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Review paper

Soil enzymes in a changing environment: Current knowledge and future directions

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ABSTRACT

This review focuses on some important and challenging aspects of soil extracellular enzyme research. We report on recent discoveries, identify key research needs and highlight the many opportunities offered by interactions with other microbial enzymologists. The biggest challenges are to understand how the chemical, physical and biological properties of soil affect enzyme production, diffusion, substrate turnover and the proportion of the product that is made available to the producer cells. Thus, the factors that regulate the synthesis and secretion of extracellular enzymes and their distribution after they are externalized are important topics, not only for soil enzymologists, but also in the broader context of microbial ecology. In addition, there are many uncertainties about the ways in which microbes and their extracellular enzymes overcome the generally destructive, inhibitory and competitive properties of the soil matrix, and the various strategies they adopt for effective substrate detection and utilization. The complexity of extracellular enzyme activities in depolymerising macromolecular organics is exemplified by lignocellulose degradation and how the many enzymes involved respond to structural diversity and changing nutrient availabilities. The impacts of climate change on microbes and their extracellular enzymes, although of profound importance, are not well understood but we suggest how they may be predicted, assessed and managed. We describe recent advances that allow for the manipulation of extracellular enzyme activities to facilitate bioremediation, carbon sequestration and plant growth promotion. We also contribute to the ongoing debate as to how to assay enzyme activities in soil and what the measurements tell us, in the context of both traditional methods and the newer techniques that are being developed and adopted. Finally, we offer our collective vision of the future of extracellular enzyme research: one that will depend on imaginative thinking as well as technological advances, and be built upon synergies between diverse disciplines.

1. Introduction

The debris of plants and microbes and their constituent carbon rich bio-macromolecules, such as cellulose, hemicellulose, pectin, chitin, lignin and tannin, necessitate recycling by aggressive consortia of microorganisms (Hedges and Oades, 1997; Gessner et al., 2010). The proximate agents of organic matter decomposition are the extracellular enzymes (EE) and their production can be viewed as a form of foraging strategy that has evolved whereby nutrient and energy supplies are aligned with demand.

The polymeric carbon and nitrogen components of plants (and microbes and animals) are both structurally complex and highly diverse, and their breakdown requires the combined activities of many different microorganisms. Depolymerisation followed by mineralization of the labile products by bacteria, archaea and fungi creates the trophic base for detritus food webs, drives global carbon

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and nutrient cycles and mediates plant production and atmospheric composition. In the process, the maintenance and essential functions of the overall microbial community are sustained. The activities and significance of enzymes, once they have been secreted or otherwise released into the soil, have been researched and debated for decades (Skujins and McLaren, 1968; Burns, 1978a, 1982; Burns and Dick, 2002; Nannipieri et al., 2002; Tabatabai and Dick, 2002; Caldwell, 2005). Advances in molecular, microscopic and analytical techniques (Bouws et al., 2008; Wallenstein and Weintraub, 2008) and some original thinking (e.g. Burns, 1983; Burns and Stach, 2002; Allison, 2005; Theuerl and Buscot, 2010) together with a growing need to understand how enzyme activities contribute to a large number of industrial, medical and environmental processes, have generated new insights into the ecology of EEs. For example, those investigating composting (Crecchio et al., 2004; Raut et al., 2008), sludge treatment (Whiteley and Lee, 2006), paper production (Ravalason et al., 2008; Witayakran and Ragauskas, 2009) and the conversion of plant materials to fermentable sugars for biofuel generation (Wackett, 2008; Wilson, 2008, 2009) all need to understand the functions and efficiency of the many enzymes responsible for lignocellulolysis. Combating the invasive activities of phytopathogens (Kikot et al., 2009) and understanding the complex enzymology of ruminant digestion (Morrison et al., 2009) also depend on a detailed knowledge of organic polymer breakdown and mineralization. Additionally, a number of potential organic pollutants, such as the polycyclic aromatic hydrocarbons and polychlorinated biphenyls, are chemically complex and poorly water soluble and require extracellular catalysis prior to microbial uptake and catabolism (Tiehm et al., 1997; Safinowski et al., 2006; Martin et al., 2009). Inevitably, the rational and successful bioremediation of contaminated soils and sediments will depend on a thorough understanding of the relevant microbial and enzymatic processes (Wackett, 2004; Asgher et al., 2008).

In the last decade, a growing concern about the potential consequences of climate change on soil processes, coupled with a desire to develop methods of improving carbon sequestration, have stimulated experimental research, modelling and theorizing (Davidson et al., 2000; Kirschbaum, 2004; Eliasson et al., 2005; Fang et al., 2005; Jones et al., 2005; Knorr et al., 2005b; Bradford et al., 2008; Zak et al., 2011). There has been particular interest in the role of EEs in soil organic matter (SOM) formation and decomposition (Theuerl and Buscot, 2010). Terrestrial soils contain reserves of organic carbon estimated at between 1500 and 1700 Pg (Houghton, 2007; Canadell et al., 2010) and lake sediments store approximately 420–820 Pg (Cole et al., 2007). Soil organic matter is an often recalcitrant complex that is both synthesized and degraded by microbial enzyme activities. The balance between these two competing processes determines how much carbon is sequestered as well as contributing to: soil aggregate structure and stability (Six et al., 2006); plant nutrient availability; water retention and soil management (Powlson et al., 2011a); microbial diversity and activity; and a host of enzymic properties that determine soil fertility and plant productivity (Bardgett, 2005; Shaw and Burns, 2006; Trasar-Cepeda et al., 2008; Powlson et al., 2011b).

Increased soil temperatures, elevated atmospheric carbon dioxide (Finzi et al., 2006) and more frequent wetting and drying cycles (Fierer et al., 2003), will change microbial community composition and possibly increase biomass and enzyme activities (Henry et al., 2005; Allison and Treseder, 2008) – either directly or following stimulation of plant growth and increases in litter deposition and root exudation. The phenomenon of soil 'priming', whereby microbes utilize labile substrates to produce EEs which may then attack the SOM pool, has again become topical because of the projected impact of elevated CO_2 on root exudation (Allard et al., 2006; Phillips et al., 2011), the size and activities of the microbial biomass (Kuzyakov, 2010) and SOM stability (Melillo et al., 2002; Jones et al., 2003). Those attempting to quantify or even manipulate the outcomes of global warming (Luo, 2007; Gillabel et al., 2010; Kleber, 2010; Macias and Arbestain, 2010) must now take into account the microbial responses, including soil enzyme activities, that may affect recalcitrant humic matter (Davidson and Janssens, 2006). A related focus is the impact of manipulated carbon sequestration, specifically in the form of biochar additions, on soil microbial and enzymatic properties (Bailey et al., 2011; Cross and Sohi, 2011; Lehmann et al., 2011).

The topicality and diverse nature of EE research is revealed by a cursory search of the literature. In 2012 there were in excess of 2500 research papers that linked extracellular enzymes and soil and, if water and sediment are added to the search criteria, the number exceeds 3000. There have now been four international conferences on 'Enzymes in the Environment', increasingly bringing together what were traditionally separate groups of researchers. The most recent meeting was in July 2011 (http://precedings.nature.com/collections/enzymes-in-the-environment) and the subject is currently the focus of an international Research Coordination Network funded by the US National Science Foundation (http://enzymes.nrel.colostate.edu).

This review summarises recent ideas concerning soil extracellular enzyme location, regulation and control, considers the potential effects of climate change and soil management, and presents a mechanistic framework to explain environmentally-driven changes in enzyme activities.

2. Extracellular enzyme function and regulation

Natural selection will tend to promote enzyme production strategies that minimise carbon and nutrient costs to the cell and maximize associated benefits (Allison et al., 2011). Thus, EE activities reflect cellular economics: expending resources to produce enzymes versus the benefit of increased availability of assimilable mineral nutrients, energy sources and low molecular mass organics. The costs to the cell are the C and nutrients required for energy (i.e. ATP), enzyme (protein) synthesis and secretion, formation and organization of membrane transporters for the uptake of products, and signal production and detection of potential substrates and nearby interacting microorganisms. A chemostat study of *Bacillus licheniformis* found that extracellular enzyme production requires 1–5% of assimilated C and N (Frankena et al., 1988). A more recent investigation of the ATP costs of protein synthesis in *Escherichia coli* (Smith and Chapman, 2010) reported that the average production cost per unit mass of the proteins that are secreted (including enzymes) is significantly lower than the costs of those retained within the cell. Because the resources contained within extracellular proteins are less likely to be recycled than are their intracellular counterparts, there must be selection towards minimizing the costs of EE production.

While cells face pressures to reduce the energetic demands of EE generation, they must maintain a high enough concentration of the reaction products to support cellular function and maintain viability. Schimel and Weintraub (2003) conducted a decomposition modelling study that incorporated enzymes as the agents of organic matter breakdown and concluded that microbes must allocate a minimum of 2% of assimilated C to enzyme production to sustain biomass. In addition, EE production was defined as the first priority in C allocation. The alternative was that the biomass increased until all available C was consumed to satisfy maintenance and growth requirements. At this time, EE production was reduced to near zero, exogenous substrate decomposition began to decrease

and microbial biomass declined. This sequence of events suggests that EEs are the pre-requisite for the success of microorganisms that rely on the degradation of polymeric substrates, and that they must prioritise C (and nutrient) allocation to enzyme production to prevent starvation. In reality, the 'break-even' point, where C allocation to enzyme production is enough to provide the C necessary for maintenance, is likely to vary in response to such factors as diffusional conditions, EE survival and sorption, the nature and availability of substrate, as well as pH and temperature. At present we lack the necessary information on how the different factors interact but, as methods for extracting proteins (Giagnoni et al., 2011) and mRNA (Mettel et al., 2010) improve, we will increasingly be able to estimate resource costs.

