The impacts of herbicides on soil biology and function

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Abstract

There is a growing awareness amongst farmers about the importance of soil for sustaining crop production and providing beneficial ecosystem services. Over the last two decades, global herbicide use has increased as farmers have shifted to more sustainable conservation tillage practices and have adopted herbicide-tolerant crop cultivars. The implications of increased herbicide use for soil biology are being questioned, but a comprehensive review on this topic is lacking. In this chapter we outline the chemistry and use of the major herbicide classes, and review the soil functions relevant to crop production. We then collate and critically evaluate the evidence for herbicide effects on soil biota and activity. In general, most studies suggest that the impacts of herbicide application on soil function are only minor and/or temporary. However, there are some instances where findings consistently suggest effects that could significantly alter soil function. These include disruptions to earthworm ecology in soils exposed to glyphosate and atrazine; inhibition of soil N-cycling (including biological N_2-fixation, mineralisation and nitrification) by sulfonylurea herbicides in alkaline or low organic matter soils; and site-specific increases in disease resulting from the application of a variety of herbicides. Issues with extrapolating these findings to broad-acre farming include the lack of a consistent framework for assessing herbicide risk to soil biology; the relevance of the magnitude of herbicide impacts compared with the impacts of other soil management practices such as tillage or crop rotation; the complexity of herbicide formulations and mixtures; and the limited number of long-term field studies.

Keywords

Agrochemical, pesticide, fungi, bacteria, nematode, soil health, plant disease, nutrient-cycling, crop production

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1. Introduction

Weeds are a significant constraint and cost to agricultural production worldwide. Estimates suggest that without weed control, yield losses could range from 29% in wheat to 47% in rice crops (Oerke, 2005). Historically, weed control was achieved manually or mechanically involving some form of soil cultivation. Although this is certainly effective for reducing weed incidence and in providing a suitable seed bed for sowing crops, the mechanical disturbance of soil also has a number of detrimental side-effects, such as increasing the risks of soil erosion (Pimentel et al., 1995) and loss of soil organic matter (Six et al., 1999). Repeated mechanical tillage also incurs relatively high labour and energy costs which impact on gross farm income (Karlen et al., 2013).

In order to overcome the adverse effects caused by repeated soil cultivation, farmers have increasingly adopted 'conservation tillage' practices whereby soil cultivation is minimised. For example, in Australia the proportion of grain growers using no-till technology has increased over the last 30 years from around 5% to over 70% (Llewellyn et al., 2012). The move towards conservation tillage has required the use of alternative weed control strategies, including a higher reliance on herbicides (D'Emden et al., 2006). The development of herbicide-resistant crop varieties, through conventional breeding or genetic modification (GM), has also promoted herbicide use but in a more selective manner to match the mode of crop herbicide resistance. Benbrook (2012) estimated herbicide-resistant crop technology led to a 239 million kilogram increase in herbicide use in the United States between 1996 and 2011, mainly through increased glyphosate use. It is also estimated that herbicide usage in the Australian grains industry increased by more than 30% from 2002 to 2012, from a value of $700M to $1.1B over that period (APVMA, 2003; APVMA 2012; data CPI and land-area adjusted). Although precise data for the total increase in the volume of herbicides applied worldwide are not readily available, herbicide use is predicted to continue to increase as food production intensifies and urbanisation continues to put pressure on availability of labour for manual weed control, especially in developing countries (Gianessi, 2013).

Unfortunately, little is known about the impact of increased herbicide use on soil biota and the ecosystem services they provide. This in part reflects the diversity of the chemicals being applied and also the diversity in soil ecological communities and function, which renders a full systematic assessment almost impossible. A review of the effects of herbicides on soil biology almost a decade ago (Bünemann et al., 2006) suggested that the database of knowledge is 'simply too small to draw sound conclusions', and a more recent review emphasised the lack of a suitable framework for the routine evaluation of pesticide effects on soil microbial communities and functions (Imfeld and Vuilleumier, 2012). Scheepmaker and van de Kassteele (2011) conducted a broad meta-analysis on the effects of chemical control agents (including fungicides, insecticides, herbicides and antagonists) on numbers of non-target microbial soil organisms by pooling different microbial count variables, such as colony forming units per g soil, nodule score, root colonization, mycorrhizal root length, induced host mortality and number of taxa. Unfortunately, herbicides were excluded from the final analysis because of limited data availability. Even the conclusions from the analysis of insecticides and fungicides lacked the resolution to make specific recommendations about individual compounds.

From the few review papers available, the emerging picture is one of compound-specific effects on particular soil functions. With regard to herbicides, adverse effects on phosphatase activity by glyphosate (Sannino and Gianfreda, 2001), inhibition of nitrification by simazine (Hernández et al., 2011) and adverse effects on pathogen-antagonistic *Pseudomonas* bacteria by acetochlor and chlorimuron-ethyl (Wang et al., 2013; Wu et al., 2009) are just some of the examples of the potential effects of herbicides on soil health as related to plant nutrition and disease.

In light of these reports, herbicides applied with the ultimate goal of maximising productivity and economic returns potentially act at the expense of ecosystem functions. Although not immediately obvious, these ecosystem services also contribute to crop health by promoting of crop stubble turnover, pathogen suppression, nutrient cycling and maintenance of soil structure. Sandhu et al. (2008) estimated that soil functions provide approximately $330 per ha per year of unaccounted, non-material value in 'conventional' agricultural systems, and up to $500 per ha in 'organic' systems. As an example, this would equate to a value of over $6

billion per year to Australian ('conventional') grain production, compared to the gross value of grain production of around $9 billion per year.

A comprehensive review of the impacts of herbicides on soil biota and functions is required to gain insight into if, or how, their use should be changed to improve productivity and economic returns. Synthesis of the current knowledge will enable farmers to identify where a change in herbicide management could protect the ecosystem services provided by their soils in crop production. In this paper, we first summarise the chemistry of the different herbicide classes and review their mode of action with regard to potential effects on non-target organisms. Second, we outline the methods used by researchers to measure herbicide-induced changes to soil biological communities. Subsequently, we evaluate the empirical evidence for direct effects of herbicides on non-target soil organisms and biological community structure, followed by effects on key ecosystem functions contributing to agricultural production. These functions include carbon (C) turnover, nutrient cycling, and the suppression of disease. We then discuss a number of factors that confound or moderate our understanding of the impacts of herbicides. Finally, we identify priority areas for monitoring and knowledge gaps that limit our current ability to draw appropriate conclusions on the impact of herbicides on soil biology and function.

2. Herbicide chemistry and mode of action

A chemical is classified as a herbicide if its toxicity towards plants is orders of magnitude higher than its toxicity toward other organisms. Within this broad grouping, however, are numerous chemical classes with distinct modes of herbicidal action. Thus, the effect of each chemically unique herbicide on non-target organisms is likely to be different and difficult to generalise. Unfortunately the exact worldwide total usage for each individual or class of chemical is not known. To help focus our review, we searched the database Scopus using the search terms herbicide AND soil AND (microb* OR function*) and ranked the herbicide classes in terms of the most to least studied. The frequency of study (Figure 1) roughly correlates to the frequency of use, based on sporadic and localised surveys (Givens et al., 2009; Grube et al., 2011; Osten et al., 2007). There is obviously a strong focus in the literature on the herbicide glyphosate, concomitant with its widespread use.

[INSERT FIGURE 1 NEAR HERE]

A further complication in predicting effects of herbicides on soil biology and function is the relationship between herbicide application rate and its concentration in the soil. Most ecotoxicological studies are conducted by constructing dose-response curves with the dose of toxicant reported as a concentration (e.g. mg L^{-1} or mg kg^{-1}). However, in practice, herbicides are applied at rates given in kg or L per ha. The herbicide concentration in soil initially depends on the depth of incorporation and the bulk density of the soil. After application, herbicides redistribute through the soil profile and concentrations decrease over time via abiotic and biotic loss and transport pathways. A major issue is the failure of many reports to explain the assumptions used to calculate the herbicide rates used in incubation studies (in mg kg^{-1}) from field rates (in g ha^{-1}). In order to give the reader some perspective, we have calculated predicted environmental concentrations (PEC) in soil for different model herbicides according to European Economic Community (2007) guidelines (Table 1). In this scenario, a surface-applied herbicide is assumed to be distributed in the top 5 cm of a soil profile with a bulk density of 1.5 g cm^{-3}. Herbicide concentrations reported in this document refer to the concentration of the active ingredient unless otherwise specified.

Chemical structures and IUPAC nomenclature for the model herbicides are available for reference in Appendix 1.

[INSERT TABLE 1 HERE]

2.1.1. *Glycine*

Glyphosate is the most widely used herbicide in the world (Duke and Powles, 2008), with more than twice the mass of the next most popular herbicide being applied in the USA (Grube et al., 2011). It is a non-selective herbicide which is taken up through leaves and shoots and is distributed throughout the plant tissues. Glyphosate is known for its high sorption affinity for soil (especially clays and other minerals) and therefore is considered essentially non-mobile in soil (Table 1). Glyphosate is not considered persistent as it undergoes biotransformation into aminomethylphosporic acid (AMPA) as its main metabolite.

Glyphosate prevents the synthesis of aromatic amino acids, by binding to the enzyme 5-enolypyruvateshikimate-3-phosphate synthase (EPSPS) and inhibiting its function (Sikorski and Gruys, 1997). Bacteria and fungi require aromatic amino acids for protein synthesis and for the production of secondary metabolites essential for environmental adaptation (Tzin et al., 2012). Direct effects on microorganisms therefore may occur via this mode of action. Indeed, glyphosate has been shown to inhibit microbial growth in pure cultures at concentrations of 0.075 g L^{-1} (Shehata et al., 2013); however to our knowledge, thorough mechanistic studies have not been conducted to determine the exact mode or prevalence of toxicity in different microbial species. Unlike plants, many microorganisms are able to tolerate or overcome the toxic effects of glyphosate (e.g. Drouin et al., 2010), presumably through up-regulated EPSPS production, modified EPSPS structures or rapid metabolism/detoxification of the glyphosate molecule.

2.1.2. *Chloroacetamides*

Chloroacetamide herbicides, such as metolachlor and acetochlor, are commonly used herbicides. These are relatively mobile and persistent herbicides, especially metalochlor (Table 1). The mode of action of these herbicides is via inhibition of cell division and elongation in plants due to interference with a number of enzymes. The fatty acid elongase enzymes required for the synthesis of very-long-chain fatty acids (VLCFAs) and geranylgeranyl pyrophosphate (GGPP) cyclization enzymes for gibberellin production are two particularly sensitive targets. Elongases are present in

bacteria, fungi and prokaryote cells, but they are a diverse family of enzymes with many different substrates and functions. We are not aware of any studies that have directly examined the effects of chloroacetamide herbicides on these enzyme systems in soil microorganisms or soil fauna. However, Bonnet et al. (2007) showed that non-specific esterase activity was a more sensitive endpoint indicator of alachlor toxicity using a model bacterium and protist, rather than population dynamics; whereas the reverse was true for the herbicides diuron and glyphosate. This suggests that specific analysis of elongase and/or GGPP enzyme activity in soil may be appropriate endpoint assays for detecting effects of chloracetamide herbicides. There is clearly more targeted mechanistic work that needs to be conducted in this area before any sound conclusions can be drawn.

2.1.3. *Sulfonylureas and Imidazolinones*

Sulfonylurea and imidazolinone herbicides are commonly used for cereal production and are effective at very low application rates compared with other herbicides. Some of the herbicides from these classes (e.g. chlorsulfuron) are relatively persistent and mobile (Table 1). Both herbicide classes act by inhibiting the enzyme acetolactase synthase (ALS), also known as acetohydroxyacid synthase (AHAS), which is responsible for the production of the branched chain amino acids leucine, isoleucine and valine. The ALS enzyme is present in both plants and microorganisms and inhibition of microbial growth upon exposure to sulfonylureas/imidazolinones is expected. Boldt and Jacobsen (1998) confirmed a toxic effect by sulfonylurea herbicides on fluorescent pseudomonads isolated from an agricultural soil. At a concentration of 5 mg L^{-1}, sulfonylurea herbicides reduced the growth rate of up to 20% of the strains tested. However, growth inhibition was relieved when branched chain amino acids were added to the culture media, implying the mode of toxicity was an inhibition of ALS as hypothesised. Similar results were observed by Nelson and Duxbury (2008) in a study of 27 diverse soil bacterial isolates, with the authors concluding that the majority of soil microorganisms contain only one functional ALS enzyme that is sensitive to sulfonylurea herbicides.

[INSERT FIGURE 2 NEAR HERE]

2.1.4. Triazines, phenylureas, amides

Triazine, urea and (phenyl)amide herbicides block the quinone-binding site in photosystem II, leading to increased production of reactive oxygen species and subsequent damage to membranes, proteins and DNA. The build-up of irreparable damage eventually results in plant death. Because the photosystem II site is specific to photosynthetic organisms, direct toxicity to (non-photosynthetic) bacteria and fungi via this mode of action is unlikely. This does not rule out the possibility of indirect toxicities through other quinone-binding sites or unknown reaction, but suggests that potential effects of this kind may occur at higher concentrations than those toxic to plants and algae. These herbicide classes tend to be mobile and persistent and have been noted for their off-site migration potential (Table 1).

