a growth medium, allowed the differentiation of microbial metabolites from other compounds in the soil. This method identified sugars and amino acids as most likely to be co-extracted with other metabolites that contained hydrogen bonding functional groups [e.g. fatty acids (FAs) and sterols]. A followup study using LC–MS identified a further 55 metabolites that interacted with iron oxides in soil, reducing access to these compounds by microbes (Swenson et al. 2015a). Metabolites from this study could be grouped into phosphate containing (e.g. AMP), dicarboxylates (e.g. fumarate), aromatic and nitrogen containing (e.g. phenylalanine), carboxylate and nitrogen containing (e.g. creatinine) and others such as thymine. Future research that draws from the work described below should allow for a more holistic approach to understanding how microbes in soil can affect soil.

3 The Rhizosphere

3.1 Map of the Rhizosphere

The rhizosphere, comprising the endorhizosphere, the rhizoplane and the ectorhizosphere (Fig. 1), defines the narrow region between roots and soil directly influenced by both root exudates and exfoliates, and associated microorganisms (Jones 1998). At the heart of the rhizosphere is the root of a plant that is undergoing symbiosis. The root surface (epidermis and outer cortex) and its adhering soil are collectively termed the rhizoplane: the interface where both microbial population and biochemical plant–microbe interactions are at their maximum. The root systems of plants serve critical roles in the provision of anchorage, water and mineral absorption and conduction, lateral movement, reproduction, metabolite synthesis and food storage centre (Kenrick 2013; Kramer and Boyer 1995; Selosse and Strullu-Derrien 2015). Roots are linear units composed of multiple regions along the root growth axis. The units—the root cap, root tip, elongation zone, root-hair zone and mature zone—are uniquely differentiable and perform distinct functions (Minz et al. 2013). Each of these units uses different libraries of metabolites for communication to other units while releasing a multitude of metabolites that modulate plant–microbe interactions in the rhizosphere (Huang et al. 2014). Root exudates, used as both substrates and signalling molecules by soil microbes, comprise of both low (e.g. amino acids, organic acids, carbohydrates, phenolics and

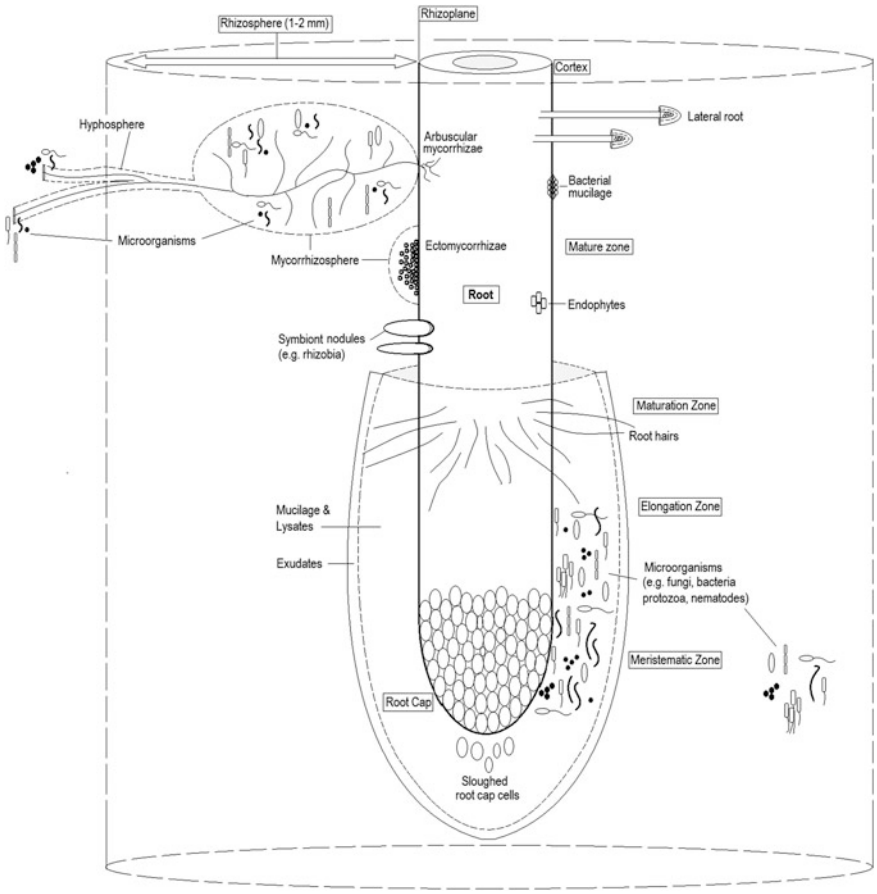

Fig. 1 Schematic representation of the rhizosphere showing commonly associated microbial associations with plant root systems

other secondary metabolites) and high molecular weight compounds (e.g. mucilage and proteins) (Bais et al. 2008; Walker et al. 2003; Ziegler et al. 2013). The nature of root exudates is observed to vary significantly between plant species. Consequently, the rhizosphere microbiome differs with both plant species and soil type (Tate 2000; Wieland et al. 2001). The influence of the rhizosphere can even extend beyond the immediate area of the plant, as discovered with metabolomic analyses of leaf litter around different tree species (Wallenstein et al. 2013; Wallenstein et al. 2010).

Apart from the root, the rhizosphere's main constituents are eukaryotic and prokaryotic microbial species, with a diversity of microbes that can range from thousands (Berendsen et al. 2012) to millions (Nihorimbere et al. 2011). The dynamic microbiome which surrounds the roots is of critical importance to

ecosystem function (Bertin et al. 2003; Sardans et al. 2011), below-ground carbon (Mendes et al. 2011) and nutrient cycling (Swenson et al. 2015b; Van Der Heijden et al. 2008), affecting overall plant fitness and soil quality (Barea et al. 2005; Minz et al. 2013). Plants spend up to half of their energy producing exudates into the rhizosphere (van Dam and Bouwmeester 2016), though it has been argued that the source of metabolites (e.g. plant vs. microbe) in the rhizosphere is still to be elucidated convincingly (Dennis et al. 2010). A major role of exudates from roots is the communication to soil-borne microbes, the most dominant class of soil biota (Van Der Heijden et al. 2008). These microbes are phylogenetically diverse (e.g. bacteria, archaea and fungi), and comprise symbionts, pathogens and saprotrophs (Balestrini et al. 2015; Reynolds et al. 2003).

Saprotrophic microbes are crucial to nutrient cycling in terrestrial ecosystems, generating the majority of nutrients required by terrestrial vegetation (Crowther et al. 2012; Schlesinger 1991). The soil microbial community is able to do this primarily via photosynthetically fixed carbon introduced to the soil ecosystem in the form of plant biomass and root exudates (Badri et al. 2013a; Dennis et al. 2010; Tate 2000). While there is competition between plants and microbes for available nutrients (Kaye and Hart 1997; Kuzyakov and Xu 2013), two key plant–microbe symbiotic associations, arbuscular mycorrhizal fungi (AMF) (Van Der Heijden et al. 1998) and root nodule symbiosis (RNS) (Li et al. 2013), impart significant benefits to both microbes and plants. AMF improve the supply of water and nutrients (such as phosphate and nitrogen) to the plant via extraradical hyphae (Kawaguchi and Minamisawa 2010; Parniske 2008). For RNS, nitrogen-fixing bacteria enable enzymatic conversion of atmospheric nitrogen into bioavailable ammonia for plant growth (Brewin 2010). Ecological benefits of symbioses are also contributed as is the case of AMF, which increases host resilience toward drought (Baum et al. 2015), decreased susceptibility to diseases (Saia et al. 2015b), improved heavy metal (Schützendübel and Polle 2002), excess salinity tolerance (Luo et al. 2009) and other abiotic factors (Habib et al. 2013).

3.2 Rhizosphere Metabolomics

The rhizosphere has undergone scrutiny using various omic techniques including genomics, metagenomics, proteomics, transcriptomics and more recently metabolomics (van Dam and Bouwmeester 2016). The principle metabolomic tools are NMR spectroscopy [Table 2, (Sardans et al. 2011)], LC–MS [Table 4, (Allwood and Goodacre 2010)], GC–MS [Table 3, (Kusano et al. 2011)], although other techniques such as capillary electrophoresis–time of flight–MS (CE–TOF–MS, Table 1) (Abhilash et al. 2012) have been utilised. For the symbiotic process to occur between microbes and plants, there must be a series of metabolites that are transferred between the two moieties (Rasmussen et al. 2012). Major bioactive metabolites found in the rhizosphere include flavonoids (Cesco et al. 2012; Cheynier et al. 2013; Narasimhan et al. 2003), phenolic compounds (Badri et al. 2013a;

Kakumanu et al. 2013; Rawat et al. 2011), exopolysaccharides (Workentine et al. 2010), antibiotics (Frisvad et al. 2004) and those participating in quorum sensing (QS) signals (De-la-Pena and Loyola-Vergas 2014; Jia et al. 2016). QS describes the process by which bacterial population density, biofilm formation and gene expression (modulating niche persistence and root colonisation) are controlled within the population through production of low-mass signalling molecules [(Braeken et al. 2008; Lima et al. 2014), Table 3]. Bacterial pathogens and symbionts are largely dependent on QS to colonise and infect their respective hosts, as in the case of *Pseudomonas aeruginosa*, which has been shown to have up to 20 % of its genes and proteins regulated via QS (Bauer and Mathesius 2004; Das and Mukherjee 2007). Other abiotic soil factors including nutrient availability (Saia et al. 2015b), soil pH (Dakora and Phillips 2002), salinity (Oikawa et al. 2011) and other environmental stressors are also known to correlate with changes in plant metabolite composition. The physiological impact of the soil microbiome on plant metabolism is receiving increasingly more attention due to changes to the climate and the need to feed an increasing population (Park et al. 2014; Sanchez et al. 2008). While assessment and identification of the full complement of rhizosphere microbes presents significant challenges, rhizosphere microbes have demonstrated a capacity to alter plant morphology, enhance plant growth and increase mineral content (Berendsen et al. 2012; Lakshmanan et al. 2014; Mendes et al. 2011). The following sections demonstrate several notable examples highlighting the use of rhizosphere metabolomics in understanding the plant–microbe interactions that underpin the practical aspects of enhancing plant performance.