Given the need for microorganisms to maximize the cost:benefit ratio of enzyme production, there is likely to be little selective advantage in the production of EEs that generate resources that are not in short supply. With physiological stress (Tiemann and Billings, 2011) or in the pronged absence of substrate, the energetic deficit resulting from constitutive enzyme synthesis and secretion would prove fatal. Under these circumstances, it is likely that the cell redirects its efforts to processes that maintain cell integrity and viability. In fact constitutive secretion of very low levels of EEs could maintain the capacity of the microorganism to detect and respond to changes in substrate availability but only in the short term (Allison et al., 2011). As with any successful regulatory process there should be a strong correspondence between resource supply and enzyme activities and, indeed, several studies (Olander and Vitousek, 2000; Treseder and Vitousek, 2001) have observed inverse relationships between inorganic P availability and phosphatase activity – although this depends on initial bioavailable P (DeForest et al., 2012). If an increase in enzyme activity in response to resource demand results in the release of excess reaction products, there may be a positive relationship between enzyme activity and resource availability. For example, Weintraub and Schimel (2005) observed the accumulation of extractable phosphate in late summer in Arctic soils and concluded that it was the result of phosphatase activity in excess of phosphate demand. This result runs counter to the economic model of extracellular enzyme production, which predicts that inducible enzyme production should stop once resource availability increases to meet demand. One possibility is that under conditions where enzyme turnover, sorption and/or deactivation rates are low, enzymes produced to fulfil demand for a particular resource may persist and continue to function after that demand has been met (Allison et al., 2007a,b).

Induction of enzyme synthesis and secretion will usually be governed by local substrate concentrations – although the substrate itself may not be the actual inducer. In addition, an appropriate density and composition of microbial degraders is a prerequisite for successful catalysis and subsequent microbial proliferation. We are increasingly aware that many (and perhaps all) microorganisms produce and respond to signals which serve to inform them of their immediate environment (von Bodman et al., 2008). For example, bacteria release and sense molecules which monitor cell densities (Fuqua et al., 1994; Redfield, 2002). This phenomenon is termed quorum sensing (Shank and Kolter, 2009) which may also serve to detect the rate at which secreted molecules diffuse away from the cell (Ekschmitt et al., 2005). It has been suggested that EE producers may use the detection of a number of autoinducer molecules to sense substrate location, quality and quantity and then to regulate EE production accordingly (Allison et al., 2007a,b). When the autoinducer concentration exceeds a certain threshold this indicates that both the substrate and the microbial population density and composition are appropriate and that gene expression for enzyme production can be induced. On the other hand, if diffusional losses are too high or the microbial community composition is inappropriate, autoinducer concentrations will remain below a threshold and inhibit gene expression. Thus, the release and detection of quorum sensing compounds may ensure that enzyme secretion is only induced or upregulated in situations where it will provide a positive return on investment. For example, it has been demonstrated that the production of extracellular hydrolases in plant pathogenic bacteria of the genus *Erwinia* is regulated by autoinducer concentration (Barnard and Salmond, 2007). Gene products in this situation are pectin methyl esterase, pectic lyase and polygalacturonase all of which help to depolymerise the protective coat of the target seed or fruit and facilitate microbial penetration and subsequent pathogenesis. Furthermore, quorum sensing (and quenching) in aquatic environments and in the rhizosphere is believed to be an important controlling process for all sorts of microbial interactions. These include the hydrolysis of organic matter (Hmelo et al., 2011) and phosphatase activities (Van Mooy et al., 2012), as well as bacterial predation by protozoa and invasions of host plants by nitrogen fixing and pathogenic bacteria (Bonkowski, 2004). Quorum sensing is a subject of intensive research and speculation (Badri et al., 2009; Mathesius, 2009; Pang et al., 2009; Babalola, 2010) but the details of its contribution to the enzymatic turnover of substrates (and the generation of microbial and plant nutrients) in soil is yet to be determined.

3. Extracellular enzyme location and microbial ecology

The locations of microbial EEs in soil (Fig. 1) and their immense range of individual properties and activities offer numerous opportunities to the microbial community to improve the efficiency of catalysis of insoluble exogenous substrates (Wallenstein and Burns, 2011).

3.1. Cell bound extracellular enzymes

Extracellular enzymes that are exported through the cytoplasmic membrane, but nonetheless remain attached to the cell, arguably have some advantages as diffusional losses will be limited and competition from other organisms for the products reduced. Furthermore, these periplasmic, mural and capsular enzymes may be configured such that their active sites are exposed and the components that are susceptible to attack by microbes and their proteases are shielded. Although this location gives EEs some protection, their scavenging abilities, in the sense of being able to operate upon substrate distal to the cell, are inevitably reduced in comparison to freely-diffusible enzymes. This suggests that, in addition to substrate diffusion and convection, cells that retain their EEs must rely on phenomena such as Brownian motion and random movement in order to make contact with their substrates and, as described above, use signals and chemical gradients to initiate and then control directed movement towards potential carbon and energy sources. The latter may be achieved *via* the process of chemotaxis which enables microorganisms to detect gradients and then move to areas of higher substrate concentration. This response would also serve to increase reaction product capture (Parales and Harwood, 2002). Not surprisingly, chemotaxis enhances the degradation of organic substrates in heterogeneous environments (Marx and Aitken, 2000) and these and other movement patterns in response to gradients in subsurface soils have been modelled recently (Centler et al., 2011).

Some Gram-negative bacteria retain EEs within the periplasm. For example, studies using *Vibrio* have found that periplasmic enzymes survive as their metabolic activities, including protein synthesis, are shutting down in response to starvation, but that secretion of the protected enzymes can be induced within minutes

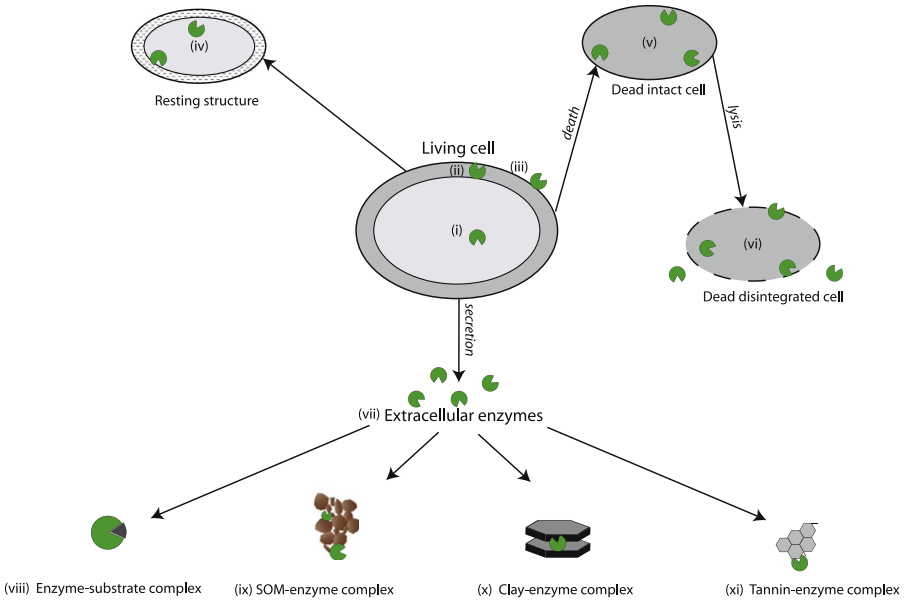

Fig. 1. Locations of enzymes in soil (adapted from Klose (2003) and Wallenstein and Burns (2011)). (i) functioning within the cytoplasm of microbial cells; (ii) restricted to the periplasmatic space of Gram-negative bacteria; (iii) located on the outer surface of cells with their active sites extending into the soil environment, contained within polysomes or retained by biofilms; (iv) situated within resting cells including fungal spores, protozoal cysts and bacterial endospores; (v) attached to entire dead cells and cell debris; (vi) leaking from intact cells or released from lysed cells; (vii) free in soil water; (viii) associated with enzyme–substrate complexes; (ix) complexed with soil organic matter by absorption, entrapment or co-polymerization; (x) sorbed to the external and internal surfaces of clay minerals; (xi) bound to condensed tannins.

(Davis, 1992; Kjelleberg et al., 1993; McDougald and Kjelleberg, 2006). It was further concluded that *de novo* RNA synthesis for enzyme production was not required and that, in addition to the existing periplasmic enzymes, rRNA and ribosomes may be present in the starved cell, allowing for the rapid resumption of enzyme production (Davis, 1992; McDougald and Kjelleberg, 2006). In fact, many Gram-negative bacteria retain their EEs in the periplasm even when not under stress. In this location the enzymes either further degrade the imported substrate *en route* to the cytoplasm or are secreted when appropriate signals are received (Kjelleberg et al., 1993). In other words, in these circumstances, enzyme synthesis and secretion are separate processes.