2.1.5. *Phenoxyycarboxylic acids*

Phenoxycarboxylic acids mimic the structure of the auxin class of plant hormones. Auxins regulate cell growth and division and thereby exert control over the shape and form of plants. They are especially important for plant nutrition as they initiate root formation and branching. There is also evidence for the role of auxins in mediating beneficial plant-microbial associations (Van Zwieten et al., 1995). When used as herbicides, phenoxycarboxylic acids disrupt the hormone balance, causing growth abnormalities and injuries such as leaf curling, tissue swelling and root splitting. Aside from plant-mediated effects, the mechanisms by which phenoxycarboxylic acids may impact on soil-dwelling organisms are unknown. High concentrations of phenoxycarboxylic acids may have direct toxic effects, whilst it is plausible that low levels may interfere with plant-microbial signalling and act to alter the structure and function of microbial communities, in particular the balance between beneficial and pathogenic organisms. To our knowledge such a hypothesis has not yet been tested.

2.1.6. Dinitroanilines

Dinitroaniline herbicides such as trifluralin and pendimethalin halt cell mitosis (division) in plants by preventing the polymerization of tubulin to form microtubules (Morejohn et al., 1987). This effect dramatically inhibits the growth of plant tissue,

particularly roots, causing seedling death. Although it was previously thought that tubulins were only present in eukaryotes, evidence suggests that the prokaryotic cell division protein FtsZ is a structural homolog (Amos and Lowe, 1998). Nevertheless, the dinitroaniline herbicides only appear to affect tubulins found in plants, algae and protozoa, but not animals, fungi (reviewed in Morrissette and Sept, 2008) or, as far as we are aware, prokaryotes. The effects on protozoa may shift the ecological balance of the soil and consequently impact on beneficial processes, but to our knowledge no such effects have been mechanistically studied. Both trifluralin and pendimethalin have a high binding affinity to soil and are persistent in nature, with half-lives of 180 and 90 days respectively (Table 1). Trifluralin shows strong affinity (binding) for soil and is susceptible to rapid volatilisation losses after application and therefore often needs to be incorporated in soil.

3. Soil biology: community structure, function and assessment

Living organisms play a critical role in the distribution, transformation, availability and sequestration of carbon, nutrients and toxicants in soil, and therefore crop production. One of the current challenges in soil science is defining which organisms contribute to, or influence, specific functions and how biological communities adapt to environmental changes without losing the ability to support plant growth and other agronomic goals. In the context of this review, we have taken an "agri-centric" stance in that our discussion focuses on the processes relevant to sustainable crop production. Although some overlap no doubt exists with processes relevant to natural ecosystems, we have deliberately avoided discussion about herbicide impacts in these systems. We should also point out that although soil biology encompasses a wide range of organisms including plant and animals, our discussion here is limited to microbial and mesofaunal communities, as well as earthworms. Mesofauna include those organisms less than 2 mm such as nematodes, collembola and mites. Earthworms have been included in this discussion because of their well established role in soil fertility and common use as a bioindicator in soil toxicity studies (Paoletti, 1999).

3.1. Biological communities and functions relevant to crop production

One of the main services provided by soil organisms is the turnover of organic matter. This process involves multiple scales of breakdown and transformation, from the macroscopic cutting and breakdown of particulate organic material, to the enzymatic cleavage of polymers into monomers as occurs in the hydrolysis of cellulose to glucose. Organic matter contributes strongly to the available water content of soils (Hudson, 1994), but the benefits to water availability appear to improve over time as the organic matter ages in soil (de Silva and Cook, 2003). Organic matter turnover also liberates nutrients for crop growth, whilst the balance between turnover and stabilisation of organic matter determines the loss of carbon from the system as dissolved organic material or gaseous molecules, mainly carbon dioxide and methane (Baldock and Nelson, 2000). As a consequence, organic matter turnover also plays a critical role in climate regulation.

Aside from organic matter turnover, soil organisms regulate nutrient availability through additional transformations of mineral and organic nitrogen, phosphorus and other elements. The N cycle is of particular importance since N is a key requisite for high crop yields and grain/fodder quality, and is strongly influenced by biological processes. Nitrogen fixation, that is the conversion of atmospheric N_2 to organic N, is carried by both free-living bacteria and also symbiotic plant-microbial associations. Symbiotic N-fixation in legume crops is especially important as contributions of N from fixation can be in the order of 100 kg N per hectare per crop (Peoples and Craswell, 1992). In terms of ecosystem services, the value of N-fixation can be readily quantified as it directly substitutes for chemical N fertiliser input. After organic (reduced) N is introduced into the soil, either through biological N-fixation or as urea fertiliser application, it is gradually mineralised to ammonium. Under aerobic conditions, ammonium will be oxidised mainly to nitrite, nitrate and smaller amounts of gases (NO, NO_2 and N_2O) in the process of nitrification. Because of the predisposition of nitrate to leaching and also denitrification to N_2O, biological processes of ammonification, nitrification and denitrification in soil strongly regulate the availability of N to agricultural crops.

The soil biology also influences the availability of P and other elements to crops. Of special importance is the symbiotic association of plants with mycorrhizal fungi. These associations mutually benefit both partners, through a flow of reduced carbon substrates from the plant to the fungi in return for other nutrients, especially P and microelements (Smith and Read, 1997). Other rhizosphere microorganisms contribute to plant nutrition through the production of organic acids that can release mineral-bound phosphates and siderophores that chelate micronutrients in the soil solution (Vessey, 2003). Members of the genus *Bulkholderia*, *Enterobacter* and *Pseudomonas* are particularly well-represented in this group of rhizobacteria (Costa et al., 2014; Khan et al., 2009)

The abilities of certain soil organisms to cause plant disease, and for some soils to suppress disease, are also important processes with direct relevance to sustainable crop production. Organisms which can cause direct crop damage include insects, nematodes, fungi and bacteria. The taxonomy of insect pests and plant-parasitic nematodes is well known and commercial diagnostic services are available for

identifying and enumerating these species in soil samples. Similarly, the most prevalent disease-causing fungi and bacteria are also well characterised, such that commercial testing for the major diseases in Australian cropping systems is available (Ophel-Keller et al., 2008). Less well known are the mechanisms by which some soils suppress the occurrence of disease, despite the presence of disease-causing organisms – but this is mainly because of the diversity of mechanisms and complexity of interactions involved (Mazzola, 2002). Such complexity means that the role of particular microbial groups or individual species in inhibiting plant pathogens is also challenging to decipher (Mazzola, 2002)

3.2. Methods for assessing community structure

One of the most difficult aspects in assessing the impacts of herbicides on soil biology and their functions is appraising which methods are the most appropriate for use and how different methods relate to each other. The complexity of this task has increased dramatically in the last few decades with the rapid development of molecular methods for monitoring community structure and function. We give a brief overview here of the methods that feature throughout this article.

In terms of assessing the community structure of mesofauna and earthworms, microscopic observation is routinely used. Although well-established, microscopy is relatively time-consuming and requires some specialist skills in identification and taxonomy (Ritz and Trudgill, 1999). Additional factors to consider include the size of the sample needed and which method of extraction will give unbiased and maximum recoveries (Neher et al., 1995). More recent molecular methods have been developed in an attempt to decrease sample processing time and cost (Chen et al., 2010; Griffiths et al., 2006). Although some evidence of bias has been detected (Donn et al., 2011), molecular methods are capable of rapidly and accurately differentiating nematode communities responding to changes in the soil environment (Donn et al., 2012).

Soil microbial community structure can be assessed through a number of different means, including culture-dependent physiological characterisation and culture-independent techniques based on nucleic acids and fatty acid profiles. Culture-dependent techniques involve growing and isolating or identifying discreet pure

colony-forming units for enumeration and characterisation (Hill et al., 2000). Such techniques are relatively inexpensive, quick and easily performed without specialised equipment. An additional benefit is that colonies of interest can be mass cultured to further explore the mechanisms behind their functioning and interactions with other organisms. Groups of interest (e.g. phosphate solubilisers, pathogens, siderophore producers) may also be enumerated through culturing on selective media. Microbial community characterisation as a whole, without isolation of individual colonies, can also be achieved through culture-dependent techniques, commonly known as community-level physiological profiling (CLPP) (Hill et al., 2000). Two of the more common, high throughput formats include the Biolog ® and Microresp ® system. The Biolog ® system involves inoculation of artificial media containing a specific growth substrate with diluted soil slurry and measuring the colour change in the media to determine growth (Garland and Mills, 1991). In contrast, the Microresp ® system involves dosing a soil microcosm with a growth substrate and using an alkaline gel to capture CO_2 respired from the soil (Campbell et al., 2003). The CO_2 is quantified colorimetrically through changes to a pH indicator in the alkaline gel.

The primary drawback of culture-dependent methods is that results can be biased towards those community members that are dominant, fast-growing and/or non-fastidious (Hill et al., 2000). Changes may also occur in the community structure and function during the cultivation period. Information gleaned through these methods is limited to a small subset of the microbial population and does not truly represent the entire community or the ecological dynamic interplay. By comparison, culture-independent molecular methods are generally more inclusive and are a powerful means to describe microbial diversity in ecosystem maintenance. However, it must be noted that some molecular methods may introduce different biases – for example, through different extraction methods or primer choice in PCR – and these should be considered when interpreting results (Hirsch et al., 2010). A number of reviews are available on the subject of culture-independent methods for microbial community and functional analysis and the reader is directed to these for a more in-depth explanation of the techniques (Hirsch et al., 2010; Rincon-Florez et al., 2013). A summary of the advantages and disadvantages of these methods is given below (Table 2).

[INSERT TABLE 2 NEAR HERE]

3.3. Methods for assessing soil biological community function

Assessing changes to soil biological community function can be achieved via macroscopic observations, measurement of chemical pools and fluxes, and microbial culture-dependent and culture-independent approaches. Many of these are advocated for use in defining endpoint toxicities in 'terrestrial model ecosystems' as described by (Weyers et al., 2004). Macroscopic observations encompass a broad range of assessments including plant root and shoot growth or form, disease symptoms, degradation of organic biomass (e.g. calico strips) and feeding activity (e.g. bait lamina). These measures are useful for longer term monitoring at higher tier scales. Similarly, measurement of pools and fluxes of chemical species, such as soil nitrate, ammonium and phosphate concentrations or CO_2 and N_2O emissions, over time can give valuable insight into changes in soil biological community function. Such measures are routinely reported in studies assessing the impact of herbicides on soil biology.

More detailed mechanistic information can be gained by applying culture-dependent and independent methods to examine specific microbial functions. Results from CLPP assays (described in section 3.2) can provide direct information about the ability of a microbial community to metabolise a specific organic compound. Another popular approach is to measure the activities of a diverse set of enzymes acting on different chemical pathways involved in C, N, P and S cycling (Caldwell, 2005). The recent development of fluorescent substrates and method formatting in microwell plates (Marx et al., 2001) has increased the sensitivity and speed of many enzyme assays, allowing for greater precision and sample throughput. However, as with culture-dependent characterisation of microbial community structure, the use of high substrate levels can induce a level of bias towards fast-growing and dominant members of the community. Additional considerations also need to be given to the environmental conditions under which assays are performed, particularly temperature and pH, as variations in these parameters can strongly influence enzyme activities (German et al., 2011; Niemi and Vepsäläinen, 2005).

Use of culture-independent, nucleic acid-based methods for functional chacterisation of soil microbial communities is becoming more frequent (Rincon-Florez et al.,

2013). Methods include quantitative polymerase chain reaction (q-PCR) of functional genes, hybridisation of functional genes in an array format, and sequencing extracts of soil mRNA (Table 3). Functional gene arrays and metatranscriptomics can give a very detailed snapshot of microbial community activity at a particular point in time, but high cost and relatively complex data processing currently limits the widespread application of these technologies. Furthermore, all three methods remain partially limited by the knowledge gaps in relating specific nucleic acid sequences to a known function.

[INSERT TABLE 3 NEAR HERE]

4. Effects on soil biota and community structure

4.1. Microbial communities

4.1.1. *Glycine: Glyphosate*

Numerous studies have found that glyphosate applied at standard application rates (PEC = 3 mg kg^{-1}, table 1) has little impact on the microbial biomass in soil, and stimulation rather than inhibition is more commonly observed (Table 4). In some cases, glyphosate has a variable effect over time, with increases, decreases or no effects being observed periodically after the initial exposure (Abdel-Mallek et al., 1994). These findings for soil application are interesting, since glyphosate at 5 mg L^{-1} (representing a maximum concentration in soil solution soon after application) applied *in vitro* reduced growth of 21 out of 22 fungal species isolated from a boreal forest soil when challenged with increasing doses of formulation (Round-up ®) (Tanney and Hutchison, 2010). This highlights the large differences between laboratory-based dose-response studies *in vitro,* compared with studies in soil where sorption and other factors likely reduce acute toxicities.