4 Exudates

Plant communication to soil microbes is almost solely conducted using metabolites called exudates (Bais et al. 2004; Bronick and Lal 2005). Exudates directly influence the structure and function of the soil microbiome and are strong mediating factors in preferential microbiome selection (Coats and Rumpho 2014). The exudates are commonly amino acids and sugars (Broeckling et al. 2008). Secondary metabolites that may be active in exudates include flavones (Redmond et al. 1986) and terpenes (Hartmann et al. 2008). The secondary metabolites mediate processes to improve nutrient uptake and microbial resistance. To date, most studies have focussed on how a single microbial species reacts to plant exudates, with only recent research taking into account the multitude of microbial species in soil (Swenson et al. 2015a). The manner in which exudates are utilised by and affect microbes varies considerably and has been extensively reviewed (Bais et al. 2006; Bertin et al. 2003; Boyce 2005; Brewin 2010; Huang et al. 2014).

A variety of positive and negative interactions with microbes have been observed occurring directed by exudates from plants (Bais et al. 2006). Positive microbial interactions include the following:

- facilitating plant growth-promoting rhizobacteria (PGPRs) and symbiosis with, among others, acids, sugars and vitamin metabolites (Ahemad and Kibret 2014);
- biocontrol of nutrient fluxes including macronutrients such as P and micronutrients including Fe (Carvalhais et al. 2011, 2013; Dakora and Phillips 2002; Valentinuzzi et al. 2015);
- isoflavanoids (Morandi et al. 1984) and phenolic acids [(Azaizeh et al. 1995; Mandal et al. 2010), Table 1] involved with vesicular-AMF symbiosis;
- alkaloids and nitrogen containing metabolites produced by endophyte symbiosis, often to counteract insect predation of plants (Rasmussen et al. 2012).

Interactions that involve defence or attack against invaders of the rhizosphere include the following:

- QS metabolites such as bradyoxetin from the soybean symbiont, *bradyrhizobium japonicum* (Loh et al. 2002), that appears to help the bacterium fight off invading microbes;
- the use of lactones such as N-(3-oxohexanoyl)homoserine lactone in the rhizosphere of ginger (*Zingiber officinale*) to defend against plant viruses using bacteria such as *Acinetobacter* and *Burkholderia* (Braeken et al. 2008; Chan et al. 2011; Cooper 2007);
- phytotoxin use against invasive plants, including metabolites that include phenolics, coumarins and quinines (Khanh et al. 2005), and antimicrobial agents such as rosmarinic acid (Bais et al. 2008; Haichar et al. 2012; Hartmann et al. 2008).

The research listed identifies that there are still many unknown aspects of exudate processes that have only been hinted at with current research efforts (Bonanomi et al. 2009; Rabie 1998). Further understanding of rhizosphere microbe–exudate interactions would increase our capacity to engineer the rhizosphere to suit particular applications, as for example the use of AMF as biofertilisers (Bonfante and Genre 2010). This strategy entails engineering ideal rhizospheric growth conditions which target particular microbes for their ability to metabolise distinct nutrients.

4.1 Rhizoengineering

Rhizoengineering entails controlling the plant's rhizosphere, a considerable challenge considering the number of different entities involved. This could be to improve plant yield [e.g. wheat (Saia et al. 2015a)] or output (with *Arabidopsis* being a plant of focus in this research) (Kabouw et al. 2012), or to use the plants to improve the surrounding environment (as was found with grasses (*Hyparrhenia hirta)* and beans (*Zygophyllum fabago*) taking up heavy metals from mine tailings

in Spain) (Padmavathiamma and Li 2007). A notable example of rhizoengineering used a metabolomics-driven approach to enhance plant–microbe interactions for bioremediation of soil contaminated by polychlorinated biphenyls (PCBs) (Narasimhan et al. 2003). In the study, an *Arabidopsis–Pseudomonas* rhizosphere model was established in which 76 % were phenylpropanoids [e.g. ampelopsin (dihydromyricetin)] of 125 identified compounds (identified by metabolic profiling of *Arabidopsis Thaliana*). The root exudates identified by LC–MS were found to create a nutritional bias for efficient rhizocolonising strain of *Pseudomonas putida* PLM2. The strain was chosen both for its ability to utilise a diverse range of phenylpropanoid compounds, and its PCB-degrading capabilities. Using a gnotobiotic system, the study showed a 90 % reduction in PCBs in flavonoid-producing *Arabidopsis thaliana* strains.

Rhizoengineering need not only be limited to *in planta* studies. Innovative efforts have been made to examine microbial community dynamics through the development of artificial root models using agarose-covered slides amended with various carbon-rich compounds (i.e. glucose, malic acid and serine) simulating root exudate composition (Ziegler et al. 2013). Such novel approaches could theoretically be modified to provide convenient models for the simulation of root–microbe metabolism. Similar research into artificial roots has utilised mucilage (Ahmed et al. 2014), a polymeric gel exuded by plants that includes metabolites xylose, glucose and uronic acids. The authors acquired mucilage from chia seeds (*Salvia hispanica* L.) to emulate maize (*Zea mays* L.) root exudate. Ostensibly, this was to identify how soil in the rhizosphere is often wetter than the roots of the plant producing the exudate. The artificial roots in this system were simplified with the assumption that chia mucilage is similar to maize. The use of artificial roots highlights the difficulties in accurately measuring metabolomics fluxes in the rhizosphere.

Another effort to undertake rhizoengineering was to accentuate microbial consumption of polychlorinated biphenyls (PCB) (Narasimhan et al. 2003). Metabolites in soil were identified and delineated between microbial and those of Arabidopsis. A number of metabolites (e.g. flavonoids, lignins, indoles, anthocyanins) identified in the plant and soil led to the realisation that phenylpropanoids could be target metabolites for rhizoengineering of the soil. This was based on the criteria that phenylpropanoid metabolites are complicated enough to be resistant to microbial degradation, thus allowing them to act as a nutrient source for bacteria that will selectively degrade PCBs.

Rhizoengineering could be an exciting new area of research. As promoted by these papers, it is expected that a mixture of different species of plants and microbes will be required to exude metabolites to the required composition to improve soil productivity. Metabolomics could be a useful approach to characterise and optimise the processes to successfully engineer the rhizosphere to suit society's needs (Abhilash et al. 2012; Bonfante and Genre 2010).

5 Metabolite Coverage Over the Lifespan of Plants

Another aspect yet to be fully elucidated by research and conducive to metabolomics analyses is studying temporal variabilities to metabolites as a plant matures and is affected by external stimuli such as climate change, or is situated in different soil types (Bais et al. 2008; Borisjuk et al. 2012). For instance, the nature of metabolite secretions from the roots of *Arabidopsis thaliana* has been found to differ over the plant lifespan which, by extension, differentially affect root microbes (Chaparro et al. 2013). Analysis of the root exudates by GC–MS identified 57 metabolites from 107 possible compounds. As the plant developed over 31 days, the metabolites showed a comparative decrease in the cumulative secretions of sugars (e.g. fructose, glucose) and sugar alcohols (e.g. glycerol) and an increase in secretion levels of amino acids (e.g. glycine, alanine) and other metabolites (i.e. organic acids, carboxylic acids, FAs and other phenolic compounds). This was noted as being suggestive of a genetically programmed developmental pattern of varied phytochemical root exudation. Rhizosphere mRNA pyrosequencing showed strong correlations between microbial functional genes involved in carbohydrate, amino acid and secondary metabolite metabolism, and metabolites secreted by *Arabidopsis thaliana* at specified developmental stages. Another metabolomic study of interactions over time between potential soil-borne pathogens *Phytophthora infestans* on potatoes showed a similar pattern of amino and organic acid metabolites concentration increase and sugar concentration decrease in response to microbial inoculation (Abu-Nada et al. 2007). Similar results have also been seen due to fungal infection of soybean (Scandiani et al. 2015) and strawberries (Valentinuzzi et al. 2015). One interesting study showed how maize uses the soil fungus *Fusarium verticillioides* to attack another fungus, *Ustilago maydis*, over a 7-day period (Jonkers et al. 2012). As time progressed, the battle between the two fungi could be monitored through the changes in metabolite concentrations of compounds such as fusaric acid and a mannosylerythritol lipid (Arutchelvi et al. 2008). Generally, however, the manner in which rhizosphere microbiome function is affected by temporally varied root exudates over the course of plant development remains largely unknown. Advances in analytical technologies that allow for real-time monitoring [e.g. portable MS (Yang et al. 2008)] may allow for a greater interest in using metabolomics to study how metabolites change over time.