Extracellular enzymes that remain intimately associated with or in close proximity to their producer cells include those contained within a polysaccharidic biofilm or capsule and those that are organized within structures attached to but protruding from the cell wall. The former may develop on the surface of organic debris following microbial attachment and enclose many different but interacting genotypes (Flemming and Wingender, 2002; Romani et al., 2008). The adhesive characteristics of biofilms allow enzyme producers to attach directly to insoluble substrates. Extracellular solubilisation of the substrate occurs at the interface and the reaction products penetrate the biofilm (Costerton, 1992) reducing the diffusional and convective losses that would occur in the absence of the biofilm. Stream biofilms have been observed to undergo a successional pattern in community composition, with increasing extracellular enzyme activities and a greater capability to degrade complex polymeric substrates over time (Pohlon et al., 2010; Van Horn et al., 2011). It is likely that such interactive biofilm communities develop on decomposing plant residues (Ekschmitt et al., 2005) and on plant roots (Morris and Monier, 2003).

The second group of cell-bound enzymes are those contained within a structure known as a cellulosome or polysome (Fontes and Gilbert, 2010). These are predominantly hydrolases and are arranged on a protein scaffold that provides stability and facilitates the efficient cleavage of polysaccharides. Cellulosomes were first described for the anaerobic thermophile *Clostridium thermocellum* and have been much researched since (Bayer et al., 2004; Gold and Martin, 2007; Bayer et al., 2008; Peer et al., 2009; Fontes and Gilbert, 2010). *C. thermocellum*, produces a large number of EEs, including endoglucanases, exoglucanses, β-glucosidases, xylanases, lichenases, laminarinases, xylosidases, galactosidases, mannosidases, pectin lyases, pectin methylesterases, polygalacturonate hydrolases, cellobiose phosphorylases and cellodextrin phophorylases (Lamed et al., 1983; Demain et al., 2005) and many of them are housed within cellulosome-like structures. The significance of polysomes in macromolecule degradation in soil awaits evaluation but, there are suggestions that anaerobic bacteria and fungi invoke this strategy when degrading lignocelluloses (Ljungdahl, 2008; Wilson, 2008). Interestingly, there is another group of EEs that act without passing into the external environment. These have been reported in some pathogenic bacteria that transfer their enzymes directly into the target cell using what is termed a Type III secretory system or injectisome (Hauser, 2009; Pastor et al., 2005). It is not known if such a conduit functions in the degradation of organic matter in soil but there are some hints in a recent paper by Cusano et al. (2011).

3.2. Diffusible extracellular enzymes

Most extracellular enzymes diffuse away from their parent cell. Some of these are more resilient than their intracellular counterparts because they are glycosylated and have disulfide bonds: structural modifications that confer thermostability, a broad pH range for activity, resistance to proteases and even modulate cell adhesion (Zhang and Ten Hagen, 2011). Furthermore, chemical defences, such as antibiotics and protease inhibitors, may be co-secreted (Kang et al., 2006) which suggests that one of their functions is to protect EEs from competitors (Schwarz and Zverlov, 2006) although Kantyka et al. (2010) warn that this conclusion is speculative. Some microbes rely on the activities of predators to control their rivals and, in the rumen stomach, protozoa may have this function (Modak et al., 2007). Of course, proteases are also subject to all the pressures that other EEs encounter.

With regard to the area from which the soluble enzyme can scavenge substrates, it has been suggested that modifying the size and structure of enzymes prior to secretion *via* cellular regulation (acclimatization) and/or selection pressures (adaptation) may influence diffusivity (Allison et al., 2011). They hypothesized that optimal enzyme diffusivity should increase as substrate availability decreases, allowing the enzyme producer to access more distant substrates. On the other hand, competition for products should exert selection towards relatively low enzyme diffusivity. This is because as distance between a cell and its extracellular enzymes increases, the amount of reaction product captured by the cell per unit of enzyme produced declines as a function of the diffusional environment, reaction product losses, enzyme and substrate concentrations, and competition for reaction products.

3.3. Immobilized extracellular enzymes

Many EEs become stabilized through association with clay minerals, humic acids and particulate organic matter and retain significant levels of activity for prolonged periods of time (Burns et al., 1972; Boyd and Mortland, 1986; Burns, 1986; Dick and Tabatabai, 1987). Bound enzymes will often have reduced activity in comparison to their free counterparts, as complexation can restrict substrate accessibility, occlude active sites and cause conformational changes (Allison and Jastrow, 2006; Nannipieri, 2006; Quiquampoix and Burns, 2007). Nonetheless, these soil colloid-immobilized enzymes represent a significant reservoir of potential activity and may even function as the first catalytic response to changes in substrate availability in soils as well as serving as the generator of signal molecules for the microbial community. Soil-bound enzymes may also be a source of substrate turnover during periods when microbial biomass is low or shut down due to stressed conditions (Stursova and Sinsabaugh, 2008). In fact, much of the total activity of some EEs may be associated with organic and inorganic colloids rather than being free in solution (Kandeler, 1990). It should be noted that some of these bound enzymes (e.g. urease, phosphatases) are not extracellular *sensu stricta* but rather they have become externalized as a result of the death and lysis of their parent cell. The turnover rates of immobilized enzymes in soils and the contribution of the various locations to the overall catalysis of specific substrates under a range of conditions, have not yet been assessed.

3.4. Product competition

Once EEs are in contact with their substrates, they have a variety of ways to optimize their activities (Hilden and Johansson, 2004). For example, fungal and bacterial cellulose binding moieties (CBMs) anchor enzymes to substrates in orientations that allow the catalytic domain (CD) to cleave the β-1,4-linkages (Boraston et al., 2003; Wilson, 2008). In addition, CBMs may detach from the substrate allowing the enzyme to slide across the fibrillar cellulose surface. This relocates the CD and facilitates the processive hydrolysis of the substrate (Jervis et al., 1997). However, even when appropriate conditions for catalysis exist and soluble diffusible products are generated, other microbes may intercept the molecules before the enzyme producing cell can benefit. Those microbes that keep their EEs close may suffer less from this but, for those depending on the activities of secreted and remote functioning enzymes, the efficiency of the process is likely to be low. This is because there will be diffusion and dilution and opportunistic microbes, which may not have invested any resources in extracellular enzyme generation, will capture the products (Allison, 2005). Notwithstanding, the apparent inefficiency of EE product generation may be part of a complex microbial community synergy: the intercepting microbes (the so-called 'cheaters') provide some direct or indirect benefit to the enzyme producers. Syntrophic relationships of this type are well described in the literature (e.g. Sousa et al., 2009; Wintermute and Silver, 2010). It may be that the benefits of extracellular depolymerisation to the overall microbial community outweigh the disadvantages to the principal enzyme generators.

4. Extracellular enzymes in soil and water

Striking similarities and sharp contrasts exist between the microbiology, enzymology and geochemistry of surface soils and aquatic sediments. Both ecosystems are macrohabitats characterized by varying proportions of solid surfaces and water. Globally, a continuum is manifest which ranges from arid deserts containing very little or no pore water, through periodically-saturated wetlands to offshore sea water with only traces of particulate matter and few solid surfaces (Stolp, 1988; Gutierrez and Jones, 2006). The fundamental difference between soil and water habitats, from the perspective of microbial activity and EE functions, is the availability of water and the continuity of the water film.

In global terms bacteria, fungi and archaea are the dominant organisms as measured by biomass and diversity (Øvreås, 2000). According to direct counts, soil typically contains between 10^8 and 10^{10} bacteria per gram (as do aquatic sediments) but analysis based on 16S rRNA give figures 2–5 orders of magnitude greater (Sandaa et al., 1999; Sogin et al., 2006). Estimates of bacterial:fungal ratios, which are important with regard to the range of EEs produced and the area over which substrates can be accessed, are difficult to arrive at (Joergensen and Wichern, 2008) but, in forest soils and litter layers, fungi may be dominant. In aquatic sediments, typically bacterial biomass dominates, although degrading leaf litter usually contains more fungal than bacterial biomass (Findlay et al., 2002; Marxsen, 2006).

Molecular techniques reveal that prokaryotic diversity has been underestimated and it is apparent that there is a great deal of enzymic potential contained within the so-called viable but non-culturable (VBNC) majority (Amann et al., 1995; Handelsman et al., 1998; Hugenholtz et al., 1998; Streit and Schmitz, 2004). Current computed estimates of soil diversity range from 5000 to more than a million 'species' (sometimes called operational taxonomic units, OTUs) g^{-1} soil (Gans et al., 2005; Schloss and Handelsman, 2006) and metagenomics is now enabling assessment of the biocatalytic potential of the large number of non-culturable microorganisms (Lorenz and Eck, 2005; Steele and Streit, 2005). The genotypic and phenotypic diversity helps microorganisms to exploit the vast range of organic carbon and energy sources, the numerous microenvironments, and the ever-changing physical and chemical properties. Soils and sediments are heterogeneous in time and space and communities not only vary with depth but also on a centimetre, micrometre and nanometre scale: microsite variations can allow very different microorganisms to coexist side by side (Ettema and Wardle, 2002; Crawford et al., 2012) and allow for close cooperation between EEs.