[INSERT TABLE 4 NEAR HERE]

Even though the effect of glyphosate on the total microbial biomass appears to be negligible, this does not rule out an effect on finer-scale population dynamics. A number of studies examining these dynamics using high-throughput and more advanced culture-independent methods are discussed below.

The addition of the recommended field-rate concentration of glyphosate (5 kg ha^{-1} corresponding to 50 mg kg^{-1}) to two different forest soils caused no major changes in microbial community structure assessed by CLPP, PLFA, and standard cultural and microscope methods (Ratcliff et al., 2006). A higher rate of 100-times the field rate concentration (i.e. 500 kg ha^{-1}), reflecting an undiluted chemical spill, produced a significant enrichment of bacteria and minimal change to the fungal community (Ratcliff et al., 2006). Similarly, glyphosate (2.5 kg ha^{-1}) had no effect on microbial diversity, as measured by ester-linked FAMEs, 14 d after application in three different seasons in soybean rhizosphere or bulk soil. Laboratory incubations showed slight alteration in community structure (FAME) 3 d after relatively high application rates of 47 or 150 mg kg^{-1}, but these values reconverged at 7 d (Weaver et al., 2007). Glyphosate application as Roundup PowerMAX ® temporarily lowered the total microbial biomass in the rhizosphere of glyphosate-resistant soybean grown in soil that had no previous exposure to glyphosate, but caused no changes in the microbial community structure as measured by ester-linked FAME (Lane et al., 2012). Repeated applications of glyphosate as Roundup PowerMAX ® (six applications of 118 mg kg^{-1} over 6 months, equivalent to 88.5 kg ha^{-1} per application) also did not significantly change the microbial community structure as measured by ester-linked-FAME (Lane et al., 2012) and a field survey could not detect any significant effect of glyphosate on total microbial biomass or fungal and bacterial biomass and ratios using PLFA (Rosenbaum et al., 2014). Single and repeat applications of glyphosate as Roundup WeatherMAX ® (49 mg kg^{-1}) had only a minor effect on FAME profiles, but clone libraries and pyrosequencing showed increases in proteobacteria, specifically Burkholderiales (Lancaster et al., 2010).

Using CLPP and DNA fingerprinting, Zabaloy et al. (2012) found little to no effect of standard glyphosate rates on the microbial community structure by either C substrate utilization or T-RFLP. Similarly, glyphosate at both conventional application rates and 10 times higher had little impact on the T-RFLP profiles of bacterial communities

and total DNA extracted from three different soils (Zabaloy et al., 2012). Application of 1.8 kg ha^{-1} of glyphosate to Roundup Ready ® corn also had no effect on the structure of the rhizosphere fungal community as measured using T-RFLP (Hart et al., 2009). Interestingly, glyphosate (50 and 500 mg kg^{-1}) applied as Roundup ® increased the CLPP diversity in both triticale and mixed triticale-pea rhizospheres 15 d after application, even though diversity change could not be detected by DGGE (Mijangos et al., 2009). At the same time, the ability of the microbial community to metabolise the surfactant Tween 20 was increased, suggesting a strong influence by the formulation constituents on community level physiological profiles. However, at 30 d after application, the differences were inconsistent between application rates and plant community rhizospheres, suggesting potential effects are short-lived and difficult to generalise (Mijangos et al., 2009). This supports the findings of Lupwayi and Blackshaw (2012) that periodic increases and decreases in the CLPP bacterial diversity occurred in corn rhizospheres and bulk soil after annual glyphosate applications over a five year monitoring period.

More recent attempts to delve into community structural changes have utilised rapidly-developing next-generation sequencing techniques, which have greater sensitivity in detecting changes to non-culturable and under-represented microbial taxons. The findings from one of the first studies to use next-generation sequencing supported earlier work using different methods by showing that repeated application of glyphosate at a standard rate (0.72 kg ha^{-1}) to maize grown on two soils had insignificant effects on microbial community structure (Barriuso et al., 2011a). However, studies by the same authors also detected transiently altered microbial community composition in response to glyphosate application, but these changes were small compared to the herbicide mixture 'GTZ' (containing 2.2 kg ha^{-1} acetochlor and 0.87 kg ha^{-1} terbuthylazine), which had a more persistent effect on reshaping the microbial community (Barriuso et al., 2010; Barriuso and Mellado, 2012). Pyrosequencing of culturable bacteria also showed a reduction in diversity caused by glyphosate, but even greater reduction caused by GTZ (Barriuso et al., 2011b). Taken as a whole, these results suggest that the application of glyphosate at or near recommended field rates has no demonstrable consistent, significant impact on soil microbial community structure.

A herbicide that has similar chemistry as glyphosate, i.e. glufosinate, appears to have a greater impact on microbial communities. Low levels of glufosinate-ammonium (1, 10 mg kg^{-1}) increased the culturable counts of cellulose degraders and ammonia-oxidizing bacteria, but dramatically reduced actinomycete counts and these did not recover even by the end of the experiment, 40 d after application (Pampulha et al., 2007). Glufosinate application (3 kg ha^{-1}) caused transient changes in the eubacterial and *Pseudomonas* population structure as detected by PCR-DGGE (Gyamfi et al., 2002) and also altered the active bacterial communities in canola rhizosphere (16S rRNA DGGE), with generally higher active populations of key groups (Sessitsch et al., 2005). However, Schmalenberger and Tebbe (2003) did not detect changes in the bacterial community structure after glufosinate application to conventional or transgenic herbicide-tolerant maize, using molecular methods. Likewise, Ernst et al. (2008) found no impact on bacterial community structure when glufosinate (0.6 kg ha^{-1}) was applied to glufosinate-resistant rape/maize. The contradictory nature of these results may be a consequence of different monitoring periods and environmental conditions, as Griffiths et al. (2008) found altered microbial diversity (CLPP and ester-linked FAME) at 6 weeks after herbicide application but no differences at 12 weeks.

4.1.2. Chloroacetanilides

Most studies examining the impact of chloroacetanilide herbicides on microbial community size and structure have focussed on the chemical butachlor. Generally speaking, butachlor application at standard rates (<10 mg kg^{-1}) was found to have little effect on the size of the soil microbial community under laboratory (Xia et al., 2011) or field conditions (Singh and Ghoshal, 2010). At these rates, effects on microbial community structure are also insignificant as determined by random amplified polymorphic DNA (RAPD) analysis (Wang et al., 2007; Wang et al., 2009), but they may have a temporary effect on functional diversity as measured by CLPP (Fang et al., 2009). Butachlor application rates higher than 10 mg kg^{-1} have been reported to cause a more marked and longer lasting reduction of soil bacterial diversity (Wang et al., 2007; Wang et al., 2009). Similarly, application of alachlor and metolachlor at rates of 10 mg kg^{-1} did not have any pronounced effect on bacterial and fungal populations (Dzantor and Felsot, 1991). In contrast, a relatively low application rate (0.85 mg kg^{-1}) of another chloracetanilide herbicide, acetochlor,

stimulated bacterial populations, while fungal growth exhibited a reverse trend (Bai et al., 2013). The microbial composition as measured by PLFA was significantly altered by acetochlor in the early stage (15 d) after application; thereafter (19-35 d), any impacts on soil microbial communities were attenuated and eventually undetectable (Bai et al., 2013). Another study examining the effect of high concentrations of acetochlor (50-250 mg kg^{-1}, relative to a PEC of about 8 mg kg^{-1}) on soil fungal communities by DGGE found that acetochlor had a transitory effect on fungal diversity, returning to background levels by 60 d (Xin-Yu et al., 2010). Nevertheless, the actual community structure had shifted at 60 d from controls in all acetochlor treatments (Xin-Yu et al., 2010). Applications of metazachlor (0.5 kg ha^{-1}) also altered the PLFA community structure in the rhizosphere of non-transgenic canola (Ernst et al., 2008), but measurements were made at one unspecified time, so no assessment of resilience could be made. Pretilachlor (0.45 kg ha^{-1}) had no significant impact on MBC or PLFA profiles in a rice paddy soil (Murata et al., 2004). As with glyphosate there was little effect of chloroacetanilide herbicides on microbial community structure and where effects were present they were not typically consistent among studies, making conclusions difficult to draw.

4.1.3. Sulfonylureas and Imidazolinones

Research into the effect of sulfonylurea herbicides on microbial populations suggests that most compounds from this class applied at conventional rates have no impact on microbial biomass, whilst higher rates may temporarily reduce microbial biomass. For example, rimsulfuron at 0.025 kg ha^{-1} had no effects on microbial biomass C (MBC), but higher doses at 10 and 100 times the conventional rate reduced MBC (Perucci et al., 1999; Vischetti et al., 1997; Perucci et al., 2000). The onset and magnitude of these effects were dependent on temperature and humidity; however, they were generally slight (<20%) and transitory. As with rimsulfuron, chlorsulfuron applied at a conventional rate (0.01 mg kg^{-1}) also had no significant effect on MBC or microbial biomass nitrogen (MBN), whilst 10 and 100 times higher doses significantly reduced MBC and MBN by around 25% and 50%, respectively (El-Ghamry et al., 2000). But unlike rimsulfuron, higher application rates resulted in sustained suppression of microbial biomass even after 45 d (El-Ghamry et al., 2000). A low rate sulfonylurea mixture (0.01 mg kg^{-1} metsulfuron + 0.01 mg kg^{-1} bensulfuron) significantly reduced MBN and MBC in the first 10 d following application, after which they recovered.

Higher rates had an even greater (negative) impact on MBN and MBC (El-Ghamry et al., 2001). Similar results were observed when bensulfuron-methyl was applied without metsulfuron at a conventional rate (0.01 mg kg^{-1}), wherein it transiently reduced MBC and MBN in the first week after application. Higher rates (10×; 100× conventional) also affected MBC and MBN and increased the time taken to return to control levels (El-Ghamry et al., 2002).

In terms of microbial community structure, bensulfuron-methyl at rates of 0.067 mg kg^{-1} and higher reduced the counts of culturable bacteria until 60 d, whereas the effects on fungi and actinomycetes were inconsistent and transient. Overall bacterial diversity measured by DGGE was only significantly affected at application rates of 0.355 mg kg^{-1} or higher in a soil with pH 7.2 (Lin et al., 2008). This differs from another study which found no significant effect of bensulfuron-methyl on bacterial diversity measured by DGGE when applied at 0.051 or 0.51 mg kg^{-1} to a soil with pH 4.7 (Saeki and Toyota, 2004). Similarly metsulfuron-methyl (1 mg kg^{-1}) had no effect on CLPP diversity (Zabaloy et al., 2008). The discrepancy between these studies may be related to soil pH, as sulfonylurea herbicides are known to breakdown much more rapidly at acidic pH (Sarmah and Sabadie, 2002). Long-term (5 or 10 yr) application of chlorimuron-ethyl to soybean fields significantly reduced culturable bacteria and actinomycetes, but increased fungal counts. Molecular profiling of bacterial and fungal DNA also indicated significant shifts in community structures of both microbial groups, corresponding to reduced diversity (Zhang et al., 2011).

Limited information is available about the effect of imidazolinone herbicides on soil microbial community structure. Imazamox (0.1 kg ha^{-1}) reduced MBC by 19-22 %, but levels subsequently recovered (Vischetti et al., 2002). Similarly, imazethapyr added at a conventional rate (0.12 kg ha^{-1}, approximately equivalent to 0.16 mg kg^{-1}) had no effect on MBC (Zhang et al., 2010b) or a temporary inhibition (Xu et al., 2013), but levels recovered within 30 d after application. Higher rates of application caused greater negative impacts, but levels also quickly recovered in the same time frame. Fluctuations in fungal and bacterial PLFAs and PLFA patterns were also observed at these rates, but they were inconsistent over time and did not persist beyond 60 d (Zhang et al., 2010b; Xu et al., 2013).

4.1.4. Triazines

An early study on the effect of the s-triazine herbicide atrazine found that application rates of 30 or 100 mg kg^{-1} to a loam soil resulted in increased populations of culturable actinomycetes, bacteria and fungi over those in non-treated soil (Percich and Lockwood, 1978). The increases were in proportion to the quantity of atrazine applied, and effects persisted for at least 2 months. Application rates more consistent with label rates (10 mg kg^{-1}, slightly more than double our PEC of 4 mg kg^{-1}, Table 1) had no significant effect on culturable populations (Percich and Lockwood, 1978). Despite no effects on numbers of culturable organisms, application rates < 10 mg kg^{-1} still appear to alter the microbial community structure. In one study, atrazine applied at 5 mg kg^{-1} to 5 different soils induced a minor temporary (3 wk) shift in microbial community as measured by PLFA (Mahía et al., 2011). Atrazine at 1, 2 and 3 mg kg^{-1} also caused a shift in the soil microbial community as monitored by DGGE: 10 d after application, some DNA bands showed increased intensity and up to 10 new bands could be detected, but by 30 d the differences were negligible (Briceño et al., 2010). A similar rate (1.5 mg kg^{-1}) of the related herbicide simazine also altered bacterial diversity in soil, by increasing the relative proportion of α- and β-proteobacteria and decreasing γ-proteobacteria up to 30 d after application (Girardi et al., 2013; Caracciolo et al., 2010). Prometryn-treated soils also showed some differences in molecular profiles (by 16S PCR-DGGE and amplified ribiosomal DNA restriction analysis) at 100 mg kg^{-1}, but the differences usually involved detection of additional bands, suggesting enrichment of prometryn-degrading organisms (Crecchio et al., 2001). However, another triazine herbicide, hexazinone did not reduce soil microbial population at 1, 2 and 8 kg ha^{-1} (Chakravarty and Chatarpaul, 1990).