6 Microbial Soil Inoculants

The interactions of beneficial rhizosphere soil microbes with root systems have pivotal roles in the growth, development and ecological fitness of their plant hosts. The prevalences of intensive farming practices that are high yield and/or quality centric are traditionally predicated on extensive use of environmentally harmful and costly chemical fertilisers (Riding et al. 2015; Wissuwa et al. 2008). Subsequently,

this has led to increased industry interest in the use of sustainable and environmentally ethical farming practices (Nihorimbere et al. 2011; Zahir et al. 2004). The use of soil inoculants or 'biofertilizers' comprising beneficial soil microbes has been observed to strongly fulfil this niche through enhancement of plant growth, biological control of plant pathogens, nutrient supply and promotion of soil productivity [(Cappellari et al. 2013), Table 1]. Examples of soil inoculants are mycorrhizal fungi, the filamentous fungi *Trichoderma* spp. and plant growth-promoting rhizobacteria (PGPR) (including, but not limited to, genera *Acetobacter*, *Azospirillum*, *Bacillus*, *Burkholderia*, *Herbaspirillum*, *Paenibacillus*, *Phyllobacterium* and *Pseudomonas*) [(Chamam et al. 2013; Saharan and Nehra 2011), Table 1].

For example, the effect of soil inoculation with PGPRs in the commercially valuable wild marigold (*Tagetes minuta*) has been assessed (Cappellari et al. 2013). Wild marigold produces an essential oil (EO) known as "Tagetes oil" sought for the preparation of high-grade perfumes. The effect of single and co-inoculation of *Tagetes minuta* with *Pseudomonas fluorescens* and *Azospirillum brasilense* on plant growth parameters and essential oil production was assessed under glasshouse conditions. *Azospirillum* has been used for growth and yield promotion in cereal plants as rice, maize and wheat (Chamam et al. 2013). Both single and co-inoculations showed an increase in shoot fresh weight by approximately 50 %. Total phenolic content of shoots was upregulated by up to twofold with total EO yield increased by 70 % in single and co-inoculated plants. Major components of the EO that significantly increased ($p < 0.05$) included nine types of terpenoids, such as tagetone and ocimenone, identified by GC–MS. While individual inoculation with either *Azospirillum brasilense* or *Pseudomonas fluorescens* increased plant growth and EO production, significant enhancement to both metrics was observed in co-inoculated plants suggesting synergy between the two bacterial genera.

PGPRs demonstrate significant capacity for plant enhancement. In some cases, the magnitude of enhancement may be both bacterial strain and plant cultivar-dependant (Chamam et al. 2013). This study showed a strain-dependent effect in the association between two types of nitrogen-fixing bacterium *Azospirillum* and Asian rice (*Oryza sativa*). The bacteria *Azospirillum lipoferum* 4B (rhizosphere-colonising strain isolated from *Oryza sativa* cultivar Cigalon) and *Azospirillum* sp. B510 (an endophytic strain isolated from *Oryza sativa* cultivar Nipponbare) were observed to significantly increase ($p < 0.05$) growth of the cultivar by up to 1.5 mg/plant over 10 days if they were used to inoculate the rice strain from which they were isolated. Metabolic profiling data from reverse-phase LC–MS demonstrated significant modification in rice secondary metabolites in PGPR-inoculated plants, with 17 flavonoids, 10 hydroxycinnamic acid derivatives and four alkylresorcinols the most affected metabolite classes. Moreover, the metabolites were unique to the cultivar with only a few compounds, such as tryptophan, found common to all cultivars. This research stands as a strong example of how metabolomics techniques can be used to directly assess the nature of host–PGPR symbiotic interactions, here being able to distinguish the physiological

responses of both rice cultivars to specific *Azospirillum* strains with opposing root colonisation strategies. In another case, similar changes in plant secondary metabolism were observed in a study investigating maize-*Azospirillum* interactions (Walker et al. 2011). Major secondary metabolic changes were exclusively observed in the roots of the Cigalon-4B pairing, predominantly for benzoxazinones and benzoxazolinones, while the endophytic B510 strain elicited a systemic response inducing metabolic shifts in both shoots and roots of both rice cultivars tested.

Compared to bacterial soil inoculants, fungal soil inoculants including *Trichoderma* spp., soil fungi that are often associated with plant root ecosystems (Vinale et al. 2008), and AMF can impart significant benefit to host plants (Contreras-Cornejo et al. 2011). Considered beneficial to plants, they protect them from disease by attacking phytopathogenic fungi. Metabolomic studies have identified that *Trichoderma* detect fungi due to the exudation of metabolites from cell wall degrading enzymes and sensing the sugars released by degradation. More importantly, *Trichoderma* releases secondary metabolites that are antifungal, including antibiotics, water soluble acids and peptaibols. The non-polar nature of the antibiotics (e.g. 6-pentyl-α-pyrone) suggests that these are used as a long-range defence, while more polar metabolites (i.e. peptaibols) are used to attack fungi at 'close quarters'. The positive relationship between *Trichoderma* and plants, which includes increasing crop productivity by up to 300 %, along with activation of the plant's defences (Woo et al. 2006), has led to the use of this fungi as a natural biocontrol agent.

Other research that has identified microbially related metabolites involved in systemic acquired resistance and pathogen protection include the following:

- salicylic (SA) and jasmonic acid (JA) (Segarra et al. 2007) in cucumbers;
- SA, JA and indole-3-carboxaldehyde (Contreras-Cornejo et al. 2011), sugars, amino acids, ethanolamine, tagatose, oxoproline, GABA and urea (Chaparro et al. 2013) in *Arabidopsis thaliana*;
- the increased production of phytoalexins (e.g. flavonoids, terpenoids, indoles) after inoculation by *Trichoderma* that showed increased root development and biomass of *Arabidopsis thaliana* over 8 days (Contreras-Cornejo et al. 2011);
- 2,4-diacetylphloroglucinol, from AMF (Wehner et al. 2010);

Another reason for inoculation has been to counteract stressors on plants such as drought or salinity. Abiotic stress adaptation has been shown to involve a range of metabolites including the following:

- rhizospheric fungi *Trichoderma harzianum* Rifai strain T-22 using lipid peroxides in tomatoes as stress biomarkers (Mastouri et al. 2010);
- drought protection of plant through the increased production of hormones such as indole-3-acetic acid, JA, SA, ethylene, auxins, cytokinins and gibberellins and amino acids like proline (Azcón et al. 2013);
- reduced concentrations of malondialdehyde and increased concentrations in proline and phenol metabolites in wheat due to salinity (Rawat et al. 2011); and,

- in the case of *Trichoderma* spp., the regulation of root system architecture that was made possible with reactive oxygen species that convert into hydrogen peroxide (Contreras-Cornejo et al. 2013; Mastouri et al. 2010; Samolski et al. 2012).

Metabolomics can also be used as a tool to find soil microbial metabolites that are a signal of diseases occurring within the soil (Bundy et al. 2009). For example, the fungi *Trichoderma* spp. (Lorito et al. 2010; Vinale et al. 2008) have been found to improve plant defences through secondary metabolite communication. A pathogen attacking a plant results in *Trichoderma* expressing amino acids to create small proteins called hydrophobins that act to coat the plant as a barrier. Other metabolites used for defence included heptelidic (koningic) acid (Itoh et al. 1980), along with isocyanide derivatives, proteins with α-aminoisobutyric acid and a range of FAs (e.g. C8, C10, C10:1).

7 Humus

Another major component within the rhizosphere is humus. Humus is a complex, amorphous and colloidal substance of natural organic matter, a condensation of phenolics and nitrogen containing compounds derived from plant and animal tissue decay (Paul 2016; Sutton and Sposito 2005). Humic substances, which until recently (Lehmann and Kleber 2015) have been considered largely the products of microbial metabolism (Manlay et al. 2007), are recognised as promoting both microbial and plant growth (Coûteaux et al. 1995; Ponge 2013). A recent study examined the changes in the metabolic profile of maize seedling root exudates by ^{1}H NMR and GC–MS following co-inoculation with the PGPR diazotrophic β-proteobacterium *Herbaspirillum seropedicae* and humic acid (Lima et al. 2014). The classes of compounds detected in maize exudates included nitrogenous compounds, FAs, organic acids, steroids and terpenoid derivatives. Substantial changes in the exudation patterns 14 days post-inoculation were observed. For instance, root exudates from seedlings exclusively treated with humic acids demonstrated differing quantities of FAs, phenols and organic acids from that of the controls. Those seedlings treated singularly with *Herbaspirillum seropedicae* or in combination with humic acids exclusively exuded a diversity of heterocyclic, nitrogenous benzilamines and polyamines. The study showed that enhanced root colonisation of *Herbaspirillum seropedicae* in the presence of humic acids could be explained by the interplay between increased endophytic colonisation of *Herbaspirillum seropedicae* and sorption of humic acids to plant cell wall surfaces (Canellas et al. 2013). Interestingly, compounds identified as possible QS-inducing agents (e.g. substituted γ-butyrolactones and 3-hydroxypalmitic acid methyl ester) were also detected.