In spite of their high numbers and great diversity, the surface area in soil and aquatic sediments occupied by living microorganisms is generally less than 5% of the total (Ingham et al., 1985) and as little as 0.001% of the potentially habitable soil volume. In fact most of the biological activity is concentrated in 'hot-spots' within aggregates, at root surfaces and on organic debris (Parkin, 1987; Nannipieri et al., 2003; Zoppini et al., 2005; Kim et al., 2008; Briar et al., 2011). However, this does not mean that the volume of soil that the microbes have an impact on is small. This is because diffusion and convection of substrates (Poll et al., 2006), signals,

metabolites and EEs, together with cell motility, will greatly expand the zones of influence.

Microbes will likely attach to sites that are favourable for replication. As growth and colony formation take place, exopolysaccharides and glycoproteins are formed, creating adhesives that help orientate adjacent clay particles, cementing them together to form microaggregates (Wright and Upadhyaya, 1998). A great variety of adhesive polysaccharides and glycoproteins are produced by bacteria and fungi which result in aggregate formation and stabilisation (Rillig and Mummey, 2006; Six et al., 2006). Bacteria also generate adhesins which enable biofilm construction and the resulting entrapment and retention of collaborative microorganisms and their EEs. Appropriate to their functions, these polymers tend to be resistant to hydrolysis (Robert and Chenu, 1992). Comparisons of communities and activities support the idea that different microbes and enzymes are involved at various stages in the development of micro- and macro-aggregates (Bailey et al., 2011) and, as a consequence, that there are variations in SOM composition and stability between the two size classes. In general, the more labile and more bioavailable SOM is concentrated in the macroaggregates and the more recalcitrant SOM in the microaggregates (Jastrow et al., 2007). All aggregate sizes provide stratified habitats for soil microorganisms and their enzyme activities (Davinic et al., 2012) but there may be large differences in the range and rates of processes performed (Miller and Dick, 1995; Dorodnikov et al., 2009).

Strong attachment to soil sediment and particulate organic matter surfaces confers several advantages for microorganisms living in competitive and generally oligotrophic environments (Gilbert et al., 1993; Lock et al., 1984; Blažina et al., 2011). These may include protecting bacteria from protozoan predation; providing higher levels of inorganic and organic nutrients than are present in the soil solution; facilitating communication with and genetic exchange between microorganisms; and allowing the accumulation and protection of appropriate combinations of EEs (Romani et al., 2008). Of course, as nutrient supplies at a surface site are depleted, microorganisms need mechanisms by which cells can disperse to new sites that may have additional resources (Gram et al., 2002). Thus, connected aqueous phases, which are intermittent in soil and the norm in aquatic environments, become critical to the success of microbes.

5. Lignin – a challenging substrate for extracellular enzymes

About half of terrestrial plant production is holocellulose, which makes cellulolysis the principle carbon acquisition pathway for decomposer communities. However, in the early stages of plant litter decomposition, rates of cellulolysis are controlled to a large extent by the availability of nitrogen whereas in the latter stages lignin is rate-limiting. The abundance and composition of lignin is a primary control because it restricts the access of enzymes to cell wall polysaccharides (Talbot et al., 2012). In addition, the concentration and composition of lignin and other hydrocarbons (e.g. waxes) in plant litter is thought to influence the quantity of carbon transferred from litter to soil organic matter and ultimately soil carbon sequestration (Theuerl and Buscot, 2010). Fungi have a major role in ligno-cellulose degradation and employ a mixture of oxidative and hydrolytic enzymes which varies among taxa. This enzyme distribution is the basis for the traditional functional classification of wood-degrading fungi as white rot (predominantly Basidiomycota), brown rot (Ascomycota and Basidiomycota) and soft rot (Ascomycota). These descriptors represent a crude gradient in the relative capacities of the fungi to degrade and mineralize lignin in their quest to obtain carbohydrates for growth (Higuchi, 1990; Rabinovich et al., 2004; Baldrian, 2006; Hoegger et al., 2006).

5.1. Ligninases

Lignin degradation is principally an oxidative process catalysed by enzymes broadly categorized as phenol oxidases, peroxidases and dehydrogenases. Phenol oxidases are enzymes that oxidize phenolic compounds using oxygen as an electron acceptor. The most studied of these are fungal laccases and their prokaryotic counterparts that have multiple copper (Cu) atoms at their reaction centre (Rabinovich et al., 2004; Baldrian, 2006; Hoegger et al., 2006; Bugg et al., 2011). The redox potential, pH optima and substrate preferences of laccases range widely because there is great variability in their polypeptide sequences and the extent of glycosylation (Baldrian, 2003; Bugg et al., 2011). Many fungi have multiple laccase genes encoding both intracellular and extracellular enzymes that serve a variety of purposes including morphogenesis and defence as well as the acquisition of C and N (Courty et al., 2009). In general, the redox potentials of laccases and other Cu containing oxidases (450–800 mv) are too low to directly oxidize the C–C and ether bonds that link the phenyl propanoid monomers of lignin. However, they can oxidize Mn^{2+} and a variety of organic molecules, including benzenediols and lipids, to create extracellular diffusible radicals (redox mediators) that can in turn depolymerise lignin (Bourbonnais et al., 1998; Leonowicz et al., 2001; Camarero et al., 2005; Strong and Claus, 2011).

Peroxidases have Fe-containing haeme prosthetic groups that use H_2O_2 as an electron acceptor. With redox potentials up to 1490 mv, they can oxidize lignin linkages either directly or through redox intermediates such as Mn^{3+} (Higuchi, 1990; Kersten et al., 1990; Rabinovich et al., 2004). The most studied peroxidases are the lignin peroxidases (EC 1.11.1.14) and the more widely distributed manganese peroxidases (EC 1.11.1.13) primarily of basidiomycetes, although these enzymes are also produced by a diverse range of bacteria (Bugg et al., 2011).

The third group of ligninases, the dehydrogenases, are primarily intracellular oxidative enzymes that transfer hydride groups from a substrate to an acceptor such as NAD+. They are generally considered substrate-specific but play a key role in the decomposition process, particularly for bacteria, which generally are the ultimate consumers and mineralisers of aromatic compounds (Masai et al., 2007; Bugg et al., 2011). However, at least a few bacteria, e.g. *Sphingomonas*, depolymerize lignin extracellularly using dehydrogenases (Masai et al., 2007). Other extracellular oxidative enzymes associated with the degradation of recalcitrant plant and microbial components include: saccharide-oxidizing enzymes such as glyoxal oxidase, galactose oxidase (EC 1.1.3.9) and glucose oxidase (EC 1.1.3.4) that reduce oxygen to H_2O_2 in support of peroxidase activity; and cellobiose dehydrogenase (EC 1.1.99.18) which reduces phenoxy radicals, quinones and metal cations, contributing to the supply of redox mediators (Zamocky et al., 2006).

Oxidase activities in soil are more dynamic than are hydrolytic activities (Sinsabaugh et al., 2008). This partly reflects their varied functions which include detoxification of phenolics and reactive metals, pigment production and antimicrobial defence (Burke and Cairney, 2002; Claus, 2003; Rabinovich et al., 2004; Baldrian, 2006) as well as the degradation of lignin and humics to obtain C, N and P (Theuerl and Buscot, 2010). High spatio-temporal variation in activity may also be a product of lower environmental stability as well as catalytic interactions with the Fe and Mn containing minerals (Huang, 1990; Rabinovich et al., 2004). In soil, phenol oxidase activity is typically measured as the rate of substrate oxidation in soil suspensions while peroxidase activity is assessed as the rate of substrate oxidation in the presence of added H_2O_2. The most commonly used substrates for phenol oxidase and peroxidase assays in soil are pyrogallol (1,2,3-trihydroxybenzene),

DOPA (L-3,4-dihydroxyphenylalanine) and ABTS (2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)). These substrates differ in redox potential (pyrogallol < DOPA < ABTS) and the extent to which their redox potential changes in relation to pH. This complicates comparisons across systems but a compilation of DOPA studies found that phenol oxidase activity tends to increase with soil pH (Sinsabaugh, 2010).

5.2. Lignocellulose decomposition

During the early stages of plant litter decomposition, accessible cellulose and other labile molecules are degraded preferentially (Berg, 2000; Moorhead and Sinsabaugh, 2006). As decomposition progresses, the depolymerisation of lignin becomes increasingly linked to microbial C acquisition (Allison, 2006; Herman et al., 2008; Berg et al., 2010; Rinkes et al., 2011) leading to shifts in microbial community composition and increases in oxidative enzyme activity (Sinsabaugh and Shah, 2011). Products of these oxidative enzyme activities (e.g. semiquinones, quinines, lipid radicals) condense with other nearby phenols, peptides and carbohydrates to form secondary humic products (Grandy and Neff, 2008) although this concept has been disputed (Schmidt et al., 2011).