4.1.5. Phenoxycarboxylic acids

The effects of 2,4-D application on microbial community size and structure are inconsistent. Devi et al. (2008) observed that 2,4-D application at conventional rates (0.5, 1, 2 and 4 kg ha^{-1}) temporarily reduced culturable bacteria and increased culturable fungi; but in another study, DGGE analysis did not detect any community shifts in soil treated with 2,4-D at 10 mg kg^{-1} (Macur et al., 2007). Moreover, the same rate (10 mg kg^{-1}) of 2,4-D butyl ester had no significant impact on culturable microbes, but did cause a significant shift in the PLFA profile of the soil microbial

community (Zhang et al., 2010a). The only consistent finding is that microbial growth-dependent methods detect higher numbers of 2,4-D degrading organisms at conventional application rates of 5-10 mg kg^{-1} (Zabaloy et al., 2010; Macur et al., 2007). Together, these results highlight the difficulties in comparing or aggregating data from studies that use different methods, and imply that the use of multiple methods for assessment purposes is prudent in order to cover methodological biases.

4.1.6. Phenylureas, amides

Data on the effect of phenylurea and associated PS-II inhibiting herbicides (e.g. propanil) on microbial biomass and structure is sparse. Linuron applied to soil at a conventional rate (4 mg kg^{-1}) did not affect numbers of culturable bacteria, fungi, nitrifiers, denitrifiers or N-fixers, but some transient (<28 d) effects were observed at higher rates (Cycoń and Piotrowska-Seget, 2007). Metoxuron at 5 mg kg^{-1} had no effect on bacterial or fungal numbers, but fungal propagules were temporarily lower at 50 mg kg^{-1} and severely curtailed at 500 mg kg^{-1}, whereas total counts of bacterial propagules were greatly increased at 500 mg kg^{-1}. Propanil at all rates (1-100 mg kg^{-1}) did not affect community structure as measured by PCR-DGGE and amplified ribosomal DNA restriction analysis (ARDRA) (Crecchio et al., 2001)

4.1.7. Dinitroanilines

Trifluralin (0.1-0.8 mg kg^{-1}) had no consistent effect on fungi, bacteria or actinomycete populations in either bulk soil or in wheat rhizospheres, although periodic increases and decreases were observed with respect to controls not receiving herbicide (Olson et al., 1984). Field application of trifluralin (1.0 kg ha^{-1}) into wheat at two different locations showed no effects on soil fungi, bacteria, actinomycete, denitrifying bacteria, and nitrifier populations (Olson et al., 1984).

4.1.8. Other herbicide groups

Other minor-use herbicides appear to have little effect when applied at conventional label rates. Mefenacet did not affect diversity (DGGE) at 0.133 mg kg^{-1} (Ye et al., 2006) and had no significant impact on PLFA profiles in a rice paddy soil when applied at 1.05 kg ha^{-1} (Murata et al., 2004). Mesotrione applied at a conventional rate (0.45 kg ha^{-1}) or 10× higher (4.5 kg ha^{-1}) did not significantly impact the DGGE bacterial community structure (Crouzet et al., 2010), nor did it alter the diversity or

structure of soil cyanobacterial communities (Crouzet et al., 2013). Napropamide only significantly affected community structure (16S-DGGE, CLPP and PLFA) at 10× the conventional rate, whilst impacts at conventional rate were insignificant or quickly returned to normal (Cycoń et al., 2013a; Cycoń et al., 2013b). Dinoseb applied to soil at 20 mg kg^{-1} did not significantly affect MBC, but 60 mg kg^{-1} significantly reduced MBC and levels did not recover by the end of the incubation (25 d) (Lin and Brookes, 1999). It is noteworthy that 60 mg kg^{-1} is a very high rate of application. Fluazifop-butyl had no significant effect on total fungal propagule populations at a standard rate equivalent to 0.6 mg kg^{-1}, but at higher rates of 3 and 6 mg kg^{-1}, it caused temporary reduction in fungal populations until 1 and 2-weeks after application, respectively (Abdel-Mallek et al., 1996). No significant impacts on microbial biomass and diversity were observed by applications of bromoxynil (3 applications each month) at 10 mg kg^{-1} (Baxter and Cummings, 2008). However, repeat applications of 50 mg kg^{-1} dramatically altered diversity as measured by PCR-DGGE (Baxter and Cummings, 2008). Furthermore, a herbicide mixture consisting mainly of bromoxynil significantly reduced culturable fungi, actinomycetes, ammonia-oxidizing bacteria and cellulytic bacteria at all levels tested (1, 10, 100 mg kg^{-1}). All except fungi returned to control levels after 40 d (Pampulha and Oliveira, 2006).

4.2. Mesofauna and earthworms

Numerous invertebrates groups influence soil and ecosystem services that contribute to plant growth and environmental systems in general. Nematodes, mites, collembolans and earthworms all play a strong role in redistributing and breaking down organic matter, recycling nutrients, regulating microbial communities and interacting with plant roots. Because they can be assessed visually, in terms of type and quantity, the effects of soil disturbances are often more clear-cut than simiilar studies of microbial communties. Nevertheless, their mobility and interactive roles at lower and higher ecological tiers means that the agronomic effects of changes to these communities is sometimes difficult to predict.

Liphadzi et al. (2005) investigated the direct effects of five glyphosate rates ranging from 0.56 – 4.48 kg ha^{-1} on soil nematode communities in a controlled growth chamber experiment and found that total nematode density and densities of individual

populations (herbivores, fungivores, microbivores, omnivores) were unaffected by all of the tested application rates. Similarly, application of glyphosate (0.9 kg ha^{-1}) had no significant impact on the number of collembola in a maize-turnip rotation up to 40 d after application (Lins et al., 2007). Indeed, in terms of overall faunal response, Reinecke et al. (2002) found that glyphosate applied at 1.1 kg ha^{-1} to a vineyard soil stimulated, rather than inhibited, bait-lamina feeding activity.

Contrasting effects of glyphosate on earthworms have been described in the literature, with differences arising from earthworm ecotypes and the nature of the study. Dalby et al. (1995) observed little effect of glyphosate on endogeic earthworms (topsoil feeders), while epigeic earthworms (surface litter feeding) *Eisenia fetida* lost approximately half their body mass after 28 d when exposed to 8 mg kg^{-1} glyphosate (Yasmin and D'Souza, 2007). In another study, the impact of glyphosate at 10, 100, 500 and 1,000 mg kg^{-1} on the earthworm *E. fetida* was a gradual and significant reduction in mean weight (50%) at all test concentrations (Correia and Moreira, 2010). In the same study, 2,4-D at 500 and 1,000 mg kg^{-1} caused 100% mortality, while after 14 days, 30%–40% mortality levels were observed at 1, 10 and 100 mg kg^{-1}. Clearly these rates are very high and the experiments were designed to assess the thresholds of adverse effects. However, *E. fetida* exhibited strong avoidance behaviour in field soil treated with glyphosate at 1.44 kg ha^{-1} (Casabé et al., 2007). Avoidance behaviour was also demonstrated when *E. fetida* were exposed to a formulation containing 5% glyphosate by mass (as isopropylamine salt); however the exact concentration was unclear (Verrell and Van Buskirk, 2004). According to Zaller et al. (2014), glyphosate also has the potential to alter ecological interactions between earthworms, mycorrhizal fungi and above ground plants, leading to reduced mycorrhizal plant-colonization and modified earthworm feeding behaviour. Because there are still many unknowns about the effects of glyphosate on meso-fauna, particularly in complex ecological systems beyond those found in many laboratory incubation studies, further research in this area is warranted.

With regards to triazine herbicides, the compound simazine was found to have no significant effects on nematode or food web structure when applied at 2.68 kg ha^{-1} (Sánchez-Moreno and Ferris, 2007), and no effect on bait-lamina feeding activity when applied at 3 kg ha^{-1} (Reinecke et al., 2002). Atrazine had no significant impact

on the number of collembola (Lins et al., 2007) or earthworms (Chelinho et al., 2010) at conventional rates; but did begin to inhibit earthworm reproduction in one soil at 20.7 mg kg^{-1}, equivalent to a conventional application rate distributed only in the top 1 cm of soil (Svendsen et al., 2008). Atrazine also elicited a dose-response relationship with avoidance behaviour, in which over 50% of worms avoided soil concentrations of 38 mg kg^{-1} – approximately 10 times higher than the PEC of 4 mg kg^{-1} (Amorim et al., 2008). Atrazine toxicity towards *E. fetida* in soils varies, with lethal concentrations to 50% of the population (LC_{50}) ranging from 15 mg kg^{-1} (Frampton et al., 2006) through to 110 mg kg^{-1} (NRA, 1997). The enchytraeid worm *Enchytraeus albidus* was more sensitive to atrazine than previous studies with *E. fetida* having an LC_{50} of 12 mg kg^{-1} with 50% impact on reproduction at 2 mg kg^{-1} (Novais et al., 2010). Terbuthylazine had no toxic effects on soil animals tested (microbes, oppioid mites, two gamasid mite species, enchytraeids, and nematodes) when applied to soil at rates of 1-53 kg ha^{-1} as the active ingredient (Salminen et al., 1996). When it was applied as herbicide preparation, acute toxic effects on enchytraeids were observed, but only at rates above 10 kg ha^{-1}.

Although some adverse effects of herbicides have been reported, the diversity of experimental systems in various published studies means that the magnitude and duration of any effects may not be predictable. For example, Amorim et al. (2008) pointed out that *En. albidus* avoidance (50% of the test individuals) to phenmedipham in an initial study (Amorim et al., 2005) occurred at a concentration of 51 mg kg^{-1}, whereas a later study using the same conditions indicated a higher sensitivity, with 50% population avoidance at 7 mg kg^{-1} (Amorim et al., 2008). The authors suggested that the sensitivity to herbicides may therefore vary even within sub-populations of the same species, since different 'batches' of individuals were used. This is further complicated by the fact that soil characteristics strongly influence the variability in responses (Amorim et al., 2008). Indeed, Griffiths et al. (2008) found that glufosinate or terbuthylazine applied at conventional application rates reduced protozoa and microarthropods (mites and collembola) in maize rhizospheres in one soil but not another.

Furthermore, even if adverse effects are observed, it is difficult to determine the exact cause of the effect. For example, although Hartley et al. (1996) found that

terbuthylazine (4 kg ha^{-1}) on two different apple orchard soils reduced earthworm numbers, they concluded that the effect was related to a lower weed density which reduced the food source, rather than direct herbicide toxicity. Pelosi et al. (2013) also found that an increase in herbicide usage correlated with decreased numbers of three different earthworms, but they could not determine whether the effects were direct, or whether they were an indirect result of the decreased OM inputs caused by conventional *versus* organic farming practices. Finally, Cheng et al. (2008) found that multiple herbicide treatments in a long-term (15 year) field experiment did not significantly affect nematode communities under turfgrass compared with equivalent controls without herbicide treatments; whereas high N fertilizer treatment did alter nematode community structure. This shows that the effects of other management practices or experimental treatments have the potential to be misattributed to the effects of herbicides *per se*.

5. Effects on soil functions

5.1. Microbial activity and C cycling

As highlighted in section 3, the addition of herbicides to the soil can have both positive and negative impacts upon different members of the soil microbial community. Depending on the balance of these impacts, which can be either direct or indirect, alterations to soil C-cycling may also evolve. Direct effects include the herbicides being toxic to the microbes, which can result in a reduction of microbial biomass, and thence soil heterotrophic respiration and activity of OM decomposing and nutrient cycling microbes. In contrast, herbicide addition can directly benefit soil microbes by providing a resource to support their growth (Panettieri et al., 2013). Herbicide impact on plants can also indirectly impact upon microbes. For example, where plant growth is suppressed, the levels of labile C input into the rhizosphere are expected to decline, which can have important consequences on those microbes that utilize root exudates. Where plants die following herbicide application, the remaining plant debris provides a resource to support microbial growth and activity. Further, a reduction in plant cover may result in an increase or decrease in soil temperature and water content, both of which affect rates of microbial activity. While not explored here in detail, herbicide impacts on plant community composition may also affect soil

microbial diversity and activity via plant mediated selection of distinct microbial communities.