There is some controversy with the metabolomic characterisation of humus, with some researchers claiming that extraction methods are creating the larger "biomolecules" from smaller soil-sourced metabolites (Schmidt et al. 2011). For example, they argue that the well-known but difficult to characterise humics are actually synthesised from smaller soil metabolites such as carboxylates that condense due to the extraction process, as evidenced by the use of non-destructive analytical techniques, and in situ observations (e.g. near-edge X-ray fine structure spectroscopy combined with scanning transmission X-ray microscopy).

8 Terroir

In the viticulture industry, the word 'terroir' describes regional influences associated with climate, soil factors and plant genotype which strongly affect varietal flavours and aromas (Styger et al. 2011). Biochemically, the same term relates to modifications to the metabolome of a plant that impart a set of discrete desired qualities, such as increased plant growth or reduced feeding by larvae (e.g. cabbage "worm" caterpillar *Trichoplusia ni*) on plants (Badri et al. 2013b). The 'terroir effect' is shown to manifest itself in cultivated grape varieties that have been resident in the same location for extended time periods (van Leeuwen and Seguin 2006), and this concept has begun to be used to describe 'microbial terroir' (del Mónaco et al. 2016). It has been suggested that this effect could likely be attributed to soil microbes or the overall soil microbiome actively adjusting metabolite fluxes in response to signals from plants (Badri et al. 2013b). In one study, GC–MS was used to assess the effect of diverse soil microbiomes applied to the roots of *Arabidopsis thaliana* on the leaf metabolome and, by extension, whether the changes in leaf metabolome influenced the feeding behaviour of *Trichoplusia ni* larvae. The study demonstrated that variations in soil microbiome composition (primarily comprised >60 % actinobacteria and proteobacteria) produced a differential response to canopy and root biomass accumulation in *A. thaliana* plants. In comparison to control soil slurries (absence of soil microbiome), all *A. thaliana* plants showed an upregulation of amino acids, phenolics, sugars and sugar alcohols in leaf material. In addition, presented evidence suggested a strong capacity of soil microbial communities to either modulate above-ground feeding behaviour of *Trichoplusia ni* or to enhance the herbivory resistance of 4-week-old *A. thaliana* plants. It is surprising that in examining what makes a quality grape for wines that soil and the microbial life has until recently been largely ignored (Burns et al. 2015). The reason behind this is that soil microbial effects are subtle versus climate [(Pereira et al. 2006), Table 2], rainfall [(Lee et al. 2009), Table 2] and soil texture (Pereira et al. 2006) which have been found to be larger drivers of terroir.

9 Nutrients in the Rhizosphere

Nutrient availability is at the heart of producing a healthy crop or pasture, and changes to the macro- (e.g. N, P and K) and micronutrients (e.g. S, Mg, Ca) affect plants and soil, with soil microbes adjusting accordingly. As the soil environment changes due to season, moisture content, pH, soil texture, etc., nutrient fluxes in soil result in changes in metabolite concentrations as microbes adapt or suffer from these changes (Lorenz and Wackernagel 1994).

Phosphorus, due to its low solubility (≤ 10 μM), remains one of the most challenging soil nutrients for plants to acquire (Smith et al. 2011). For instance, organic P is thought to be made up of predominantly inositols, DNA, RNA and phospholipids (Nash et al. 2015). Often complexed to the minerals that make up the soil (e.g. iron and aluminium), they can be difficult for soil microbes to access (Jones 1998). One study examining organophosphorus metabolites used glucose-6-phosphate with labelled ^{33}P or ^{14}C atoms to identify how microbes are affected by deficiencies in nutrients (Heuck et al. 2015). The authors determined that soil microbes prefer utilisation of this metabolite as a C rather than P source with even complete uptake of the sugar resulting in excretion of P. The addition of the additional organophosphorus compound revealed an increase in microbial activity, which seemed to level off after 66 h, although this was not further examined to see if this was due to other nutrient deficiencies (i.e. N) or specific to fungi or bacteria. A metabolomic analyses might have also revealed the type of C containing compounds that were being taken up. Increase in P availability to the host plant is one reported benefit of mycorrhizal root colonisation, which plants do through the expenditure of energy and organic metabolites to organisms such as AMF (Smith and Smith 2015). The trade off to acquire P has been observed in tomato roots from AMF *Funneliformis mosseae* and *Rhizophagus irregularis* colonisation experiments [(Rivero et al. 2015), Table 4]. The authors observed that in acquiring P, the use of AMF led to significantly greater N in mycorrhizal roots ($p < 0.05$). However, shoot and root biomass, root/shoot ratio and total C were not significantly altered. The cost to the plant appeared to be the reduction in phenyl alcohols and vitamins, along with some amino acids (i.e. tryptophan, tyrosine, phenylalanine, alanine and leucine). However, increases in root concentration of intermediaries to amino acid (i.e. phenylalanine and tyrosine), sugar, carboxylic acids and fatty acid metabolites revealed the benefit of the interaction between plant and fungi, from the increased uptake of nutrients to the improved stress response of the colonised plants.

Nitrogen has been the major driver of increases in pasture and crop production since the 1950s. Due to the success of inorganic N used to increase plant biomass, our knowledge of organic N is surprisingly limited (Warren 2013, 2014). Characterising organic N metabolites that are produced by microbes is also a nascent field, and soil metabolomic data would certainly advance the knowledge base of this important nutrient. To date, characterisation of amino acids in soil has been the most studied group of metabolites that are associated with microbes, with

fumigation of samples often used to determine microbial metabolites (Heuck et al. 2015; Swenson et al. 2015b; Warren 2015). However, there has been progress in identifying key metabolites that microbes use in the soil.

AMF are also important soil microbes that have been studied for understanding the movement of organic N. However, the study of N fluxes between plants and AMF has been limited compared to the current focus of understanding the transfer of C from plants (Hodge and Fitter 2010). Until recently, it was thought that AMF received most N from its host plant, with transport generally occurring through the metabolite arginine. However, it has been shown that AMF also seek out and promote decomposition of organic compounds for the acquisition of N, with a substantial concentration retained within arbuscular mycorrhizae structures for their own growth. As N limitation is reported to reduce the benefits of AMF symbioses (particularly with excess P) (Huang et al. 2014), a comparable study examined the effect of AMF field inoculation (either single or co-inoculated with PGPRs) on the root metabolome of durum wheat (*Triticum durum* Desf.) under N-limited, P-rich conditions [(Saia et al. 2015b), Table 4]. Metabolomics was used to determine how AMF or plant growth-promoting rhizobacteria (PGPR) affect wheat growth. Despite low N and high P soil conditions, plant growth doubled when adding AMF at the expense of amino acids and saturated fatty acid concentrations in roots. However, with the addition of PGPR, the acids were retained. Overall, 118 metabolites were identified and using the Kegg database (http://www.genome.jp/kegg/) metabolic pathways were delineated from 83 identified compounds. Multivariate analyses showed separation by treatment effects, with amines and unsaturated FA conserved in the AMF treatments. Organic acids correlated with AMF + PGPR treatments versus the control samples, which were also correlated with P-containing compounds, saturated FA, carbohydrates and amino acids. An interesting observation was the increase in xylitol, indicating a strong interaction between AMF and wheat.

A recent paper has shown how a multi-omics approach can lead to an improved understanding of fungi and plant interactions (Larsen et al. 2016). The experiments were designed to identify the signalling metabolites between aspen trees (*Populus tremuloides*) and the mycorrhizal fungi *Laccaria bicolour*. Combining transcriptomics, metabolomics and genomics, the authors were able to identify a number of biochemical processes related to communication between the plants and fungi. Metabolites identified from the fungi and correlated to plant gene regulation included amino acids and phosphor sugars related to biochemical pathways for the biosynthesis of aromatic compounds, plant hormones, plant metabolites including quinines and their precursor, and metabolites related to plant terpene biosynthesis. These metabolites were identified as being involved with fungal communication with the aspen to modulate cell adhesion, defence response and cell wall modification, presumably to facilitate the symbiosis between the two. A similar multi-omics approach has also been reported for novel compounds such as nanoparticles that can potentially exhibit unknown and potentially hazardous effects (MacCormack and Goss 2008).

Other examples of enhancement of nutrient availability and the metabolites involved include the following:

- the suppression of soil pathogens and production of auxins, peptides, ketones and terpenes (López-Bucio et al. 2015) by *Trichoderma* spp. protecting chickpeas (Rudresh et al. 2005) so as to increase P uptake; and,
- interactions between fungi and plants that express lipids and trehalose that result in microbe (e.g. *Glomus versiforme*) mediated exchange of nitrates, phosphates and amino acids [particularly arginine (Smith and Smith 2015)] from soil to plant (Bonfante and Genre 2010). This two-way interaction includes the release of metabolites such as strigolactones from plants into the rhizosphere.