Because oxidative enzyme activities mediate both the degradation and creation of the most recalcitrant components of detrital organic matter, they are closely linked to soil carbon sequestration (Theuerl and Buscot, 2010). Nitrogen addition increases decomposition rates for litter with low lignin and N contents and slows rates when there are high contents of lignin and other secondary compounds (Berg and Matzner, 1997; Fog, 1988; Knorr et al., 2005a). The latter responses have been linked to losses in phenol oxidase activities (Carreiro et al., 2000; Allison and Vitousek, 2004; Grandy and Neff, 2008). In soils, chronic N enrichment has been associated with a progressive loss of microbial biomass and reduced rates of respiration (DeForest et al., 2004; Frey et al., 2004; Zeglin et al., 2007; Treseder, 2008; Eisenlord and Zak, 2010). These responses are generally accompanied by net declines in phenol oxidase and peroxidase activities and increases in soil organic matter (Matocha et al., 2004; Sinsabaugh et al., 2005; Prescott, 2010).

Many of the recent advances in our understanding of the enzymology of lignin degradation have been driven by the goal of producing biofuels from lignocellulosic plant residues (Rabinovich et al., 2004; Masai et al., 2007; Bugg et al., 2011; Badieyan et al., 2012). These developments, which provide good examples of the expanding world of EE research, have generated new information concerning the diversity of laccases, peroxidases, dioxygenases and dehydrogenases (particularly among bacteria) and include thermostable and alkaline active enzymes (Ye et al., 2010).

As knowledge of the enzymology of lignin degradation expands, the opportunities for ecological studies of soil microbial communities increase. The number of investigations that use molecular methods to examine the distribution of potential lignin-degrading organisms in soils and their responses to disturbances associated with anthropogenic N deposition and climate change is growing rapidly. To date most of the research has focused on fungal laccase genes (Luis et al., 2005; Lauber et al., 2009; Kellner et al., 2010; Christ et al., 2011) because of limited information for primer design. Despite this restraint, some long standing questions such as the relative contribution of mycorrhizal and saprotrophic fungi to degradation of recalcitrant soil organic matter can finally be addressed (Talbot et al., 2008; Pritsch and Garbaye, 2011; Cheng et al., 2012).

6. Extracellular enzymes in a changing world

The climate is changing as the concentrations of CO_2 and other greenhouse gases in the atmosphere increase, resulting in global warming and altered precipitation patterns. Because the activities of enzymes in natural environments are controlled by both abiotic factors (e.g. temperature, water potential, pH) and biotic processes (e.g. enzyme synthesis and secretion) they are likely to be responsive to atmospheric warming and more frequent and extreme variations in precipitation patterns. These changes will have important consequences for ecosystem functions such as decomposition, nutrient cycling and plant–microbe interactions, which will ultimately affect productivity and net C balance.

6.1. Temperature and moisture changes

At the soil microenvironment scale, changes in enzyme activities may be small because substrate availability will continue to be a limiting factor as will the restricted diffusion of substrates and enzymes. These constraints are likely to be more the rule than the exception in many soils although there is, as always, a caveat: with dehydration the now discontinuous water films may function as hotspots of activity as substrates and enzymes become more concentrated.

Only a few studies have measured potential enzyme activities in field experiments that have included soil warming treatments. In a boreal forest, microbial biomass decreased as soil temperature was raised by 0.5 °C, but there was little effect on enzyme activities (Allison and Treseder, 2008). In an old-field, 1–4 °C warming did not have a consistent effect on EE activities (Bell and Henry, 2011) despite an increase in microbial biomass. In another old-field experiment, there were also no long-term effects of experimental warming on EE activities (Steinweg, 2011). Due to the counteracting effects of enzyme production and degradation, it is difficult to predict the effects of warming on enzyme pools. Furthermore, the temperature sensitivities of enzymes, and thus the dynamics of nitrogen and carbon mineralization, are not the same (Luxhoi et al., 2002; Koch et al., 2007) and may be influenced by climate change in different ways.

Changes in the abiotic environment, due to short-term weather or long-term climate drivers, will affect enzyme activities in complex ways that are difficult to predict. For example, activity increases with temperature (up to some optimum which usually exceeds temperatures recorded in their native environment) and so climate warming will at least theoretically increase the rate of enzymatically-catalysed reactions – assuming that enzyme pool sizes remain constant (Wallenstein and Weintraub, 2008). On the other hand, microbes may decrease enzyme synthesis and secretion in response to warming (Allison, 2005; Wallenstein et al., 2012). Furthermore, enzyme denaturation rates (and the degradative activity of extracellular proteases) could also increase with warming (Wallenstein et al., 2011) which would counteract any changes in enzyme pool sizes, suggesting that either microbes decreased enzyme production per unit biomass or enzyme turnover increased. Clearly, it is difficult to make generalizations with regard to the effects of warming on enzyme pools.

Frequent and intermittent drying of soils introduces both matric and osmotic stresses and this will impact community composition and enzyme activities as microbes focus on synthesizing osmolytes and on intracellular maintenance strategies (Schimel et al., 2007; Chowdhury et al., 2011). In re-wetted soils, increased organic matter availability was recorded probably due to organic matter release by physical disruption of soil aggregates and/or cell lysis caused by osmotic shock (Fierer and Schimel, 2002). The newly-released organic matter, which resulted in a short-term pulse of available C and N, gave rise to rapid microbial growth and enhanced soil carbon and nitrogen mineralization (Fierer and Schimel, 2003). As a proportion of this released organic matter could be polymeric, the pulse in CO_2 production in soil after re-wetting is probably

linked to the activity of EEs that transform this organic matter to labile C (Miller et al., 2005).

Soil texture and moisture determine both the release and impact of root exudates (Song et al., 2012) and the rates at which enzymes, substrates and reaction products diffuse. When soil moisture is low, *in-situ* EE activities are also low (at least at the scale of bulk soil) although some microsites may exhibit high activity as solute concentration increases within pore spaces. Prolonged droughts are likely to decrease enzyme production resulting in lower measured activities when moisture returns. However, a counter argument is that enzyme turnover is slower in dry soils, so pool sizes could potentially increase during drought if enzyme production proceeds even at low rates. Steinweg (2011) found that EE activities actually increased during periods of drought, suggesting that not only did production continue but also that enzyme turnover rates were slowed down.

In many regions, changing seasonal precipitation patterns will give rise to increased stress associated with hydration/dehydration events. Because rewetting sometimes results in a pulse of microbial biomass turnover (Fierer et al., 2003; Schimel et al., 2007) many intracellular enzymes could be released into the environment, some surviving long enough to create a temporary increase in EE activities. On the other hand, decreased biomass could lead to a decrease in EE production and a decline in the relative abundance of different types of enzymes (Weintraub et al., 2007). Prolonged precipitation can result in increased EE activities (Henry et al., 2005; Clark et al., 2009; Henry, 2012) although this may be at least partially a response to enhanced plant growth and rhizodeposition (Bell and Henry, 2011).

Increasing drought frequency and duration represents as much an emerging problem for aquatic ecosystems as it does for soils. Insufficient attention has been given to the responses of sediment microbial communities to drying–rewetting cycles, especially in ephemeral waters that undergo a more extended period of drying. Desiccation alters the chemistry and mineralogy of the sediment and kills up 75% of microbes (Qiu and McComb, 1995). In semiarid regions drought events have major impacts on benthic community functions, including delaying litter decomposition and C-uptake (Fonnesu et al., 2004; Amalfitano et al., 2008). Dry period length was negatively related to non-specific esterase activity, used as a measure of total enzyme activity in sediment (Larned et al., 2007). Some EEs may be stable when adsorbed in dry soil although, in certain circumstances, tolerant cyanobacteria and lichens are likely to make a contribution (Miralles et al., 2012). Similarly, in benthic cyanobacterial mats and river sediments, phosphatase was not affected by the progressive reduction of water availability (Sirová et al., 2006; Zoppini and Marxsen, 2011) although β-glucosidase, aminopeptidase and lipase activities decreased (Marxsen et al., 2010). Following rewetting, a rapid recovery of reduced microbial functions was observed but with differences in the response of the various EEs (Romani and Sabater, 1997; Marxsen et al., 2010).

Microbial degradation of organic matter deposited on sediment plays an important role in nutrient cycling in the aquatic environments (Zoppini et al., 2010). In particular, sediments of temporary waters, during the period of drought, represent a link between the aquatic ecosystem and soil. Following rewetting of dried river sediments, the activities of aminopeptidase and phosphatase increased rapidly in the water column (Fazi et al., 2008) and, after 28 h, both enzyme activities were similar to those found in low land river water (Wilczek et al., 2005) and other freshwater systems (Marxsen and Witzel, 1990). The lower hydrolysis rates of organic phosphorus with respect to proteins and the increasing ratio of aminopeptidase to phosphatase activity indicated a slower P-cycling with respect to N-cycling. The persistence of phosphatase in dry sediments (Sirová et al., 2006; Zoppini and Marxsen, 2011) could, therefore, influence the metabolism of the planktonic microbial community as P will be made available soon after re-inundation.

The large initial flush of mineral phosphorus and nitrogen observed in rewetted sediments is also a common feature of rewetted soils. Microbial communities contribute significantly to this nutrient pulse *via* different mechanisms. For example, the preservation of enzymes during the period of drought, even in conditions prohibitive for microbial metabolism, can significantly contribute to nutrient release, thereby creating an excess of labile organic matter and nutrients ready to be taken up by bacteria and fungi upon rewetting.