Soil microbial activity, in the context of soil C cycling, can be measured in many ways. The most common measures in the context of herbicide impacts on soil microbes are heterotrophic respiration, the activity of enzymes involved in soil C cycling, and organic matter decomposition and mineralization. In the following section these factors are considered for different herbicide classes.

5.1.1. Glycine

Generally, glyphosate applied at conventional rates (0.5-5 kg ha^{-1}) does not significantly reduce respiration. More commonly, respiration is either unaffected (Pereira et al., 2008; Houston et al., 1998; Busse et al., 2001; Wardle and Parkinson, 1991; Zabaloy and Gómez, 2008) or is stimulated (Means et al., 2007; Araújo et al., 2003), especially at higher application rates (Wardle and Parkinson, 1990b; Lancaster et al., 2006; Eser et al., 2007). Interestingly, in one study glyphosate application to virgin soil stimulated respiration yet application to a soil with previous exposure had no effect on respiration (Lane et al., 2012), whereas another study found the reverse was true (Zabaloy et al., 2012). This highlights the need for further studies that directly compare soils with different herbicide application histories. In one of the few cases where glyphosate application (5 kg ha^{-1}) to field plots temporarily reduced microbial respiration, such a response only occurred in plots where weeds were present (Wardle and Parkinson, 1992).The authors, suggested that the impact of glyphosate was therefore a plant-mediated response, rather than a direct impact of the herbicide on soil microorganisms (Wardle and Parkinson, 1992).

Literature reports on the impacts of glyphosate on other enzyme indicators related to soil C cycling usually follow a similar pattern to respiration measurements: that is, no change (Wardle and Parkinson, 1991) or an increase (Means et al., 2007; Araújo et al., 2003; Wardle and Parkinson, 1990b; Zabaloy et al., 2008; Panettieri et al., 2013). Interestingly, Haney et al. (2000) showed that glyphosate significantly stimulated soil microbial activity in a dose-dependent manner as measured by C and N mineralization, but did not affect soil microbial biomass at any rates. Although it was not clear whether higher C mineralization resulted from the breakdown of the

herbicide or the native soil organic matter (Haney et al., 2002), Panettieri et al. (2013) speculated that increased activities of enzymes such as dehydrogenase and β-glucosidase are probably a result of glyphosate acting as a source of easily available C. As far as we know, such a hypothesis is yet to be confirmed.

In contrast to the studies above, Damin et al. (2012) found that glyphosate application to a black oat cover crop slowed down the breakdown of plant residues. These authors hypothesised that this resulted from a change in the C:N content of the residues during the glyphosate-induced plant senescence, rather than the herbicide inhibiting decomposer organisms. Abdel-Mallek et al. (1994) also found that plant-applied glyphosate inhibited the breakdown of broad bean residues, but accelerated the decomposition of wheat biomass. This further emphasises the complexity of interactions and that the impact of glyphosate on plant residue breakdown may be regulated by litter quality.

5.1.2. Chloroacetanilides

As with glyphosate, chloroacetanilide herbicides appear to have few negative impacts on respiration and general measures of microbial activity. Conventional application rates of alachlor (10 mg kg^{-1}), metolachlor (10 mg kg^{-1}) and butachlor (2.5 kg ha^{-1}) did not affect soil dehydrogenase activity (Dzantor and Felsot, 1991; Subhani et al., 2002), whilst four rates of butachlor (5, 10, 50, 100 mg kg^{-1}) increased dehydrogenase activity in a dose-dependent manner for 3 weeks (Xia et al., 2011). Both the biomass and activity (respiration) of soil microbes were also enhanced with the application of pretilachlor (Kumar et al., 2012). One exception is the chloracetanilide allidochlor, which reduced dehydrogenase activity over a period of 3 weeks when applied to soil at a rate of 10 mg kg^{-1}.

However, in contrast to the microbial activity measures mentioned above, there is some evidence that chloracetanilides can disrupt other processes involved in C-cycling. In one study, the application of alachlor and metolachlor (at standard and high rates) reduced the number of cellulolytic microorganisms (especially bacteria) and their ability to colonize the cellulosic substrate (Sahid and Yap, 1994). These herbicides delayed the decomposition process, which recovered when there was no

residual activity in the soil, after 12 weeks of incubation (Sahid and Yap, 1994). In another study, butachlor application significantly reduced CH_4 production in an alluvial rice soil in a dose dependent response (from 5-100 mg kg^{-1}), even at the lowest level of application (Mohanty et al., 2004). This is interesting since metolachlor applied at 4.7 kg ha^{-1} had no significant effects on CH_4 emissions in an aerobic shortgrass steppe soil (Kinney et al., 2004). These results highlight the importance of measuring specific functional activities, as opposed to more general measures such as respiration or dehydrogenase activity; but also reveal the importance of compound- and site-specific interactions, which make generalisation difficult even with a chemical class. In many cases other factors such as soil moisture or nutrient status will have a much stronger influence on measures of microbial activities than herbicides (Muñoz-Leoz et al., 2012).

5.1.3. Sulfonylureas

Most studies have found also that sulfonylurea herbicides applied at conventional rates have no impact on respiration or other activity measures, whilst higher rates often lead to transient inhibition or stimulation (Table 5). Nevertheless, there are some exceptions to these general trends. For example, whereas metsulfuron-methyl applied at a range of concentrations (0.01-10 mg kg^{-1}) to an *acidic* soil had little impact, a low rate application of 0.01 mg kg^{-1} to an *alkaline* soil depressed CO_2 evolution (Zabaloy and Gómez, 2008). Similarly, Sofo et al. (2012) also observed minor reductions in cumulative respiration from an alkaline soil, 30 d after application of prosulfuron and triasulfuron at conventional rates. It is noteworthy that sulfonylurea herbicides being acidic are more mobile and bioavailable in neutral to alkaline conditions (Sarmah et al., 2000).

[INSERT TABLE 5 NEAR HERE]

The impacts of sulfonylurea herbicides on methane oxidation and cellulose decomposition activity have also been studied. The application of a mixture of nicosulfuron, atrazine and dimethenamide did not significantly alter the soil methane oxidation rate or the abundance of methane-oxidizers in another study (Seghers et al., 2005). However, as reported above for responses to glyphosate application, the

application of bensulfuron-methyl to a virgin soil inhibited cellulytic microbes, but did not do so in a soil that had received historical applications (Gigliotti et al., 1998).

Among other ALS inhibitor herbicides, similar to sulfonylureas, Imazaquin (0.14 kg ha^{-1}) also had no effect on soil microbial biomass, soil dehyrogenase or hydrolase activity when applied to field-grown soybean (Seifert et al., 2001). In both field trials and laboratory experiments, the field rate of imazethapyr (0.05 kg ha^{-1}) had no adverse effects on the microbiological processes tested, but at 10× and 100× higher rates, the herbicide decreased dehydrogenase activity and increased hydrolyase, protease and catalase activity (Perucci and Scarponi, 1994).

5.1.4. Triazines

There is little evidence to suggest that triazine herbicide significantly ihnibit microbial activity or C-cycling when applied at recommended rates. Moreno et al. (2007) found that atrazine only affected microbial activity (respiration, dehydrogenase activity) at levels greater than 100 mg kg^{-1}, wherein increases, rather than decreases were observed. Furthermore, atrazine applied at a conventional rate (5 mg kg^{-1}) to five different soils had no significant effect on β-glucosidase activity (Mahía et al., 2011). The related herbicide terbuthylazine (4 kg ha^{-1}) also had no effect on soil respiration on two different apple orchard soils (Hartley et al., 1996), and did not influence soil respiration or straw decomposition when applied at 10 kg ha^{-1} (Hantschel et al., 1994). In contrast, simazine (2 mg kg^{-1}) or dinoterb (1.75 mg kg^{-1}) had no short term effects on MBC or straw turnover, but in the longer term (after 33 d) both herbicides reduced degradation and respiration of added straw C (Harden et al., 1993). Conversely, atrazine (4.48 kg ha^{-1}) stimulated C and N mineralization, but it could not be determined if this was from the herbicides or the native soil organic matter (Haney et al., 2002). Similarly, Briceño et al. (2010) observed significantly elevated respiration from in sieved pasture soil treated with low levels of atrazine (at 1, 2 and 3 mg kg^{-1}), but only for the first 10 d after application. These examples highlights the need for temporal studies of herbicide effects on soil microbes, as well as dose responses, in order to understand potential integrated effects over longer time periods as herbicides dissipate.

5.1.5. Phenoxycarboxylic acids

The addition of low rates of 2,4-D at 0.5 mg kg^{-1} to soil microcosms produced only minor and transitory effects on microbial respiration (Zabaloy and Gómez, 2008), and higher rates (5 mg kg^{-1}) showed transient effects on other measures of microbial activity, inhibiting hydrolase activity and stimulating dehydrogenase activity in the short-term [<24 d] (Zabaloy et al., 2008). Even higher rates of 2,4-D (50 mg kg^{-1}) were reported to reduce a larger suite of enzyme activities (acid and alkaline phosphatases, arylsulfatase, urease, protease and b-glucosidase) but all had recovered by 15 d after application, except protease (Bécaert et al., 2006). Aerobic degradation of cellulose and cellobiose were not impacted by conventional rates of bentazon or MCPA, but higher rates (100 times) reduced turnover (Schellenberger et al., 2012).

Finally, triclopyr for control of woody weeds had no impact on soil enzyme activity or substrate induced respiration, whereas the alternative management practice of tree cutting enhanced beta-glucosidae and phosphatase activity (Souza-Alonso et al., 2013).

5.1.6. Phenylureas

Niemi et al. (2009) observed negligible effects on the activity of a variety of enzymes in fallow soil treated with linuron at standard rate of 0.7 kg ha^{-1} and also at 7 kg ha^{-1}. Linuron and metoxuron applied at a range of rates (5, 50 and 500 mg kg^{-1}) only had an inhibitory effect on CO_2 evolution at 500 mg kg^{-1}, with some minor reduction also found for linuron at 50 mg kg^{-1} (Grossbard and Marsh, 1974). Linuron (4, 20, 100 mg kg^{-1}) temporarily stimulated substrate-induced respiration in dose-dependent manner (<7 d), but dehyrdogenase activity was repressed at the higher rate of 100 mg kg^{-1} (Cycoń et al., 2010). Chloroxuron, diuron, fluometuron, metobromuron and monuron added to soil at 500 mg kg^{-1} caused an initial stimulation of CO_2 production, followed by indications of inhibition (Grossbard and Marsh, 1974). Although one study found that diuron at all levels above 1.67 mg kg^{-1} reduced microbial activity (measured by microcalorimetry) in a dose-dependent manner (Prado and Airoldi, 2001), the duration of this experiment was only 2 d and it is unknown if the microbial activity rebounded after this time, making it difficult to compare to other data sets.

5.1.7. *Dinitroanilines*

Trifluralin reduced respiration at all doses of 16, 32, 64 and 96 mg kg^{-1} at 20°C, but no consistent effects were found at higher temperature (Aka Sağlıker, 2009). The effects of trifluralin and 12 of its soil-formed metabolites on the decomposition of radio-labelled glucose, protein and cellulose were determined using $^{14}CO_2$ evolution from soil as a measure of decomposition. Trifluralin increased ^{14}C-glucose mineralization rates, but these increases could be eliminated by providing additional. Trifluralin had no inhibitory effect on the mineralization of protein or cellulose, but five of the metabolites inhibited glucose mineralization. None of the trifluralin metabolites affected protein mineralization. Seven trifluralin metabolites increased the rate of cellulose mineralization when applied at rates exceeding those that would be expected in soil. After considering the rate of metabolite application and the

magnitude of the responses observed, these compounds are expected to have no major effects on the microbial population (Boyette et al., 1988)

5.1.8. Others

Most studies have found limited effects of other herbicide classes on general measures of microbial activity at conventional application rates, including pendimethalin, difenzoquat (both 0.5-5 mg kg^{-1}) or thiobencarb (2.5-25 mg kg^{-1}) (Atlas et al., 1978); mesotrione (0.45 mg kg^{-1}) (Crouzet et al., 2010); propanil (5 mg kg^{-1}) (Kyaw and Toyota, 2007); and dalapon at 2.6 or 26 mg kg^{-1} (Greaves et al., 1981). Similarly, ethofumesate did not affect dehydrogeanse activity at conventional rate of 5 mg kg^{-1}, but reduced activity at 50 mg kg^{-1} whilst also increased the metabolic quotient (Muñoz-Leoz et al., 2013). Application of asulam also had no or minor effects at 16 mg kg^{-1} on C turnover, but 160 mg kg^{-1} significantly inhibited respiration of cellulose-amended soil decomposition processes in soil (Marsh, 1980).

The general trend of limited impacts of herbicides at conventional rate on microbial activity is supported by a study conducted by Lewis et al. (1978). They surveyed the impact of 25 herbicides and herbicide mixtures applied at commonly used rates, finding no effects on respiration, assayed by CO_2 evolution and dehydrogenase activity, in either silty clay loam or loamy sand. Organic matter decomposition, determined by the amount of CO_2 evolved and inorganic N formed from decomposing alfalfa tissue, was also unaffected. Moreover, selected herbicides (trifluralin, linuron, dinoseb) at concentrations 100-fold higher than the recommended rates did not affect alfalfa decomposition (Lewis et al., 1978).