Tree litter is another important source of nutrients on which soil microbes can feed. Research has shown that microbial communities adapt to the tree they are under (Ayres et al. 2009). A study of leaf litter revealed that different tree species litter, monotypic stands of trembling aspen (*Populus tremuloides*), lodgepole pine (*Pinus contorta*), and Engelmann spruce (*Picea engelmannii*), resulted in different populations of microbes (Wallenstein et al. 2010). A number of metabolites were identified that varied significantly between the tree species. Although the metabolites were only identified by retention time and mass, it was suspected with comparison to other studies that the metabolites were products of soil microbes or fungi. A followup study using pyrolysis molecular beam MS (py-MBMS) was able to determine that the metabolites were a mixture of lower mass (<137 m/z) carbohydrates, phenols and lignin monomers combined with higher mass (m/z = 252–706), lipids, alkanes, alkenes and FA (Wallenstein et al. 2013).

This research was expanded to analyses of typical oak, beech and grassland soils, and likewise identified that soil microbes were adapting to tree species differences, expressed through changes to a range of soil metabolites [(Liebeke et al. 2009), Table 2]. Grassland and beech forest soils were found to be less diverse than oak forest. This research contrasted that with improvements to soil quality when using soil that the microbes were collected from compared with artificial substrates, due to the variety of metabolites were available for each of the three groups of soil. Glutamic acid was the predominant amino acid (approximately 70 μM in concentration) in oak and in the top three for the other two soil types (although at lower concentration). Leucine and valine were also common to all three, and of the other amino acids, only the oak soil sample had sulphur containing methionine. Amino acids were attributed to degradation of proteins in the soil from microbial decomposition. The most concentrated sugar was trehalose, again being an order of magnitude greater in concentration in oak compared to the other soil samples. Other sugars and organic acids were identified with the total mass of these compounds revealing the richness of the oak soil metabolites (136 μM vs. 7 μM for grassland and beech), a similar ratio as found for the physicochemical analyses (e.g. soil organic carbon). Gram-positive and Gram-negative bacteria grew exponentially in the oak soils, and the types of soil metabolites seemed to indicate what biochemical processes were being used by the bacteria that were found.

10 Extracellular Enzymes

As the previous sections attest, there is no doubt that it can be difficult to identify microbial metabolites from the myriad of chemical compounds in soil (Ponomarova and Patil 2015). This becomes more problematic when sampling away from the microbial powerhouse of the rhizosphere. One example is extracellular enzymes (EE), enzymes released by microbes or plants into the soil to facilitate biochemical processes (Wallenstein and Weintraub 2008). These processes include degrading recalcitrant fractions of SOM for uptake by the microbes, secretion of metabolites to "sense" what predators or prey are in the immediate environment, and release of antibiotics to attack other microbes (Burns et al. 2013; González-Fernández et al. 2015; Wallenstein and Weintraub 2008). For example, EE from fungi produce metabolites such as the lactone-based botcinolides and the terpene-based botrydial compounds (González-Fernández et al. 2015). These metabolites are thought to be excreted to attack plants through decomposition of plant cell walls followed by nutrient acquisition from the plants. EEs from soil bacteria and fungi are known to consume carbon-rich biomolecules such as chitin (Roberts and Selitrennikoff 1988), lignin (Burns et al. 2013), tannins (Joanisse et al. 2007) and pectins (González-Fernández et al. 2015; Tepper and Anderson 1990). Sugars and amino acids from glycoproteins are also found on microbial adhesives (Wang et al. 2014) from EE-producing microorganisms after consumption of the plant and microbial debris. Plant litter is also a rich source of nutrients for EE-producing microorganisms. Monitoring of lignin decomposition has revealed the production of metabolites such as quinines and radical lipids that may potentially form humic compounds in soils with access to phenols, peptides and carbohydrates (Schmidt et al. 2011).

Quantitatively and qualitatively, there is still ambiguity in the sources and consumption of metabolites in soil. Whether the source is via a combination of EE excretion, plant decomposition or experimental error, metabolomics techniques may be amenable to characterising the macromolecules produced by EEs (e.g. polysomes). A potential solution described in the literature involves attaching coloured marker molecules to EEs in a dilute soil slurry, as the enzymes tend to stay fixed to the soil and unavailable for analyses through typical extraction techniques (Burns et al. 2013). Limitations of the current methodology include no knowledge of enzyme turnover rates, limited number of fluorescent markers available to attach to a limited number of functional groups and selection of only those enzymes capable of being stabilised in the slurry, versus in situ soil samples.

Metabolomics has been suggested as a way of removing these limitations by detecting the entire metabolome of a soil sample. Both pyrolysis-GC-IRMS and LC–MS analyses have provided examples of how this might work. For instance, a general survey of EE metabolites involved soil from the USA and Germany (Liebeke et al. 2009). Analyses of soil involved both untargeted metabolomics of GC–MS and ^{1}H-NMR of SOM, while targeting metabolites of microbe *Bacillus licheniformis* for comparison. A range of FAs, sugars, amines, amino acids and organic acids were identified. Most metabolites were species dependent though

metabolites acetic, fumaric, aspartic and glutamic acid, glycine, proline, serine, glucose and sucrose were found ubiquitously across the varied agricultural types.

11 Anthropogenic Effects on Soil Microbes

It is argued that we are in the Anthropocene age (Bundy et al. 2009; Desai et al. 2010; Rockström et al. 2009), so it is not surprising that soil microbes have had to adapt to human endeavours, some of which can be deleterious to the environment. Estimates made in 1998 include up to 100 000 chemicals which were available for purchase (Rockström et al. 2009), many of which will end up in the environment. Since then, material synthesis and engineering has advanced greatly, and so it should be no surprise that many of these compounds end up affecting the soil microbial food chain [(Simpson and McKelvie 2009), Table 2]. Indictor animals such as earthworms are often used to determine soil health (Rochfort et al. 2009; Whitfield et al. 2013), but there is an increasing amount of research into the effects of synthetic chemicals on soil microbes (Hernandez-Soriano and Jimenez-Lopez 2014). The use of microbes to monitor or remediate contaminated sites is one of particular interest (Desai et al. 2010; Jones et al. 2014). A soil metabolome would allow for the ability to look across a range of biogeochemical factors that may affect soil health. The discussion here will focus solely on metabolomics research as it relates to microbes.

Agricultural changes of soil from pristine forest to farm land has been a major change to the environment that has been occurring for thousands of years (Foley et al. 2005). Changes occurring in soil management have been described through the lens of metabolomics (Singh 2006). For instance, land use provided an opportunity to use NMR metabolomics combined with mid-infrared spectroscopy (MIR) to identify the effect of land management across four different regions of Victoria, Australia (Rochfort et al. 2015). Comparing soil samples from relatively untouched native land and adjacent farm land of oats or wheat, ^{1}H-NMR was able to identify a series of signals from lipid, terpene and sugar. The study identified that these metabolites could be differentiated by NMR due to different concentrations depending on land use, whereas soil location was differentiated using MIR. This is similar to a ^{1}H-NMR study of various mine sites across England (Jones et al. 2014) which identified a similar series of metabolites, with differences between compounds thought to have occurred due to a different solvent extraction system (methanol for this study versus deuterium oxide). In both cases, the assumed microbial source of these metabolites was explored, using a target microbe, *Bacillus subtilis*, for the Australian study and the identification that most soil metabolites were microbially based for the English analysis. Labelling techniques may give enhanced information, as was shown in the effects of microbes feeding on mine waste. Metabolite confirmation of mine waste and its effects on microbes was conducted using stable isotope labelling (Mosier et al. 2013). Using this method with ^{15}N labelling allowed for the identification of 80 metabolites from 3500

metabolite features that included artefacts, non-biological metabolites, adducts, etc., the latter of which were considered predominantly microbial.

How soil microbes are able to adapt to new environments and unusual metabolites has found use in regions away from their original habitat, as for example in recovering petroleum oil (Arora et al. 2014). For example, the use of mutant bacteria known to be resistant to metalloids, *Pseudomonas pseudoalcaligene*, was used to remove polychlorinated biphenyls (Tremaroli et al. 2009). To identify the mode of action, metabolomic studies identified that thiols were oxidised when the microbe reacted to the metal. ^{1}H-NMR combined with multivariate analyses showed that wild-type and mutant bacteria resulted in changes to concentrations of amino acids (i.e. glutamate, aspartate, glycine, histidine, tryptophan and tyrosine), betaine and NAD+. *Pseudomonas pseudoalcaligene* was found to be resistant to other toxic compounds, including caffeine, sulphates, streptomycin and chlorinated compounds.