6.2. The rhizosphere priming effect and elevated carbon dioxide

Elevated atmospheric CO_2 levels do not affect microbial activities directly because concentrations are naturally much higher in soils than in the atmosphere. However, microbial communities are strongly affected by the many plant responses to elevated CO_2 which can include increased water use efficiency (Morgan et al., 2011), increased rhizodeposition of labile C (Phillips et al., 2011) and accelerated nutrient uptake to facilitate increased plant productivity (Larsen et al., 2002; Finzi et al., 2006). When plant rhizodeposition increases, microbial biomass often increases, which could elevate overall enzyme production and respiration, although free air carbon dioxide enrichment experiments showed no dramatic effects (Finzi et al., 2006). However, Weintraub et al. (2007) reported that, although microbial biomass supported by rhizodeposition increased the production of peptidases to hydrolyse enzymes and other proteins (and thereby acquire N), it did not increase the production of enzymes to degrade cellulose and other C-rich substrates. Similarly, Dorodnikov et al. (2009) found that, of the enzymes they measured, only chitinases increased in response to elevated CO_2, supporting the notion that rhizosphere microbes allocate C towards producing enzymes to acquire N. Furthermore, in a calcareous grassland, the activities of chitinases, peptidases and phosphatases increased in response to elevated CO_2, but C-degrading enzymes did not respond (Ebersberger et al., 2003). Finally, tree girdling (which eliminates rhizodeposition) of beech decreased protease and cellulase activities but increased oxidative enzymes that degrade resistant SOM (Kaiser et al., 2010). Overall, these findings suggest that cellulases are not driving increased mineralization of soil organic carbon in response to increased rhizodeposition. Yet, there is clear evidence that labile substrate additions can stimulate the decomposition of more resistant soil organic matter, even after the substrate is depleted, which must be the work of extracellular enzymes (Kuzyakov, 2010).

Phenol oxidase and peroxidase activities have also been linked to the impacts of climate change with potentials for both positive and negative feedbacks on soil carbon (Freeman et al., 2001, 2004; Sinsabaugh, 2010). Peatlands, which store large quantities of carbon, appear to be enzyme-limited systems where low oxygen availability and low pH restrict oxidative activities thereby allowing accumulation of phenols that inhibit hydrolytic activity and microbial substrate utilization. This, in turn, contributes to organic matter accumulation (Freeman et al., 2001, 2004). At the other extreme, arid ecosystems appear to be substrate-limited. In these situations, several factors interact to limit SOM accumulation: photodegradation of surface litter reduces soil inputs; alkaline pH lowers the redox potentials of phenols; and aridity stabilises oxidative enzymes (Henry et al., 2005; Collins et al., 2008; Acevedo et al., 2010).

Soil microbial responses to environmental change are complex, especially as the many different climatic factors interact. The net effect on EE activity depends on how both enzyme production and

turnover are affected by changes in temperature, moisture and the stoichiometry of available resources.

7. Measuring extracellular enzyme activity

The methodologies adopted for soil enzyme measurement are not universal and this often creates difficulties when making comparisons. Here we summarise the major issues, make some recommendations, and urge greater uniformity in the design and conduct of assays.

For the most part, the conflicts arise because of the two contrasting philosophies that inform the choice of the enzyme assay protocol (Burns, 1978a,b; Tabatabai, 1994). The first is the classical approach where conditions (e.g. pH, temperature) are optimized and constant, and substrate dilutions are used to determine kinetics. The second attempts to mimic the soil environment in order to approximate potential enzyme activity under natural conditions (Saiya-Cork et al., 2002). The two approaches may produce markedly different results. Ultimately, the methodology adopted must depend on the research question. If the focus is enzymological, where the properties of the enzyme itself are of concern, then the '*in vitro*' approach is appropriate. On the other hand, the so called '*in situ*' alternative is useful if the research questions are ecological in origin, for instance understanding microbial function in the context of the soil and its overall environmental or agricultural significance (Dick, 1994). In this latter case, EE activity measurements become an environmental variable. Obviously, these two approaches are not mutually exclusive and a hybrid can sometimes be useful (Marx et al., 2001; Wallenstein and Weintraub, 2008). However, when deciding on the most appropriate methodology it is essential that any customization is justified and the results that follow must be amenable not only to internal but also to external comparisons. Regardless of the approach, full details of the sampling and storage methods must also be described. Only with the consistent use of comparable if not identical methods can we improve our understanding of organic matter degradation at larger spatial and temporal scales and confidently conduct quality meta-analyses of a large amount of data from a variety of sources. Recent detailed protocols and discussions concerning the methodology of soil enzyme assays can be found in DeForest (2009), Dick (2011) and German et al. (2011).

7.1. Sampling and storage

One of the logistical constraints when measuring soil enzyme activities is that inevitably the soil is disturbed during collection, preparation and storage. These manipulations have the potential to change enzyme activity by influencing microbial activity, enzyme immobilization, availability of substrate, etc. As such, measuring enzymes shortly (e.g. within 24 h) after sampling is preferable (ISO10381-6, 2009; ISO/TS22939, 2010). Of course, this is not always possible and storage is necessary. Keeping freshly collected soils cool (5 °C) but not frozen during transport and storage appears to have the least impact on enzyme activity for temperate soils (Lee et al., 2007; DeForest, 2009) but not for tropical soils where long term storage at 22 °C had the least impact (Turner and Romero, 2010). For long term storage freezing is preferable to drying and −80 °C is best for organic soils (Wallenius et al., 2010). Another consequence of storage is that it can increase the quenching with fluorescence of the popular methylumbelliferyl substrates as a function of storage time (both after freezing and air during) and organic matter content (Wallenius et al., 2010). One conclusion from methodological studies is that there are no unequivocal guidelines because the influence of temperature or duration of storage is enzyme and soil specific (Bandick and Dick, 1999; DeForest, 2009a; Lee et al., 2007; Turner and Romero, 2010; Wallenius et al., 2010). However, storing soils after air-drying reduces the activity of many enzymes (Burns, 1978a,b; Bandick and Dick, 1999; Lee et al., 2007; Wallenius et al., 2010) and should be avoided. In the absence of studies of many soil types from different ecosystem and climates, it is not possible to state definitively if differences in storage compromise the comparability of results. Nonetheless, we recommend that samples should be analysed as soon as possible after collection and that full details of storage conditions must always be reported.

7.2. Assay parameters

Two of the most frequently used enzyme assays are: (i) colourimetric using *p*-nitrophenol (*p*NP)-linked substrates or L-3,4-dihydroxyphenylalanine (L-DOPA) (Sinsabaugh and Linkins, 1990; Tabatabai, 1994); and (ii) fluorometric using 4-methylumbelliferone (MUF) or 7-amino-4-methyl coumarin (AMC) linked-substrate (Darrah and Harris, 1986; Marx et al., 2001). Each assay has advantages and disadvantages but, over the last decade, with the adoption of microplates (typically 96 wells), the fluorometric methods have become more common. This is because they facilitate the rapid measurement of large numbers of samples and are considered more sensitive than are the colourimetric methods (Marx et al., 2001; Saiya-Cork et al., 2002; Drouillon and Merckx, 2005). Nonetheless, one possible disadvantage of using MUF is that an alkali (e.g. NaOH) is typically added at the end of the incubation period to increase the low levels of fluorescence typically recorded in acidic solutions (Marx et al., 2001; Saiya-Cork et al., 2002). This prevents repeated measurements over time and makes it more difficult to determine kinetics. Furthermore, once the alkali is added the fluorescence of free MUF (i.e. not linked to MUF-substrate) which is used as a reference standard, increases at a greater rate when compared to MUF-linked substrates (DeForest, 2009). Therefore, the calculated enzyme activity will decrease with increasing time between adding NaOH (German et al., 2011) and reading the plate until it becomes constant sometime after 10–20 min. Thus, the calculated enzyme activity will be approximately 30% greater when the plate is read at one minute when compared to six minutes. However, it is unclear if this difference is universal or dependent on buffer pH. Recently, German et al. (2011) tested MUF based enzyme assays in acid soils without the addition of an alkaline solution and had no difficulty in detecting accumulation of MUF released by enzyme activity - despite the alkaline fluorescence optimum of MUF. Thus, they recommend not adding alkali unless the pH is below 4.5 or MUF is difficult to detect.

7.3. Substrates, buffers and pH

Another issue with the *in situ* approach to enzyme assays is that some protocols include only one concentration of standard and substrate. While this does facilitate higher throughput, it adds the challenge of ensuring that the single substrate concentration used is appropriate. Methods utilizing a single substrate concentration add relatively high amounts with the intention of having non-limiting substrate and, thereby, measuring V_{max} as a proxy for enzyme pools. However, it is rare that the substrate concentration is tested in order to determine saturation (German et al., 2011). For example, the commonly assumed appropriate substrate saturation concentration for a MUF-linked substrate is 200 μmol L^{-1}, but this might be insufficient in soils with the very high enzyme concentrations usually associated with an abundance of available carbon or severe nutrient limitations (ISO/TS22939, 2010). Therefore, we recommend that substrate saturation curves be determined in all soils prior to assays.