Nevertheless some exceptions are apparent. In one case, fluazifop-butyl application at a range of concentrations generally stimulated the decay of calico buried in herbicide-treated soil, compared to controls (Abdel-Mallek et al., 1996). Napropramide (2.25 mg kg^{-1}) reduced dehydrogenase activity when applied at conventional rates, but the duration of this effect was only 14 d (Cycoń et al., 2013b). Mesotrione application affected active chlorophyll concentrations in soil, suggesting a reduction in C-input by surface crusts (Crouzet et al., 2013), but the impact of this on the entire soil profile and plant growth is unlikely to be significant in cropping soils. More importantly, Pampulha et al. (Pampulha et al., 2007) observed that glufosinate-ammonium (1, 10

or 100 mg kg^{-1}) dramatically reduced dehydrogenase at all levels by over 50%, without recovery by the end of a 40 d soil incubation. Sessitsch et al. (2005) also found that canola receiving 3 kg ha^{-1} of glufosinate-ammonium had lower rhizosphere activities of invertase, urease and phosphatase, but only at the third sampling time, two months after application. A lack of studies disputing or offering explanations for the findings of Pampulha et al. (2007) and Sessitsch et al. (2005) suggests that further work should be conducted to better understand the impacts of glufosinate on soil microbial activity.

5.1.9. Conclusions

Herbicide application can affect soil respiration, emissions of other greenhouse gasses, rates of organic matter decomposition, C and N mineralization, enzyme activities and substrate utilization patterns. What is not clear, however, is the nature of the responses of these variables to herbicide application. That is, the magnitude and direction of responses differ widely between studies. Despite this, it is clear that effects can be modulated by the identity and dose of the herbicides, the timing of application, and soil type. Standardized testing of herbicide impacts on soil biota would greatly enhance our understanding of their impacts, and may provide insights into the mechanisms that underpin these responses.

5.2. Nitrogen cycling

5.2.1. Glycine

Data from a number of studies suggests that glyphosate applied at conventional rates has little impact on N cycling in soil. Glyphosate had no direct effect on N-fixation when applied to different soils at rates of 2.6 kg ha^{-1} (Muller et al., 1981) or 1.25 kg ha^{-1} (Angelini et al., 2013). Furthermore, an in vitro study found no inhibition of 122 rhizobial isolates by the herbicides atrazine, glyphosate, MCPA, paraquat, imazethapyr, linuron or metolachlor at field rates, equivalent to 3.7 kg ha^{-1} (Drouin et al., 2010). A functional dose-response study by Martensson (1993) predicted that heterotrophic or cyanobacterial N-fixation would only be inhibited at levels of 400 mg kg^{-1} or higher, representing levels of approximately 100 times the recommended dose.

Glyphosate has variable, but generally minimal effects on N-mineralization (ammonification). Stratton and Stewart (1991) found that glyphosate stimulated N-mineralization by 50% at concentrations ranging from 140 to 550 mg kg^{-1} when applied to agricultural or forestry soils, respectively. N-mineralization activity was also stimulated in forest litter that had been exposed to glyphosate during spraying. However, these rates are relatively high and may not be representative of standard practice. At more conventional levels of 10 mg kg^{-1}, Tu (1994) found that glyphosate had no impact on N-mineralization in an agricultural soil. Damin et al. (2012) found that glyphosate (1.44 kg ha^{-1}) application to a cover crop (black oat) slowed down the mineralisation of N from these plant residues, which resulted in reduced N uptake by the subsequent maize crop – but the authors suggest that this finding was related to the effect of the glyphosate on the C:N ratio of the black oat crop, rather than a direct effect on the soil microorganisms responsible for N-mineralization.

With regards to nitrification and denitrification, Muller et al. (1981) found that glyphosate application (4.2 or 18 mg kg^{-1}) to two different soils had no direct effect on either process. Stratton and Stewart (1991) also found that glyphosate applications had little effect on nitrification or denitrification, and predicted that levels of 1000-2000 mg kg^{-1} would be required to inhibit nitrification and 450 mg kg^{-1} could inhibit deniftrication. These represent levels at least 100 × greater than predicted environmental concentrations after application at label rates in Australian broadacre cropping systems. Although Tu (1994) observed a slight reduction in nitrification caused by application of 10 mg kg^{-1} glyphosate, this effect was only temporary and levels of nitrate had returned to control levels 3 weeks after treatment. By contrast, Kyaw and Toyota (2007) observed that glyphosate at 2 kg ha^{-1} significantly reduced N_2O emissions by 20-90% in two different soils amended with organic matter in the form of rice straw or chitin. This result deserves further study, especially since reduced denitrification could be seen as a positive rather than negative consequence in terms of reduced greenhouse gas emissions.

5.2.2. Chloroacetanilides

The chloroacetanilide herbicides have been reported to have a mixed effect on N-cycling, depending on the specific herbicide, agricultural system or pathway under

investigation. For example, butachlor application at conventional rates of 1.5-2 kg ha^{-1} increased the number of N-fixing organisms in rice paddy soil in two different studies (Das and Debnath, 2006; Yen et al., 2013). Chen et al. (2009) used DGGE of *nifH* gene fragments to show that butachlor at 0.15 and 1.5 kg ha^{-1} caused shifts in the diversity of diazotrophs, resulting in an initial suppression and then enhancement of acetylene reduction (N-fixation). Any increase in N-fixation is unlikely to be caused by stimulation of N-fixing cyanobacteria, as Kumari et al. (2012) found that butachlor reduced N-fixation by cyanobacterial mats from multiple soils and reduced cyanobacterial diversity. Moreover, other chloroacetanilides herbicides including metolachlor and alachlor have been shown to reduce diazotroph numbers and N-fixation in aerobic soils (Angelini et al., 2013; Pozo et al., 1994). Although this discrepancy between butachlor and other chloracetanilides could be due to the specific differences in their chemical structure, it is equally possible that the effects of chloroacetanilides on N-fixation vary depending on the oxygen status of the soil, since butachlor is most often used for weed control in flooded rice paddies. Such a hypothesis is yet to be tested.

The effects of chloracetanilides on other N-cycling pathways are generally quite limited. Application rates up to 10 mg kg^{-1} of butachlor either have no effect on urease activity and N-mineralization (Wang et al., 2007; Singh et al., 2012; Wang et al., 2009) or temporarily stimulate urease activity (Xia et al., 2011). There is some evidence that higher concentrations (>50 mg kg^{-1}) of butachlor, alachlor or acetochlor can inhibit urease activity (Wang et al., 2007; Wang et al., 2009), denitrifying bacteria (Pozo et al., 1994) or nitrifying bacteria (Li et al., 2008), respectively, but such levels are unlikely to occur in soils after single applications at recommended rates.

5.2.3. Sulfonylureas

Of all the herbicide classes, evidence suggests that the sulfonylureas pose the greatest risk to nitrogen cycling process (Figure 3). Martensson (1993) reported that the lowest observable effect level (LOEC) on both heterotrophic and cyanobacterial N-fixation for a sulfonylurea herbicide, chlorsulfuron, was 0.2 mg kg^{-1}. Repeated application of a related herbicide, chlorimuron-ethyl (0.03 kg ha^{-1}), over 5-10 years to soybean crops significantly reduced the number and diversity of N-fixing bacteria (Zhang et al., 2013). The authors demonstrated that accumulation of the herbicide had occurred as a

result of its relatively long half-life, so it is possible that levels had approached or exceeded the threshold identified by Martensson (Martensson, 1993). By contrast, application of bensulfuron-methyl to two soils (one with no history of application, one with) at rates of 0.016 or 0.16 mg kg^{-1} did not affect counts of N-fixing colonies, but the rapid half-life of bensulfuron-methyl (1-3 weeks) could explain the lack of effect (Gigliotti et al., 1998).

As with N-fixation, there is evidence that sulfonylurea herbicides can impact on mineralization and nitrification at recommended or slightly higher rates. El-Ghamry et al. (2001; 2002) found that a low rate (0.01 mg kg^{-1}) of sulfonylureas metsulfuron-methyl and bensulfuron-methyl had no impact on N mineralization, but higher rates (0.1 mg kg^{-1}) transiently reduced N mineralization up to 10 d after application. Application of bensulfuron-methyl at recommended levels (0.06 kg ha^{-1}) or 10 times higher (0.5-0.6 kg ha^{-1}) significantly reduced nitrification in some soils, but usually only temporarily (Saeki and Toyota, 2004; Gigliotti et al., 1998). A conventional rate of cinosulfuron (0.04 mg kg^{-1}) also temporarily reduced nitrification activity over a period of 1 month (Allievi and Gigliotti, 2001). Repeated application of chlorimuron-ethyl (0.03 kg ha^{-1}) over 5-10 years to soybean crops also significantly reduced the number and diversity of nitrifying bacteria, which translated to a strong reduction in nitrification potential as measured by a functional biochemical assay (Zhang et al., 2013). Finally, Das et al. (2011) found that a single doses of bensulfuron-methyl at 0.35 kg ha^{-1} also reduced N_2O emissions.

[INSERT FIGURE 3 NEAR HERE]

5.2.4. Triazines

The effects of triazine herbicide on N-cycling have not been studied as intensively as other high-use herbicides. Cortina et al. (2010) reported that simazine application (0.75 kg ha^{-1}) significantly reduced N fixation in biological soil crusts, but the practical impacts of this on the entire soil profile is not clear. Simazine applied at 10 mg kg^{-1} did not affect N mineralization (Tu, 1994), but the related herbicide atrazine caused a short-term (6 wks) inhibition of N-mineralization in 5 different soils when applied at 5 mg kg^{-1} (Mahía et al., 2011). A mixture of two other triazine herbicides (terbutryne 34% plus terbuthylazine 15%) at 3 kg ha^{-1} also decreased N mineralization

in two soils, but increased it in one other soil (Kara et al., 2004). This herbicide mixture (Kara et al., 2004) and simazine applied at 10 mg kg^{-1} (Tu, 1994; Hernández et al., 2011) also temporarily inhibited nitrification. Higher levels of simazine (50 mg kg^{-1}) completely inhibited nitrification (in fertilized soil) by inhibiting the growth of specific ammonia-oxidising bacteria, as determined by DGGE (Hernández et al., 2011). Metribuzin also inconsistently affected nitrification in different soil types, with inhibition of nitrification in one sandy soil and one organic soil and stimulation in one clay soil; however, all effects were transient and not detected consistently over time (Junnila et al., 1993). Hexazinone at 15 mg kg^{-1} did not affect N-cycling processes but significantly affected nitrification when applied at 10 times these levels (Vienneau et al., 2004)

5.2.5. Phenoxycarboxylic acids

Limited data are available, but 2,4-D has been shown to have some toxicity towards heterotrophic N-fixation with a lowest observable effect concentration (LOEC) at 21 mg kg^{-1} (Martensson, 1993). In contrast, cyanobacterial N-fixation was less sensitive with a LOEC of 210 mg kg^{-1} (Martensson, 1993). 2,4-D application (50 mg kg^{-1}) reduced urease and protease activity in short term but urease recovered by 15 d after application and protease was only slightly lower (Bécaert et al., 2006). 2,4-D at 2.25 kg ha^{-1} had no effect on nitrification, but 9 and 36 kg ha^{-1} showed a marked inhibition of nitrate accumulation especially during the first half of the incubation period (32 d)

5.2.6. Phenylureas, amides

Some effects of phenylurea herbicides on N-cycling have been reported, but always at levels higher than would be expected from a recommended application rate. For example, linuron only reduced plate counts of N-fixing heterotrophs and nitrification at 400 mg kg^{-1} and had no effect at lower levels of 4, 20 or 100 mg kg^{-1} (Cycoń and Piotrowska-Seget, 2007; Cycoń et al., 2010). Other reports confirm the negligible effect of a range of phenylureas on N-cycling processes at expected environmental concentrations (<50 mg kg^{-1}), but provide additional evidence for the inhibition of nitrification at higher levels [>50 mg kg^{-1}] (Corke and Thompson, 1970; Grossbard and Marsh, 1974; Tu, 1993).

5.2.7. Other herbicide classes

With regard to other herbicide classes, only a few reports have identified discernible impacts on N-cycling at conventional rates. Napropamide significantly reduced plate counts of nitrifying and N-fixing bacteria, as well as net nitrification, at conventional (2.25 mg kg^{-1}) and higher (22.5 mg kg^{-1}) rates, and none of these measures had rebounded to control levels after 28 d (Cycoń et al., 2013b). Other herbicides, including bentazon applied at 10 mg kg^{-1} (Allievi et al., 1996), and ethofumesate at 5 mg kg^{-1} (Muñoz-Leoz et al., 2013), did not affect N-cycling processes but significantly affected nitrification when applied at 10 times these levels.