11.1 Engineered Nanomaterials (ENM)

Novel compounds, often of the size or smaller than the microbes themselves (e.g. nanometre), are increasingly being developed, used and disposed of, on soils. The design and use of engineered nanomaterials (ENM) is in part because they have unusual and useful properties (e.g. strength and conductivity) that are different from the bulk compound found in nature (Dinesh et al. 2012). ENM, so called to distinguish them from natural soil nanoparticles (e.g. colloids), are receiving increasing attention in agricultural and environmental literature. As production of ENM increases to meet the demand of high-tech materials, these products are more likely to end up in soil. Products with ENM include sunscreens, cleaning products and therapeutic goods. Some ENM, such as zero-valent Fe, are widely used in parts of the world for cleaning up toxic chemicals (Lee et al. 2008). Until recently, the effect of ENM on the environment had not been systematically studied, in part due to the cost of manufacture, the similarity in size and composition of natural colloids (Klaine et al. 2008), as well as the physicochemical aspects of the soil (MacCormack and Goss 2008). Various studies have shown that ENM are potentially hazardous in a laboratory setting, but there are few field studies (Dinesh et al. 2012; Johansen et al. 2008). To date, ENM have been predicted to be in the environment (i.e. surface waters) in concentrations of 0.8 ng/L for carbon-based ENM up to 10 μg/L for Ag-, Ti- and Zn-based compounds (Maurer-Jones et al. 2013). No such study has been conducted for concentrations of ENM in soil.

Adding ENM to soil to see how they affect the biosphere has been the main method of determining their effects [(Jin et al. 2014; Johansen et al. 2008; Shah and Belozerova 2009), Table 1]. This has enabled studies of how the dosage of ENM affects the soil environment. As ENM are in a solid matrix, it can be difficult to ascertain the dose actually received by microbes, and so studies may overestimate their soil concentration. Although it is well recognised that they have the potential

to cause pollution in soil through accumulation, ENM have particular properties that can make it difficult to determine their interactions with denizens of the soil matrix, including microbes. This includes aggregation into larger particles and adsorption to minerals within the soil. It is inevitable that nanoparticles are found in soil due to accidental release as they have already been detected in marine and airborne environments.

This lack of knowledge means that these compounds are an unknown threat to the soil and its microbial community. A recent review of 10,000 papers on ENM found that despite the explosion of research on human health, the consensus on their toxicity is at best, weak and often misleading (Krug 2014). As to be expected, research on ENM and their effects on soil and its inhabitants are even less clear. Beyond health and environmental aspects, ENM are also being explored as intermediaries and markers in microbe communication to help researchers identify disease rates in soil (MacCormack and Goss 2008).

Carbon fullerenes and nanotubes are arguably the most well-known ENM. They are also increasingly finding their way into the environment, and into soil (Berry et al. 2016). However, studies to date find limited interactions with soil microbes (Pettibone and Louie 2015). Two papers by researchers in South Korea highlighted how microbes are affected by carbon nanotubes in their environment (Jin et al. 2014; Jin et al. 2013). The metabolic profile of the microbes, revealed through phospholipid fatty acid analyses (PLFA), showed that microbial FA typically changed abundance depending on whether the soil were treated with powdered SWCNT (single-walled carbon nanotubes) or solvent suspended SWCNT. Generally, as the concentration of SWCNT increased, the biomarker fatty acid (i.e. odd chained and/or hydroxyl grouped and/or cyclic unsaturated) metabolite concentration for Gram-positive bacteria, Gram-negative bacteria and fungi significantly decreased. This effect on microbes occurred for at least 25 days. Increases in other types of FA (e.g. iso-branched) led the authors to believe that microbes change their lipid composition to defend against SWCNT. Another study of bacteria and protozoa and how they are affected by C60 fullerene (Johansen et al. 2008) noted that systematic biases may be an issue leading to difficulty in comparing results with other research. In particular, using pure solvents versus soil samples meant that other chemical characteristics of fullerenes are not taken into account when the effects on microbes are analysed. However, as was found in studies with cleaner conditions, fast-growing microbes suffered significantly upon addition of C60 to soil (p = 0.004–0.033). The bacteria eventually recovered from fullerene exposure. Suggested reasons for this included absorption of C60 to soil or other particles that coated the ENM and minimised contact with microbes.

Metal ENM have a longer history than carbon ENM, and have been used, sometimes unknowingly, since ancient times [i.e. gold nanoparticles for decoration of ancient Roman sculpture (http://phys.org/news/2013-08-goblet-ancient-romans-nanotechnology.html)]. Despite their more extensive history, research into their interactions with microbes in the soil is limited. Like other ENM, metal ENM analyses are complicated by the number of naturally occurring nanoparticles or similar minerals already present in the soil. It has been suggested that labelling

metal ENM with unusual isotopes could improve the analyses of these particles (Klaine et al. 2008). Most research of metal ENM has shown that they act as bactericides (Lee et al. 2008). As these compounds have been used for water treatment and fabric formation, some of the pollution may end up in soil (Stefaniuk et al. 2016). Measuring microbial responses to metal ENM have usually focussed on their toxicity through the use of fatty acid methyl esters (FAMEs) analysis. For instance, a study on microbial response to Pd, Cu, Si and Au ENM used FAMEs typical to microbes to determine how microbes survived in their presence [(Sasser 2006; Shah and Belozerova 2009), Table 1]. Despite using two comparative concentrations of metal ENM in soil over 15 days (0.013 and 0.066 % w/w), no significant change in these microbial biomarkers metabolites was seen. However, it was speculated that ENM may exhibit an indirect effect through interaction of compounds required by microbes. This was shown in a concurrent experiment where the growth of lettuce seeds was reduced in the presence of Pd and Au ENMs. One positive review discussed how microbes could be used to create metal ENM for commercial use, based on microbes being able to create metal organic complexes such as iron oxide magnets, metal phosphate medicines and transition metal catalysts (Lloyd et al. 2008).

11.2 Heavy Metal Contamination

Microbes such as AMF can be affected by heavy metal contamination (Karimi et al. 2011), and it has been shown that the metals reduce microbial biomass. Metal wastes reaching soils are another environmental issue (Maurer-Jones et al. 2013; Simpson and McKelvie 2009). Similarity in atomic size to nutrients, some heavy metals can access microbes through channels designed to diffuse and transport cations (Singh et al. 2016). This similarity has been put to use with Cu used as a fungicide that has been shown to improve crop yield (Dhawi et al. 2015). As metals cannot be degraded, microbial action tends to involve immobilising the contamination so that its toxic effects are mitigated (Azcón et al. 2013). The success of microbes and plants in resisting radiation effects (Stone 2009) and reducing concentrations of toxic metals has been identified in places of recent disasters such as the Chernobyl and Fukishima nuclear accidents (Aung et al. 2015; Geras'kin et al. 2008). It has been noted that continuous exposure to heavy metals can result in tolerance forming in microbes as they employ metabolic strategies to reduce metal toxicity though reduction of the metal's oxidation state or coordination of metabolites to soluble metals ions (Jones 1998; Karimi et al. 2011). For instance, the microbe *Klebsiella mobilis* CIAM 880 was able to release metabolites that bind with Cd to promote plant growth in soil with high cadmium concentrations, precipitating the metal (Nies and Silver 1995; Pishchik et al. 2002). This was seen in the plant having a larger root system and increased exudate concentrations released into the soil.

The compound that probably typifies pollution since the industrial era is lead (Nriagu and Pacyna 1988). One metabolomics study identified metabolites that changed in concentration due to mycorrhizal microbe influence attenuating the plants response to Pb contamination (Souza et al. 2014). The analyses identified a series of amino acids that may complex heavy metal and remove the metal's ability to affect the plants. The amino acids include asparagine, histidine, proline and glutamine. Using the AMF *Glomus etunicatum*, it was possible to identify potential microbial interactions with plants (e.g. N and carbohydrate metabolism) that offer protection from Pb. Pb was also identified as a factor in metabolomics analyses of a series of mines in the UK [see the "Community Metabolomics" chapter by Jones et al. (2014) this book]. Using ^{1}H-NMR, metabolomics was used across 11 sites that mined Pb and Zn (Jones et al. 2014). A simple methodology with minimal extraction meant that, along with the microbial community, soil and invertebrates were also sampled. The authors examined specific groups of metabolites (nucleotides, sugars, lactate and amino acids) using multivariate statistics. They were able to show that there were sites that had similar metabolic profiles and lower Fe concentrations even though the mines were otherwise different in geochemical makeup. The authors proposed that monitoring metabolomics could act as an early warning to hazardous pollutant levels before any visible effects were seen. PLFA was used in the Czech Republic to determine how mining waste materials fly ash and mine digestate that contained high Pb, Si and Zn concentrations affected soil microbes (Garcia-Sánchez et al. 2015). Organic compounds' concentrations, including phenolics, were identified as increasing under both contaminants, with the researchers identifying this via increased microbial activity, particularly fungi, under the digestate treatment (9.3 ± 1.4 μg/kg after 60 days, $p \leq 0.05$). Other compounds found due to contamination included carbohydrates, carboxylic and amino acids, amines and polymers, although the researchers cautioned that the metabolomics and genomics analyses could not differentiate between active and passive microbes in the soil. A timed study showed that after two months, amine compounds had become preferentially consumed. Digestate contaminated soil was shown to result in a preference for microbes to consume carboxylic acids taken from the SOM. The PLFA analyses found that metabolites associated with fungi were most attenuated by the digestate and fly ash. Metabolites associated with Gram-negative bacteria were also affected. The digestate increased the concentration of Gram-positive bacteria after two months. Fly ash was found to be beneficial to soil community structure, with the metabolites associated with most types of microbes increasing. Biofilms in mines have also proven to be a rich source of metabolomic data on how microbes are affected by various metal wastes that included sulphate, iron, zinc, copper and arsenic (Mosier et al. 2013).