The use of buffer is standard in enzyme assays as it is important to both poise and maintain pH during the incubation because the concentration of H^+ will influence enzymes, substrates, and cofactors by altering ionization and solubility (Tabatabai, 1994). The type of buffer depends on the general enzyme assay philosophy and the desired pH value. For the *in vitro* approach, the optimum pH is best achieved using universal buffer at several pH values. However, this must be evaluated in advance if the soil has not been assayed previously because the pH optimum for each enzyme may be soil specific (Margesin and Schinner, 1994; Turner, 2005). For the *in situ* approach, it is common to select a buffer with a pH similar to that of the bulk soil solution. Caution must be exercised when using strongly acidic or alkaline buffers because some fluorometric substrates become unstable (ISO/TS22939, 2010). Niemi and Vepsalainen (2005) reported that most substrates were stable between pH 4 and 8, but not those used for chitinase (4-MUF-*N*-acetyl-β-D-glucosamide) and phosphatase (4-MUF phosphate). In both cases the substrates spontaneously dissociates from the MUF above pH 6.5 and below pH 5.0. While controls help to account for this, the problem decreases resolution of the assay by reducing the signal to noise ratio: a cause of concern when enzyme activities are low and difficult to detect.

7.4. New directions in soil enzyme assays

Until recently, our understanding of the ecology of EEs has been limited by conventional methods. In soils and aquatic systems, we have only been able to measure enzyme activity by detecting degradation of the target substrates and the generation of the products. In recent years, new technologies, approaches and even entirely new scientific fields have expanded our ability to study enzymes in the environment. For example, genomic tools allow us to interrogate the genetic potential for the production of specific enzymes in complex microbial communities (Raes et al., 2007; Wilmes and Bond, 2008) and transcriptomic tools enable us to examine controls on the expression of enzyme-coding genes (Morozova et al., 2009) In addition, emerging proteomic methods allow us to directly detect the presence of enzymes in the environment (Wilmes and Bond, 2009) as well as providing information about the organisms that produced them (Harrington et al., 2007; Raes and Bork, 2008; Wallenstein and Weintraub, 2008). The relative frequency of enzyme-coding genes in an environment should indicate the potential for production of those enzymes. However, there are numerous challenges to detecting and quantifying enzyme-coding genes. First, many of these genes are not well conserved and many enzymes have alternate forms with the same function (Kellner et al., 2007). This variety can only be addressed by using multiple primers or degenerate primers to detect the different versions of each gene or using metageomic approaches that do not require primers. In a pioneering study, Kellner et al. (2010) used a wide variety of primers to detect a range of lignocellulolytic and chitinolytic enzyme-coding genes in an organic forest soil, and their results highlight the importance of ascomycetes in producing EEs.

Most enzyme-coding genes have only been sequenced for a limited number of microorganisms and examples of these include chitinase (Metcalfe et al., 2002; LeCleir et al., 2004; Xiao et al., 2005), laccase (Luis et al., 2004) and proteases (Fuka et al., 2008). In general, the relative abundance of enzyme-coding genes does not correlate strongly to enzyme activities, suggesting that the detection of a gene does not necessarily indicate that it is expressed in that environment (Hassett et al., 2009). The gene indicates potential function but it is the presence of an mRNA transcript that informs that the gene has been expressed and should, therefore, correlate more closely with enzyme production rates than gene copies. However, Edwards et al. (2011) studied the effect of N deposition on the relative abundance of laccase and cellobiohydrolase gene transcripts and concluded that gene expression did not explain differences in enzyme activities. This suggests that other factors, such as enzyme persistence and turnover, must be considered in interpreting differences in enzyme activities between treatments, sites and sampling dates. Nonetheless, metatranscriptomics using mRNA and cDNA offers great promise for understanding microbial enzyme expression in soil (Damon et al., 2012; de Menezes et al., 2012; Haichar et al., 2012).

8. Manipulating extracellular enzyme activities for ecosystem services

Given what we know about EEs, from the molecular processes of their generation and how their externalisation is controlled to their locations and activities in the environment, the question arises: do we have enough information to manipulate activities in order to promote specific ecosystem services? The answer to this depends on our level of understanding regarding the particular enzyme(s) involved and the large number of abiotic and biotic factors affecting activity. Building upon the ideas of Kremen (2005), identifying the key determinants of EE activity is a necessary first step towards managing beneficial ecosystem services that are catalysed by enzymes (e.g. bioremediation, C sequestration, and plant growth promotion). Examples of abiotic and biotic factors that influence EE activity have been described in this review and elsewhere (Allison et al., 2007), but much research remains to be done before we can apply this knowledge to the rational manipulation of soil enzyme activities. For example, should we manage environments to promote the abundance, gene expression and/or protein assembly by organisms that generate the desired enzyme(s) or should we attempt to create optimal conditions for the activity of EEs functioning broadly independently of cells? In this context, spatial and temporal studies are particularly valuable for identifying factors controlling EE activity which, depending on the EE in question, might be pH, substrate availability, microaggregate distribution, microbial nutrient demand, microbial biomass and/or community structure (Allison and Jastrow, 2006; Sinsabaugh et al., 2008; Dorodnikov et al., 2009; Kang et al., 2009; Baldrian et al., 2010; Hill et al., 2010; Kaiser et al., 2010; Stromberger et al., 2011). Other factors may be important as well but remain unidentified because of methodological limitations. A good example is our inability to directly measure EE concentration in soils, although recent advancements in proteomics should make this possible in the not-too-distant future (Wallenstein and Weintraub, 2008). However, if manipulation is our overall goal, then it is important that manageable determinants are identified. The next necessary steps would be to design and implement practices that promote EE activity and, subsequently, ecosystem services. Here we outline several potential strategies for EE activity manipulation, which include influencing organisms and their enzymes already present in the environment, and bioaugmenting the soil by introducing microorganisms and their enzymes. We also discuss plant based strategies to deliver enzymes or enhance microorganisms through rhizosphere interactions.

8.1. Manipulating indigenous microorganisms and their enzymes

Environmental conditions and substrate availability can be altered to stimulate the production and activity of indigenous microbial enzymes. This is termed 'biostimulation' and is an established bioremediation practice that enhances indigenous microbial activity and contaminant degradation through additions of nutrients, electron donors and/or electron acceptors to alleviate

resource limitations (Miller et al., 2010). Another rather different example arises from the proposal by Jastrow et al. (2007) to modify the soil physicochemical environment to promote fungal growth and their EE activities for C sequestration. Their recommendations included: growing perennial plant species to alter the quality and quantity of plant C inputs (and thus rhizosphere activities); minimizing physical disturbances, such as tillage, which reduces fragmentation of hyphae and increases fungal biomass; and amendments to maintain a near neutral soil pH to optimize many EE activities. One consequence of increased fungal biomass and activity could be immobilization of plant residue C within relatively recalcitrant biomass components (Bailey et al., 2011).

8.2. Bioaugmentation with microorganisms

Depending on the desired ecosystem service, it may be that the appropriate EEs are not present or do not exist in high enough concentrations to be effective. Under these circumstances, bioaugmentation would be necessary. This option poses additional challenges including: identifying and culturing the most appropriate strains; harvesting their cells or enzyme extracts; delivering these products to the environment; and subsequent manipulation of the environment to promote survival, growth and expression of the desired activities. Nonetheless, this strategy has been applied successfully in the field and examples relevant to EE activity include bioaugmentation of contaminated soils with white rot fungi, usually in a sawdust-based carrier, to facilitate degradation of pentachlorophenol (Lamar et al., 1994) and polycyclic aromatic hydrocarbons (Hestbjerg et al., 2003). Ford et al. (2007) provide a methodology for the growth and inoculum preparation of white rot fungi for this purpose while Novotný et al. (2004) give details on the activity of several lignin-degrading EEs produced by white rot fungi and utilized for bioremediation.

Bioaugmentation of agricultural soils with plant growth promoting rhizobacteria (PGPR) can enhance plant nutrient availability, stimulate plant growth and induce resistance to disease (Ryan et al., 2009). In P-limited soils, an important ecosystem service provided by EEs is mineralization of phosphate from organic matter by phosphatases (Dodd et al., 1987; Rodriguez and Fraga, 1999). For example, arbuscular mycorrhizal fungi (AMF) are common soil inoculants and seed treatments that benefit plant growth by acquiring and translocating soil P into host roots. Moreover, AMF may acquire P from organic sources due to the activity of external phosphatases localized on the hyphal wall (Joner and Johansen, 2000). Recent reports have also documented improved plant growth in the presence of bacterial (*Bacillus bacterium* for maize) and fungal (*Sporotrichum thermophile* for wheat) inoculants that expressed extracellular phytase (Idriss et al., 2002; Singh and Satyanarayana, 2010). Increased phytase activity in soil would be extremely beneficial for alleviating plant P deficiencies, as phytic acid accounts for a large part (20–50%) of the total organic P pool (Richardson et al., 2001).

There remain many challenges to be overcome for soil bioaugmentation to be a successful and widely adopted technology (Dutta and Podile, 2010; Tyagi et al., 2011). For example, homogenous distribution of the inoculants can be difficult, although cost-effective and environmentally friendly technologies are being developed to aid in chemotaxis of bacteria towards pollutants in soil (Harms and Wick, 2006). Distribution is less of a problem with fungi than bacteria due to their filamentous growth. Combinations of fungal and bacterial inoculants are worth considering, as many bacterial species are known to form biofilms on AM hyphae (Artursson et al., 2006; Toljander et al., 2006) and thus can 'hitch a ride' as the fungal hyphae spread through soil (Wick et al., 2007). Once mixed with soil, inoculated organisms must successfully compete for space and resources with a multitude of microorganisms already established and survive long enough to produce appropriate levels of EEs for ecosystem services to be significantly affected. Combinations of biostimulation and bioaugmentation may be appropriate in certain circumstances (Silva et al., 2004) where rate limiting steps in a sequence of degradation events have been identified.