5.3. Cycling of P and other elements

Herbicide application to plants or soils may impact on P acquisition by plants by directly influencing plant metabolism or via alterations to the soil microbial and/or fungal communities that have been implicated in either P cycling or P uptake by plant roots. Herbicide impacts on plant vigour and root growth due to general toxicity of the herbicide on plants, or specific root pruning effects such as those observed following sulfonylurea herbicide application (Rengel and Wheal, 1997; Robson and Snowball, 1990), can reduce the uptake of a range of soil immobile nutrients due to reduced root exploration of the soil volume. While these impacts are important considerations in managing plant nutrition, they are not discussed in detail here as the focus of this review is the impacts of herbicides on soil biology and function.

The two major mechanisms by which herbicides can influence soil biological processes involved in P uptake by plants are 1) perturbation of microbial/fungal communities involved in organic P turnover in soils and 2) potential reduction of root colonisation, or performance, of mycorrhizal fungi that are involved in P uptake in many crop species.

4.4.1 Organic P turnover

Most studies investigating the effects of herbicides on P turnover in soils have quantified soil acid and/or alkaline phosphatase enzyme activities to assess the capability of a soil to maintain its capacity to mineralise organic P forms into the inorganic P species that are absorbed by plant roots. Studies that provided adequate information are summarised in Table 4, but differences in herbicides, application

rates, soil type and experimental conditions make it difficult to draw and firm conclusions about the results. Moreover, studies with the same herbicide (e.g. butachlor) often had contrasting results (Table 6). Even within any given study it is difficult to draw logical conclusions: for example, Perucci et al. (2000) found a significant decline in acid and alkaline phosphatase activity at 7d (10-15%) at 5 mg kg^{-1} soil yet at 50 mg kg^{-1} soil there was a significant increase in alkaline phosphatase activity at 7d (5-10%) but no change in acid phosphatase activity.

[INSERT TABLE 6 NEAR HERE]

In the only field study we found that investigated the impact of herbicides on soil functions involving P turnover, Das and Debnath (2006) examined the impact of four herbicides on populations of phosphate-solubilising organisms in the rhizosphere of rice plants. Significant increases in the populations of phosphate-solubilising organisms in the rhizosphere of rice plants following applications of butachlor, oxadiazon and oxyfluorfen at their recommended field rates (2.0, 1.5, 0.4 and 0.12 kg ha^{-1}, respectively) were observed. However, because weeds were not controlled by hand, the reduction in weed competition in plots receiving herbicide led to larger plants and increased yields. Despite the conclusion of the authors, we contend that it is not possible to determine whether the increase in P-solubilising organisms in the rhizosphere was due specifically to the herbicides or due to greater availability of carbon in the rhizosphere in the larger plants.

4.1.2 Mycorrhizas

Fungicides are known to inhibit mychorrhizas but the impact of herbicides on mycorrhizal colonisation and survival is not well understood. Most studies have focussed on ectomychorrizae because of their importance in silviculture or arbuscual mycorrhizas (AM) because they colonise a wide range of agriculturally important plants (Cavagnaro, 2008). Many studies on herbicide-mycorrhiza interactions have been conducted in laboratory conditions and the results have varied from stimulated growth to suppression of growth depending on the rate of active ingredient used and specific fungal species investigated (reviewed by Trappe et al., 1984).

The results of field and greenhouse studies are also variable: for example, studies investigating a range of herbicides including trifluralin, alachlor, diazinon, triclorpyr, imazapyr, chlorsulfuron, sulfometuron and glyphosate at a range of rates up to 2× field rates found no effect colonization of roots by arbuscular mycorrhizal fungi (AMF) or ectomycorrhizal fungi of crop plants or pine trees, respectively, even when plant growth was retarded (Burpee and Cole Jr., 1978; Busse et al., 2004; Chakravarty and Chatarpaul, 1990; Mujica et al., 1999; Pasaribu et al., 2013). In contrast, glyphosate at 0.8 and 3 mg kg^{-1} reduced mycorrhizal spore counts when applied directly to the soil (Druille et al., 2013a, 2013b) but had no effect when applied to plant foliage (Druille et al., 2013b). The same authors found reduced root colonisation by AMF when the plants were treated with glyphosate, which coincided with a

reduction in photosynthetic capacity of the plants. Given that no reduction in AM colonisation was found in glyphosate-resistant soybean roots when the plants were treated with glyphosate (Mujica et al., 1999; Powell et al., 2009), this may suggest that the reduced colonisation was due to a reduction in photosynthate supply to roots rather than a direct inhibitory effect of glyphosate on the AMF. Similar questions surround the results of Ramos-Zapata et al. (2012): paraquat reduced AM colonisation in maize roots when used over a 13-year period in comparison to other cover crops and mulch treatments, but there is no indication of whether this was a direct effect of paraquat or due to reduced carbon inputs to soil for 13 years in the inter-row where paraquat was sprayed.

5.4. Pathogens and disease incidence

The interaction of herbicides, pathogens and crop plants has the potential to either increase or decrease the incidence of disease and subsequent yield decline through a number of mechanisms (Figure 4). Kortekamp (2011) recently reviewed these interactions and the reader is directed to this review for a more in-depth discussion, particularly with respect to the herbicides glyphosate and glufosinate. We briefly summarize the pertinent findings of Kortekamp (2011) below and provide an update in terms of more recent literature and additional detail on some other herbicide classes.

[INSERT FIGURE 4 NEAR HERE]

5.4.1. Glycine

As highlighted by Kortekamp (2011), the application of glyphosate *in vitro* can inhibit a variety of soil-borne pathogens, including *Sclerotium rolfsii* (Westerhuis et al., 2007), *Pythium ultimum* and *Fusarium solani* f. sp. *pisi* (Kawate et al., 1992), *Fusarium solani* f. sp. *glycines* (Sanogo et al., 2000), *Nectria galligena* (Burgiel & Grabowski, 1996) and *Rhizoctonia solani* (Lancaster et al., 2008). However, the effect of glyphosate *in vitro* appears to have little relevance to the severity of disease in whole-plant bioassays or in the field. In fact, increased disease severity after glyphosate application has been observed in sugarbeet inoculated with inoculation with *Rhizoctonia solani and Fusarium oxysporum* (Larson et al., 2006); sugarcane

infected with *Pythium arrenomanes* (Dissanayake et al. 1998); ryegrass as a result of increased *Pythium* root rot (Kawate and Appleby, 1987); soybean infected with *Phytophthora megasperma* f. sp. *glycinea* (Keen et al., 1982), bean with *Colletotrichum lindemuthianum* (Johal & Rahe, 1990), tomato with *Fusarium* spp. (Brammal & Higgins, 1988) and grapevine with *Cylindrocarpon* sp. (Whitelaw-Weckert, 2010).

Because glyphosate inhibits the synthesis of aromatic amino acids that are a key component of many plant defence compounds, increased disease could occur via indirect effects of the herbicide on plant health or pathogen-resistance, which subsequently allows for greater colonization and/or pathogenisis by the disease-causing organism (Kortekamp 2011). This is supported by the observation that glyphosate application increased tissue levels of shikimate (the precursor to aromatic amino acids) even in glyphosate-resistant wheat varieties (Larson et al., 2006). It should be borne in mind that this effect can also occur in weed species, leading to additional agronomic considerations. For example, glyphosate increased *Fusarium* density in both glyphosate-sensitive and –resistant types of the weed species *Amaranthus rudis* (Rosenbaum et al., 2014), and in this sense could assist in speeding up weed control (Baley et al., 2009). However, increasing the susceptibility of either crop or weed species may also accelerate the build-up of pathogen density over time, leading to a higher risk of pathogen outbreak in non-resistant crops.

In other cases, as Kortekamp (2011) points out, there also is evidence that glyphosate can upset the balance of the soil microbial community and reduce the innate suppressiveness of the soil to pathogen dominance. For example, glyphosate (at 0.8, 1.2 and 2.4 kg ha^{-1}) inhibited the growth of Pseudomonads and indole-acetic acid producing microorganisms, concomitant with increasing *Fusarium* infection in soybean roots in a dose-dependent manner (Zobiole et al., 2011).

5.4.2. Chloroacetanilides

Acetochlor application at rates of 50-250 mg kg^{-1} significantly altered the structure of soil fungal communities, with a temporary increase in pathogens and reductions in common non-pathogens (Xin-Yu et al., 2010; Xin-Yu et al., 2010). Increasing acetochlor concentrations (50, 150, 250 mg kg^{-1}) also reduced the number and

diversity of culturable Pseudomonads showing antagonism towards *Rhizoctonia* (Wu et al., 2009). Because whole-plant assays were not conducted, it is difficult to speculate on whether these effects would translate to increased plant disease, but these results certainly suggest an increase in the risk of pathogen infection.

5.4.3. Sulfonylureas and other ALS inhibitors

In one of the earliest studies on the interaction of herbicides with disease in cereal crops, Rovira and McDonald (1986) followed up on field observations of farmers and agronomists who observed high incidences of poor barley growth – with symptoms typical of root rot – in alkaline soils treated with chlorsulfuron in the previous season. Through controlled-environment experiments with *Rhizoctonia solani* they found that chlorsulfuron at the equivalent of 2.5 g ha^{-1} (0.004 mg kg^{-1}) significantly increased root disease caused by *R. solani* in wheat and barely, but chlorsulfuron did not increase the incidence of the disease take-all, caused by *Gaeumannomyces graminis* var. *tritici,* in wheat (Rovira and McDonald, 1986). Lee et al. (2012) also found that sub-lethal doses (<20% recommended rate) of two other ALS inhibitor herbicides, imazamox and propoxycarbazone-Na, reduced barley growth and increased *Rhizoctonia solani* disease symptoms. The authors suggested that sub-lethal rates of herbicides and *R. solani* could alter the severity of injury symptoms, possibly owing to the herbicide predisposing the plant to the pathogen (Lee et al., 2012). Similar results have also been observed for soybeans. Bradley et al. (2002) found that the ALS-inhibitor imazethapyr increased the severity *Rhizoctonia* root and hypocotyl rot of soybeans compared to the no-herbicide control in a number of different environments. Some cultivars were clearly more susceptible than others to this interaction. Long-term (5 or 10 yr) application of chlorimuron-ethyl to soybean fields of near-neutral pH have also been observed to increase the prevalence of *F. oxysporum, R. solani,* and *P. sojae* (Zhang et al., 2011). In contrast, field application of chlorsulfuron to wheat growing in acidic soils reportedly has little impact on the severity of *Rhizoctonia* root rot (Wong et al., 1993). These results strongly suggest that farmers using sulfonylurea or other ALS-inhibiting herbicides should monitor crops growing in fields previously treated with ALS-inhibitors for disease symptoms, particularly in alkaline soils where herbicide residues are more likely to persist.

5.4.4. Triazine and other PSII inhibitors

When propazine was used continuously for 5 years as a pre-emergent herbicide in a large *Pinus radiata* nursery where the soils were not believed to be conducive to *Phyrophrhoru cinnamomi* root rot, disease appeared after 2 years and rapidly increased in intensity despite attempts to control it with fungicides. The disease and the fungus virtually disappeared within one season when chlorthal dimethyl replaced propazine (Marks and Cerra, 1991). Follow-up experiments found that propazine increased the numbers of spore-forming bacteria which appeared to stimulate sporangia formation by *Phytophthera cinnamomi*, and that the chemical may also have damaged root tissue and increased root susceptibility to infection. By contrast, chlorthal dimethyl had a negative effect on all microbiota and helped suppress *P. cinnamomi* (Marks and Cerra, 1991). It would be of interest to see if other triazine herbicides have similar effects, and to examine these potential mechanisms in other plant-pathogen systems.

In contrast, research into another PSII inhibitor, metribuzin, found that it had no effect on five selected plant-growth promoting organisms at conventional and higher rates (Myresiotis et al., 2012), and even stimulated the growth of the pathogen-inhibitory strain *Streptomyces corchorusii* (El-Shanshoury et al., 1996). The combination of metribuzin with the *Streptomyces* strain inhibited *Fusarium* and decreased disease incidence in tomatoes (El-Shanshoury et al., 1996).

5.4.5. Other herbicides

Three protoporphyrinogen oxidase inhibitor herbicides – azafenidin, sulfentrazone and flumioxazin – were investigated for their effects on *Pythium* root rot in sugarcane. All three herbicides inhibited the *in vitro* mycelial growth of *P. arrhenomanes*, *P. aphanidermatum*, and *P. ultimum*, but the effects in soil were inconsistent (Daugrois et al., 2005).