Cadmium contamination is also a concern in soil as it affects the life cycles of most species [(Sarry et al. 2006), Table 2]. Cd has been found to affect people through kidney damage after consumption of contaminated food, with the suspicion that the metal's affinity to thiols is deleterious to organisms. This includes yeasts and fungi that are found in a variety of forms in agriculture (Sláviková and Vadkertiová 2003). Yeast under the effects of cadmium was studied using

metabolomic and proteomic techniques (Lafaye et al. 2005). The research identified that the typical sulphur pathway in yeast for producing glutathione increased in rate and the sulphur amino acid concentration reduced by 30 % as the microbe attempted to remove cadmium. Proteome and metabolomic results were correlated when Cd was used, but other treatments (e.g. sulphur starvation) that also result in increased glutathione production were not, indicating an independent pathway is initiated when Cd contamination is present.

A controlled study of increasing Fe concentrations was used to determine if the microbe *Pseudomonas stutzeri* RCH2 was affected by metal-poor or metal-rich soils (Swenson et al. 2015a). The idea was that Fe will affect the microbe's metabolite output as the competition for sites on either the metal or carbon of SOM in soil is changed. As expected, increasing the Fe concentration increased the sorption of all metabolites including those with phosphate, N-containing and carboxylate functional groups. The research identified that concentration change in metabolites was correlated to the charge of the anion (e.g. $Phosphate^{-} - Fe^{+}$) when it came to sorption to Fe. The authors felt that it was important to conduct metabolomic analyses to ascertain the rates of sorption due to a typical mix of microbial metabolites, which may differ due to competing interactions compared to when separate metabolites being tested. While the analyses did not bring many surprising results (i.e. phosphates and dicarboxylates adsorb strongest to Fe), this paper is a rare example of the application of metabolomics to understand the holistic system of soil–microbe interactions.

11.3 Organic Contaminants

Research on organic compound contamination has been presented that offers not just problems but solutions using metabolomics of soil microbes. For instance, metabolomics was used to determine how microbes might be able to recover petroleum from reservoirs that are not cost effective by traditional extraction techniques (Arora et al. 2014). This study used microbial enhanced oil recovery of an oil well on soil samples from India to determine how efficient the microbes were and what decomposition products they produced during the extraction. Using indigenous hyperthermophilic *Clostridium* sp., they tested how these microbes may extract oil at high temperature sites (>91 °C). The use of water from the oil well site ensured no contamination of microbes from elsewhere in the soil. Metabolites collected included biosurfactants, organic acids, solvents, exopolysaccharides and volatile FAs. Following a targeted analysis of the metabolite mix from different groups of bacteria, the researchers were able to optimise conditions to increase the concentration of metabolites that would be suitable for extraction, including sugars (particularly sucrose), nitrates and ammonium metabolites (particularly urea). Metabolomics has also been used to better understand the bioremediation of soils or soil models (Singh 2006). NMR studies have generally used soil samples rather than solutions, although there are metabolomic studies using liquid-state NMR to

monitor microbial degradation of xenobiotics. For instance, in one study on how *Mycobacterium* biodegrades morpholine, piperidine and thiomorpholine, scientists were able to use enriched carbon and nitrogen compounds to identify the metabolites formed as the bacterium consumed the antibiotics [(Delort and Combourieu 2001), Table 2].

Persistent organic pollutants (POP) are another source of contamination that can affect all species, including soil microbes (Wania and Mackay 1996). These are small compounds with aromatic rings and potentially halogenated functional groups. While it has been thought that condensation of these compounds into soil is a better alternative to POP being airborne, the effect on organisms is still a problem. Polycyclic aromatic hydrocarbons (PAH) are one target of active research, particularly on contaminated sites such as oil fields and fires (Seo et al. 2009). One study identified a series of metabolites isolated from the microbe *Sinorhizobium* sp. C4 that was extracted from soils contaminated with another PAH, phenanthrene (Keum et al. 2008). After determining that the mode of degradation was ring opening, the authors used untargeted analyses to monitor polar metabolites such as FAs and polyhydroxyalkanoates as the bacteria were fed phenanthrene. This was detrimental to the microbe, with more than 70 % of these metabolites decreasing in concentration after being fed phenanthrene. POP eradication via soil microbes is another area of active research. Polychlorinated biphenyls (PCBs) have had a 90 % removal rate over one month when placed in the rhizosphere of Arabidopsis (Narasimhan et al. 2003). The expression of phenylpropanoids increased by over 100-fold compared to control samples which the authors linked to the breakdown of PCBs. It was proposed that by adding to the soil, 10–100 fold the current concentration of the microbe *Pseudomonas* spp., the current estimates of PCBs in soil [328 000 tonnes (est. 1988)] could be significantly removed. A consensus is that soil microbes in the rhizosphere have adapted to using PCBs as a source of carbon and energy, utilising exudates to degrade them, through the use of metabolites such as biotin, thiamine, amino acids and isoflavanoids (Jha et al. 2015). The two major metabolic processes identified from this research were anaerobic dechlorination and aerobic biodegradation.

Sometimes, as in the case of pesticides and antibiotics, chemicals are deliberately applied to soil. How soil microbes are affected is often overlooked. Pesticides have a long history in agriculture and so their effects on non-target organisms have been overshadowed by the benefit to plant yield and productivity (Imfeld and Vuilleumier 2012). Antibiotics in agriculture, either by accident or design, are becoming a real concern due the resistance of many bacteria that infect humans (Di Marco et al. 2014), even when their primary use is intended to promote animal growth (Horrigan et al. 2002).

Pesticide reduction through improved efficiency of soil microbes such as AMF has been proposed (Baum et al. 2015) with *Trichoderma* spp. providing an excellent example of how this can be achieved (Vinale et al. 2008). It should be noted that studies have shown that the replacement of conventional pesticides with "biopesticides" must be a gradual process as mycorrhizae concentrations have decreased while synthetic fertilisers were applied (Imfeld and Vuilleumier 2012;

Ruzicka et al. 2012). Metabolomics can monitor how biopesticides function by observing concentrations of carbohydrates, lipids and n-acetylglucosamine, among others (Baum et al. 2015). Other secondary metabolites from plants that also reduce the need for pesticides include phenolics, alkaloids and coumarins. For instance, phenolics have been shown to reduce weed growth (Khanh et al. 2005). Another example is the effect of 2,4-dichlorophenoxyacetic acid (2,4-D) on *Escherichia coli* (Bhat et al. 2015). Previous conflicting research identified that there were significant disturbances to soil microbe communities. Using GC–MS for the analysis [combined with analyses using scanning electron microscopy (SEM) and atomic force microscopy (AFM)] the authors were able to determine a specific pathway attributed to 2,4-D exposure. This involved a combination of attenuation of oxidative phosphorylation, ABC transporters, peptidoglycan biosynthesis, glutathione metabolism and purine/pyrimidine metabolism, and an increase in amino acid, protein, sugar and starch metabolism. There was also a significant reduction in the concentration of metabolites associated with membranes and cell walls.

In regards to antibiotics, the decreasing effectiveness of current medicines can be balanced with the discovery of new antibiotics in soil created by microbial action, as in the example of teixobactin (Ling et al. 2015). For instance, one compound that is rapidly losing effectiveness as a medicine, tetracycline, has been examined and found to affect the rhizosphere leading to a loss of exuded metabolites (e.g. phenols, flavonoids) by up to 48 % (Di Marco et al. 2014). Similar research looking at maize and its interactions with AMF found up to sixfold increases in carbohydrates, amino acids and phenolics in the soil when the antibiotics Cefotaxime and Trimethoprim were added, showing how deleterious antibiotics can be (Azaizeh et al. 1995). Research detailing how microbes and plants can themselves act as antibiotics is demonstrated by a recent study showing that *Bacillus subtilis* was inhibited when placed in soil samples from wheat farms and untouched forest (Rochfort et al. 2015). Correlating data between the lipids, terpenes and sugars in the soil was able to be matched with the antibiotic effects against the microbe. Metabolites such as fulvic acids, isochromantoxins, organic acids and xanthocillins have been identified in a wide ranging study of microbes including *Aspergilloides*, *Furcatum* and *Penicillium* (Frisvad et al. 2004). It should be noted that due to technological limitations, the metabolites of these well-known microbes may be misidentified.

11.4 Climatic Change Effects

Potentially, the greatest anthropogenic effect of the current century is how the planet responds to an increasingly variable climatic pattern of weather (Edenhofer et al. 2014). Metabolomics has shown promise as one way to quickly quantitate metabolites that microbes exude in response to climate stressors (Simpson et al. 2012). Expected changes in weather and climate have led to the call and use of metabolomics techniques to understand how the denizens of the land will adapt (Ahuja et al. 2010). Climatic effects on soil microbes are expected as changes in

temperature along with water and nutrient concentrations become more variable (Rennenberg et al. 2009).