8.3. Bioaugmentation with enzymes

The above discussion is focused on bioaugmentation with bacteria and fungi for EE production *in situ*, but another option is to add EEs directly to the soil. Exciting developments in bioremediation, C sequestration and plant growth promotion are underway that would circumvent the need to promote microbial growth and activity *in situ*. For example, immobilized carbonic anhydrases are being tested (in reactors at this stage) for their feasibility for sequestering CO_2 into bicarbonate (Ramanan et al., 2009) and, in a proof-of-concept study, purified phytase improved maize seedling growth when P was supplied in the form of phytate (Idriss et al., 2002). Challenges to be overcome include the loss of EE activity over time, which may occur as enzymes become immobilized by or entrapped within clays, organic matter or organo-mineral complexes (Gianfreda and Rao, 2004) or degraded by proteases and metabolised by microorganisms. This is an economic issue because of high production costs which can be prohibitive unless the enzyme has prolonged activity (Duran and Esposito, 2000). Immobilization of EEs on ceramic supports, porous resins and nanoparticles may be useful for extending their activity, and this has been studied for the bioremediation of polyaromatic hydrocarbons, pentachlorophenol and pesticides in water and soils (Duran and Esposito, 2000; Gianfreda and Rao, 2004; Acevedo et al., 2010; Ginet et al., 2011; Husain and Ulber, 2011; Nicolucci et al., 2011). Another technology is one that associates enzymes with an agent such as chitosan to form cross-linked enzyme aggregates. Its potential has been demonstrated for laccases, which tend to have low specific activity when stabilized on solid supports (Arsenault et al., 2011). Some older ideas regarding constructing stable synthetic humic-enzyme complexes (Sarkar and Burns, 1984) may be worth revisiting.

8.4. Plant based strategies

One promising development is the proposed utilization of plants as vectors for EEs to promote ecosystem services. Rhizomediation, for example, is a technology that uses growing plant roots to distribute bacteria throughout the soil and into otherwise impermeable soil layers whilst, at the same time, generating root exudates that stimulate growth and activity of contaminant degraders (Kuiper et al., 2004). Another approach is rhizosphere engineering, where plants are genetically modified in a way that influences the composition and activity of the rhizosphere community (Ryan et al., 2009). With genetic manipulation, it may be possible to bypass the direct involvement of microorganisms through plant expression of bacterial or fungal genes that encode for EEs. This strategy is being evaluated for multiple uses such as the improvement of soil P availability by transgenic plants that express fungal or bacterial phytase (Richardson et al., 2001; Zimmermann et al., 2003; George et al., 2004, 2005). Bacterial genes encoding EEs that degrade organic contaminants have also been introduced into plants, giving them the ability to degrade polychlorinated biphenyls (PCBs) and explosives such as TNT and RDX (Francova et al., 2004; Macek et al., 2008; Abhilash et al., 2009). As with all research, the undoubted potential of transgenic technology can only be realised after studies to determine the

efficacy of the desired process and any side effects in a wide range of soils and in association with many plants. It is worth noting that least one study has found no detrimental impact of phytase-transgenic tobacco on rhizosphere microbial community structure (George et al., 2011).

Extracellular degradation of complex organics can occur if: (i) natural or anthropogenic substrate is present and accessible to the microbe and/or its EEs; (ii) externalized enzymes are protected and survive; (iii) correct combination of enzymes and microbes are assembled; and (iv) appropriate chemical and physical conditions exist for catalysis. There are several strategies, not necessarily mutually exclusive, that may serve to minimize the costs of enzyme production and maximize the benefits. However, much remains to be learnt about how these mechanisms are employed to balance the resource costs and the acquisition benefits to extracellular enzyme producers.

9. Conclusions: scientific advances, synergies and research priorities

There is little doubt that progress in EE research will be accelerated by stepping outside the restrictive boundaries of a narrow research discipline and collaborating with others working in what we sometimes perceive as unrelated fields. Synergies with other disciplines are already accelerating advances in technological developments and their adaption to soil enzyme studies. For example, soil and aquatic enzymologists are becoming increasingly aware of their common research aims and objectives (Sinsabaugh et al., 2009; Arnosti, 2011) and the numerous opportunities offered by collaboration. In fact, many of the differences between soil and water habitats are simply a matter of scale. Perhaps most significantly environmental enzymologists are working with mathematicians to incorporate enzymes into biogeochemical and ecosystem models (e.g. Moorhead et al., 2012).

We urgently need more information on microbial regulation of enzyme synthesis, secretion and survival in order to predict how enzyme pool sizes, stability and activities are likely to change with changes in temperature and precipitation frequency. There is increasing evidence that root exudation rates will increase with elevated atmospheric CO_2 (Phillips et al., 2011) altering the relative availabilities of labile C and nutrients, particularly N, in soils (Weintraub et al., 2007; Kaiser et al., 2010). This has the potential to modify heterotrophic soil respiration which, in turn, may further increase atmospheric CO_2 concentrations. However, we need more detail as to how microbial enzyme production and turnover are altered by changes in root exudation and plant litter deposition in order to be able to predict changes in the net ecosystem exchange of CO_2. Furthermore, changes in root exudates and the alteration of microbial enzyme production is likely to have an impact on stable soil organic matter and this needs urgent study (Schmidt et al., 2011).

As our knowledge regarding EE function, regulation and expression increases, the purposeful manipulation of EE for ecosystem service becomes possible. Strategies range from manipulating 'natural' organisms and their enzymes to employing genetically modified organisms. While remaining controversial, transgenic technology will enable the use of plants and microorganisms to deliver EEs to the root zone that promote a wide range of plant growth promoting and remediation processes. The rhizosphere activities will range from improving the already highly integrated mycorrhizal and legume–rhizobia associations to the control of plant pathogens and pests and the regeneration of degraded and polluted soils. Much of our knowledge will spring from interacting with those who are investigating how microbes and plants communicate with each other in soil (Badri et al., 2009; Mathesius and Watt, 2011) coupled with an appreciation that EE activities are at the heart of this complicated conversation. A mass balance that considers the bioenergetics of enzyme synthesis and secretion, substrate degradation and product assimilation in the context of individual and community microbial growth is much needed.

Soil is a complex and dynamic biological system and it is difficult to link specific taxa to metabolic processes and determine which microbial genotypes are responsible for the production of certain EEs. There is a pressing demand to understand the relationships between genetic diversity and community structure and function (O'Donnell et al., 2001; Suenaga, 2011; van Elsas and Boersma, 2011) and because all EEs have corresponding genes, they represent an ideal model system for linking microbial identity to specific and critical ecosystem processes. In this context advances in proteomics, metabolomics and transcriptomics hold great potential. While the detection of a gene transcript suggests that production of an enzyme has been initiated, the process of translation is a critical control point, and thus direct detection of proteins and enzymes is the ultimate tool in understanding metabolic controls on enzyme production. Identification of proteins has long been possible with immunological techniques but these are difficult to apply to soils and other environmental samples (Chourey et al., 2010). However, the advent of mass spectrometry based proteomics allows for the simultaneous detection of many different proteins, and has recently been applied to soils, litter and other similar matrices (Lacerda and Reardon, 2009; Dill et al., 2010). Proteomics techniques offer great but as yet unproven potential to elucidate many facets of the regulation and ecology of EEs. In addition, the nanoscale visualisation of enzyme locations and their reactivity with substrates may soon be revealed using, for example, atomic force and scanning electrochemical microscopy (Casero et al., 2010; Koley et al., 2011; Rennert et al., 2011).

We conclude this review by identifying nine key research priorities, which we present as what we hope are thought-provoking questions.

1. Where are EEs located in the soil matrix, what contribution does each location make to the overall turnover of a substrate and how do these contributions change with time?
2. How are EEs regulated by the soil biotic and abiotic environment, and what is their contribution to global biogeochemical processes?
3. Can we link EE production to specific taxa or communities?
4. How does the ability to produce specific enzymes affect the fitness of microbial populations and associated community structure?
5. Are laboratory based assays indicative of activities in the field and can we develop nanoprobes for insertion into soil to permit *in situ* measurements in real time?
6. How are changing environmental conditions likely to impact on EE activities and will there be an overall effect on soil organic carbon deposits?
7. Can we integrate EE activities into theoretical and applied models of ecosystem function (vertical integration) and biomics (horizontal integration)?
8. Can we develop a soil enzyme index that can be used as a reliable measure of soil fertility and health?
9. How do we manipulate soil EE activities to achieve effective generation of plant nutrients, control pathogens and pests, and clean up and regenerate degraded and polluted environments?

Some of these questions have been with us in one form or another for a while, but new approaches and ideas are beginning to provide answers. Many advances will arise from collaborations,

adapting methods and adopting the new technologies outlined in this review. Others will spring from our increasing understanding of microbial ecology and how plants, microbes and soils interact. The outcomes will provide mechanistic and regulatory insights into extracellular enzyme functioning at the molecular, cellular, community and ecosystem scale.

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