Salinity is one climate change issue that is already common. Salinity is increasing and the replenishment of water tables is difficult. Exacerbating this problem is research showing that replenishment of water tables is more difficult as temperature rises (Lozupone and Knight 2007). Metabolomics approaches have predominantly examined plants, but microbial interactions with plants due to salinity have also been examined. Studies of a variety of plants (i.e. *Arabidopsis thaliana*, *Lotus japonicus* and *Oryza sativa)* seem to suggest metabolites used to communicate between microbes and plants in the rhizosphere will be significantly affected (Sanchez et al. 2008). These metabolites include organic and amino acids, along with sugars. This may be mitigated depending on the plant species, as was found in the case of the highly salt-tolerant Brassicaceae, *Thellungiella salsuginea*, where there appeared to be little change regardless of salinity levels [(Lugan et al. 2010), Table 3].

One issue with salinity is that the high salt concentration in these soils can interfere with the sample, leading to loss of data through ion suppression (Oikawa et al. 2011). To address this issue, the authors used capillary electrophoresis–mass spectrometry (CE–MS) in combination with solid-phase extraction (SPE) to selectively remove up to 17 cations from soil solution. They were also able to differentiate between plant and microbial cations. Optimising the method with a model soil solution of 78 organic and 12 inorganic compounds, the method was then used on soil solutions from rice farms. Significant differences between soil with and without rice were obtained, with microbial metabolites such as histamine and tyrosine present only in the absence of plants, and leucine, isoleucine, phenylalanine and serine significantly more concentrated without plants.

Climate change will probably result in increased periods of drought, and irrigation is one method to ensure crop survivability. This results in a dry–wet event that can lead to various stresses to both plant and microbial life (Kakumanu et al. 2013). In particular, a change in osmotic potential between the soil and intracellular contents of microorganism can result in reduced life expectancy of the microbial biomass. Besides K^+, which acts as a regulator of ionic strength in cells, amino acids, carbohydrates, quaternary amines and tetrahydropyrimidine are regulated and maintained within microbial cells when the amount of water available is reduced. Fungi have been found to preferentially accumulate polyols while bacteria have a preference for amino acids and sugars. Other factors that may improve drought resistance are a robust AMF that will increase N uptake to plants (Baum et al. 2015),

At the end of drought when rains have come, the sudden influx of fresh water generally results in a flush of microbial activity (Kakumanu et al. 2013). It has still not been determined whether this is due to the lysis of microbes, a change in equilibrium of microbes as the concentration gradient changes, or other reasons. The authors reasoned that they could quantify metabolites that are released when the concentration gradient changes. It was found that the accumulation of

metabolites appeared more related to keeping energy-rich compounds at hand than to regulate osmotic pressure. Metabolite composition of sugars and polyols in drought and non-drought prone areas revealed that microbial lysis was unlikely to have occurred when soils were dry. The authors speculated that metabolite accumulation during droughts had more to do with energy and nutrient conservation, and production of survival metabolites, such as exopolysaccharides.

12 Tools for Microbial Soil Metabolomics

When looking for microbial metabolites, it is often necessary to utilise as many methods as possible to identify the source of metabolites. Attempts at a broad understanding of microbes in soil have also been elucidated through model communities (Ahmed et al. 2014; Ziegler et al. 2013) or plants (Ahmed et al. 2014). However, extrapolating laboratory results to the field can be risky and increasing the types of data collected can be useful. Correlations with other parameters such as genomic data or physicochemical parameters can be helpful in understanding why metabolites are present (Larsen et al. 2016; Rochfort et al. 2015). Recent combined analyses have shown that a single analytical technique can give an incomplete picture of microbial fluxes (Larsen et al. 2016).

One common enabling technology compatible with metabolomics methods is stable isotope labelling, which has been shown to identify a variety of soil functions of soil microbes, from rhizosphere signalling molecules to nutrient-associated metabolites [(Gunina et al. 2014; Haichar et al. 2012; Watzinger 2015), Tables 3 and 4]. Probably, the most common method to identify metabolites of microbial origin is to feed labelled substrates which produce digested products that are easy to identify (Heuck et al. 2015). Sugars are an important energy source for microbes, and this has led to metabolomic studies to determine how sugars are taken up by soil microbes [(Apostel et al. 2015), Table 3]. Using ^{13}C-labelled glucose and ribose with PLFA to identify microbe type, a loamy soil in Germany was dosed to determine sugar uptake by the soil. Initial decomposition of the sugars occurred within 3 days. Glucose concentration then decreased by 50 % between 3 and 10 days, while ribose remained relatively constant. The position of the carbon on the sugars was important, with the majority of carbon being incorporated from glucose C-2 (approximately 90 %) and ribose C-5 (approximately 70 %). PLFA identified the majority of detected microbes taking up sugars were Gram-negative bacteria while Gram-positive bacteria and actinomycetes incorporated the greatest concentration of labelled sugars into their PLFA (0.2–0.4 %). Other microbes found to take up labelled sugars included protozoa, VA-mycorrhiza, anaerobes and fungi. Gram-negative bacteria were also found to take up the greatest concentrations of labelled carbon, while Gram-positive bacteria appeared to prefer sugars from older SOM. A similar study sought to measure the use of plant versus soil organic carbon by microbes in maize, wheat and rye farms, also in Germany (Kramer and Gleixner 2006). Following the rate and mechanism of these metabolites allowed the

researchers to determine that glycolysis and the pentose phosphate pathway were parallel processes and characteristic to the soils studied. Initially, both glucose and ribose were consumed at similar rates. After 10 days, concentration decreases in the C-5 labelled ribose showing the preference by microbes to incorporate this metabolite over glucose, presumably due to the formation of RNA and DNA. Data for other labelled sugars showed a complex series of reactions occurring in the soil that include glycolysis, pentose phosphate and gluconeogenesis metabolic pathways. PLFA data revealed a preference for the ^{13}C substrates to be taken up by Gram-negative bacteria, known to be the dominant species around the rhizosphere, while methanotrophs were found to consume non-plant material, despite its proximity.

The flux of a series of amino acids was determined using labelled compounds (Gunina et al. 2014). Compared to fungi, sugars were more efficiently taken up by bacteria, especially glucose and sucrose, if the concentrations of these metabolites were low in the soil (Gunina et al. 2014). Ribose uptake was similar to glucose for Gram-negative bacteria. The pentose phosphate pathway for these bacteria was also characterised by the uptake of xylose. In contrast, fungi appeared to have a preference for larger, more complex sugars along with decomposition of glucose to form triacylglycerols. Acetate, a common metabolite in soils due to it being sourced in plant litter and cattle slurry, was found to be incorporated preferentially into Gram-negative bacteria.

Using these labelled metabolites helped the authors to identify that Gram-negative bacteria were the most efficient at using low molecular weight metabolites, due to the preference of this type of bacteria to use the anabolic pentose phosphate pathway. Fungi and filamentous microorganisms were found to be better utilisers of acidic and complex organic compounds like palmitate and double-bonded FA. Carbon-13 labelling allowed for comparison between microbial and soil metabolites in a series of experiments investigating SOM accessibility of nutrients (Swenson et al. 2015b). Comparing samples of soil that had been fumigated versus untouched soil samples, it was possible to identify the labelled metabolites of the microbe *Pseudomonas stutzeri* RCH2. Extracellular metabolites were found to be more likely to be detected than intracellular ones, indicating that additional steps such as adding salts to reduce osmosis may not be required. As minimal processing was a goal of this research, water was used as an extractant, a solution the authors point out is not ideal for the study of metabolites such as FA or sterols. Despite this, the labelled metabolites identified included amino acids and analogues, nucleobases such as uracil, and a series of organic acids.

13 Conclusion

Soil is a rich, complex ground for metabolomics research. The majority of microbes within soil are still a mystery, but metabolomics is beginning to reveal their secrets. Even with the advances in understanding how soil microbes interact with plants

(Mendes et al. 2011), significant challenges remain in characterising the spatial separation and metabolic compartmentalization of differing metabolic pathways and metabolites to their respective source—plant, microbe or systemic metabolic response. Metabolomics techniques will help to holistically understanding how humans have changed the soil environment; hopefully we will learn how to sustain not just the soil but the environment that microbes interact with, whether it be in the ground (Holland 2004), waterways (Lozupone and Knight 2007) or sky (Conrad 1996). As advances in research occur, many of the mysterious interactions between biology, geology and chemistry will become apparent.

The knowledge acquired from the interactions between soil microbes will then allow for specific manipulation of soil from improving crop yield to soil remediation (Mosa et al. 2016). Soil microbes are potential vectors that could be manipulated and eventually synthesised to help with soil management. As humanity comes to master concepts like rhizoengineering, metabolomic techniques may be needed to monitor the efficiency of designed microbial consortiums, as they are used to inoculate soil for improved soil health and productivity (Jia et al. 2016; Zhang et al. 2015). While by no means the only technique to understand microbial life (Desai et al. 2010), metabolomics has the advantage of being able to cast a wide net over the biological, chemical and geological interactions occurring in soil.

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