The application of diquat + paraquat, glyphosate and trifluralin (all at 1 mg kg^{-1}) to unsterilized field soil increased take-all caused by the fungus, *Gaeumannomyces grarninis* var. *tritici* Walker by 13.0%, 16.6% and 10.8% respectively, while no effect on disease was recorded in sterilized soil treated with the same herbicides. The herbicides tested had no effect on the saprophytic growth of the pathogen with the exception of glyphosate, which increased pathogen growth in unsterilized soil. The

application of diquat + paraquat and glyphosate to unsterile soil had no effect on the numbers of actinomycetes. The diquat+paraquat treatment, however, increased populations of fungi while the glyphosate decreased the numbers of bacteria. The proportion of soil fungi antagonistic to the pathogen was reduced in glyphosate-treated soil. Dicamba, chlorsulfuron and chlothal dimethyl did not influence pathogenicity (Mekwatanakarn and Sivasithamparam, 1987)

6. Additional considerations for impacts within agricultural systems

6.1. Active ingredient *versus* formulation

Commercial products may contain a wide variety of substances in addition to the active ingredient in order to improve stability, mixing, dilution and application (Tominack 2000). Some examples include solvents, surfactants, emulsifiers, dispersants, binders, wetting agents, fillers, preservatives or other compounds with specific functions (Tominack 2000). One of the more common hurdles in ecotoxicology is extrapolating the effects of an active ingredient (e.g. glyphosate) to the effects of a formulated product (e.g. Round Up ®). Systematic toxicological assessment of every component found within the range of herbicide products is too costly and time consuming; thus, researchers are usually restricted to comparing the active ingredient against one or more commercial formulations. Studies of this nature are reasonably common in human and aquatic toxicology, but those focussed on risks of herbicides to soil biota are relatively rare.

In one study, the herbicide formulation Callisto ® had about a 30% greater effect than its active ingredient mesotrione in reducing chlorophyll concentrations and decreasing the diversity of cyanobacterial populations in soil (Crouzet et al., 2013). In another study, soil treated with the formulated herbicides penoxulam or sulcotrione were more frequently avoided by earthworms than the corresponding rates of the active ingredients (Marques et al., 2009). Salminen et al. (1996) conducted dose-response experiments for terbuthylazine and its formulation Gardoprim ®. Terbuthylazine had no toxic effects on the soil animals tested (microbes, oppioid mites, two gamasid mite species, enchytraeids, and nematodes), whereas Gardoprim ® had acute toxic effects on enchytraeids, with a no-observed effect level (NOEL) of 10 kg ha^{-1}, and both

gamasid mites (NOEL 24 and 50 kg ha^{-1}). These three studies each suggest that formulated herbicide products are generally more toxic than their corresponding active ingredient. Although this concurs with the general findings from other ecotoxicological studies, there are always exceptions to this rule. For example, the formulation of glyphosate had marginal and inconsistent effects on the growth of five bacterial species (some isolated from soil) *in vitro* relative to the active ingredient (Sihtmäe et al., 2013).

6.2. Herbicide mixtures

Herbicide mixtures are often used for weed control in order to target multiple weed species with a single application. The sheer number of possible combinations of different herbicides makes an assessment of the toxicity of specific mixtures extremely difficult. Only very few studies have attempted to assess the toxicity of herbicide mixtures as compared with individual active ingredients. Das et al. (2011) found that single doses of bensulfuron-methyl or pretilachlor alone at conventional rates reduced both N_2O and CH_4 emissions; but when applied together, this effect was absent or was reversed. Lupwayi et al. (2009) also examined the effect of numerous herbicide combinations on bacterial diversity and substrate utilisation profiles in canola cropping systems. They found a number of significant differences, but could not make any general conclusions about why particular herbicide combinations caused greater disturbances.

6.3. Chronic effects of repeated applications

One of the biggest concerns for landholders and scientists is the possibility of a gradual decline in soil quality caused by long-term, repeated herbicide applications (Barman et al., 2014; Strom, 2013). Biological shifts may not be detectable in short-term laboratory or glasshouse experiments and a lack of control and investment in long-term field studies means potential effects may be overlooked or misattributed to other factors. Despite these difficulties, some data documenting the chronic effects of repeat applications are available.

To date, there is little evidence to suggest that long-term, repeat applications of glyphosate to soil causes negative shifts in soil microbial communities or functions. Biederbeck et al. (1997) found that long-term (21 years) of glyphosate or paraquat application in a wheat-fallow rotation had no deleterious effects on soil microbial populations (bacteria, actimonycetes, fungi, nitrifiers, denitrifiers), nor on microbial biomass or potential C or N mineralization. Long-term field monitoring of repeated glyphosate applications (9-13 yrs) at 3 kg ha^{-1} to pine plantations on three different soil types also revealed no detectable effects on basal respiration, metabolic quotient, total bacteria, metabolic diversity or mineralizable N (Busse et al., 2001). More recent investigations did not detect significant changes to microbial community structures in unplanted microcosms receiving six applications of glyphosate over 6 months (Lane et al., 2012) or in maize fields receiving annual glyphosate applications of 0.72 kg ha^{-1} over 3 years (Barriuso et al., 2011a). Perie and Munson (2000) observed that annual glyphosate (2 kg ha^{-1}) applications for 4 yrs reduced soil organic C by 46%, total N by 15%, and acid phosphatase activity by 64% in a forestry soil; but concluded that these impacts likely represented an indirect effect of reduced weed growth in topsoil.

There is some evidence to suggest that long term applications of atrazine can induce significant changes in the microbial population. Atrazine applied at 4 kg ha^{-1} annually for 24 years permanently reduced the numbers of anaerobic bacteria, spore-formers and cellulolytic microorganisms, and temporarily reduced the nitrifying, amylolytic and denitrifying microbial groups. Atrazine also temporarily enhanced numbers of ammonifying and proteolytic organisms and permanently increased the number of Azotobacter. As a result of the long-term elimination of the direct vegetative cover and the concomitant loss of organic matter in the atrazine-treated soil, the phosphatase, saccharase, β-glucosidase and urease activities of this soil were reduced by 50 % or more (Voets et al., 1974). Seghers et al. (2003) also found shifts in the bacterial community structure in soil under a maize monoculture after 18 years annual application of atrazine (0.75 kg ha^{-1}) and metolachlor (2 kg ha^{-1}). Targeted 16SrDNA PCR-DGGE showed that herbicide treated soil had a similar structure of Acidobacteria, ammonia oxidizers and actinomycetes, but three methanotrophic phylotypes were absent in chronically exposed soils. Interestingly, q-PCR and functional assays showed that the abundance and activity of methanotrophs was not affected, suggesting a functional resilience even though community structural changes

occurred. An additional study from the same plots showed that long-term atrazine application had not affected the endophytic bacterial community within the maize roots (Seghers et al., 2004). Long-term (7 years) field trials showed that soil from atrazine-treated maize plots had significantly higher microbial biomass carbon than most other plots in the final year of treatment, apparently due to increased levels of atrazine-tolerant weed spp dominating the plots (Wardle et al., 1999). In the same trial above, herbicides (atrazine or sulfonylureas) did not exert any consistent detrimental effects on nematode communities and the nematode fauna in the herbicide treated plots tended to have greater diversity (as indicated by the Shannon-Weiner index) than that in many of the other plots. Because effects were only apparent after at least 3 years, the authors recommended that to evaluate the relative effects of different agricultural practices in the long-term it is necessary to sample until the ecosystem has achieved some degree of equilibrium rather than monitoring only initial cropping cycles (Yeates et al., 1999). In a similar study on the impacts of ground vegetation management strategies in a kiwifruit orchard on the composition and functioning of the soil biota, most of the results could be explained by the fact that differences in the amount of basal resources were likely to be present, rather than other components of intensification such as cultivation or herbicide (simazine plus glyphosate) application (Wardle et al., 2001).

There is also evidence that repeat applications of sulfonylurea herbicide may impact on soil microbial communities, particularly those involved in N-cycling. Long-term (5 or 10 years) repeated application of chlorimuron-ethyl significantly reduced the number and diversity of N fixing and nitrifying bacteria. Denitrifyers were also reduced in number but increased in diversity. Higher herbicide residues were also detected in plots of repeat application, suggesting incomplete dissipation over a cropping season could be an indicator of chronic risks. Long-term (5 or 10 years) application of chlorimuron-ethyl to soybean fields also significantly reduced culturable bacteria and actinomycetes, but increased fungal counts. Application of chlorimuron-ethyl increased the prevalence of *F. oxysporum, R. solani,* and *P. sojae* (Zhang et al., 2011). Forestry plots receiving sulfometuron-methyl (0.057-0.113 kg ha^{-1}) treatments annually for 4 years had higher inorganic N, and extractable P, than the other treatments, suggesting higher mineralization rates without consequent immobilization e.g. by weeds in control plots (Arthur and Wang, 1999)

Only a single study reporting the effects of repeat applications of phenylurea herbicides could be found. In that study, annual application of diuron (2 kg ha^{-1}), diuron plus linuron (2 + 3 kg ha^{-1}) and chlorotoluron (5 kg ha^{-1}) for 10 years to an orchard significantly reduced culturable heterotrophs and altered the soil bacterial community structure as measured by16s rDNA DGGE and CLPP (El Fantroussi et al., 1999). These shifts in community structure equated to a loss, rather than a gain, in species diversity in all herbicide treated plots.

Data on the effects of repeated applications for other herbicide classes are also scarce. With respect to phenoxy-acid herbicides, five applications per year of 2,4-D (4.5 kg ha^{-1}) over a four year period decreased culturable bacteria in soils, but had no significant effect on culturable fungi or actinomycetes (Breazeale and Camper, 1970). However, Duah-Yentumi and Johnson (1986) found that repeat application of another phenoxy-herbicide, MCPA, at a rate of 1.68 kg ha^{-1} for 22 years, reduced the number of culturable actinomycetes but had no effect on other microbial groups, including aerobic and anaerobic bacteria, yeasts or fungi.

Similar inconsistencies have been found for repeat applications of dinitroaniline herbicides. Trifluralin applied annually for 4 years at 1.12 kg ha^{-1} decreased bacterial and fungal colonies but increased actinomycete counts (Breazeale and Camper, 1970). Conversely, Moorman and Dowler (1991) observed that repeat applications over 7 years of trifluralin (0.56 kg ha^{-1}) or alachlor (2.24 kg ha^{-1}) to soybean or maize monocultures, or both sequentially in crop rotation, did not have any consistent or lasting effects on culturable microorganisms. Moreover, the herbicide treated crops did not suffer from yield decline.

Repeated applications of herbicide mixtures to a canola (Glufosinate plus Clethodim)-barley (Tralkoxydim, Bromoxynil plus MCPA) rotation in Canada showed no significant effects in the first two years on MBC, CLPP diversity or β-glucosidase activity. A positive effect on diversity was observed in the 3rd year in canola, but minor negative effects on MBC, CLPP diversity or β-glucosidase were observed in some soils in 4th year (Lupwayi et al., 2010). The authors suggest cumulative effects

may be more important, but complex experiment design makes it difficult to determine.

Overall, it is difficult to make general conclusions about the long-term effect of repeated herbicide applications. As Zhang et al. (2011) proposed, the evolution of chronic effects after repeat herbicide applications could result from a build-up of herbicide residues. This hypothesis is supported by data from the study of Baxter and Cummings (2008). They found that repeated applications of bromoxynil (three applications each month) at 10 mg kg^{-1} fostered bromoxynil breakdown and did not significantly affect diversity, but repeat applications of 50 mg kg^{-1} inhibited breakdown and dramatically altered diversity as measured by DGGE. This suggests a threshold beyond which toxicity build-up may occur.

6.4. Comparison against other weed control systems

Because some form of weed control is commonplace to prevent yield loss in cropping systems, a fair assessment of the effect of herbicides on soil biota should also account for the potential impacts of other weed management strategies. The primary alternative to herbicides for weed control is the use of tillage.

Herbicide-treated plots growing maize or asparagus were not significantly different to hand-hoed or cultivated plots in terms of respiration or turnover of organic amendment (ryegrass litterbags) (Yeates et al., 1999; Wardle et al., 1993). In a 21 yr field experiment, Biederbeck et al. (1997) compared the impacts of a zero-tillage system using the herbicides glyphosate or paraquat against a conventional tillage system. They found that the zero-till/herbicide system had no long- or short-term effects on soil microbial populations or C or N-mineralization, whereas conventional tillage had a negative impact on most soil characteristics. Similarly, Carter et al. (2007) found that glyphosate effects on soil biological properties in a 3-year potato rotation were periodic, inconsistent and considered to be ecologically negligible compared to greater effects of tillage on soil structure. These authors further speculated that the periodic reductions in microbial activity were related to reduced plant biomass rather than direct effects of the herbicide on soil biota.

Simpfendorfer et al. (2002) also analysed yield declines in wheat under direct drilling with herbicides as compared with conventional tillage, and found that reduced yields were not related to herbicides but to root-inhibitory pseudomonads prevalent in undisturbed soil as compared with cultivated soil. In a broad assessment of the effect of herbicides relative to other management practices, Steenwerth et al. (2002) used multivariate analysis to analyse the microbial community profiles associated with different agricultural soils. They found that herbicide use was correlated with particular microbial PLFAs, but that these PLFA signatures were also strongly associated with fertiliser use and cultivation, suggesting an overall system effect rather than a specific herbicide-induced effect.

Overall, these results highlight the importance of considering the effects of herbicide use within a systems context. Although a particular herbicide may have not have a direct effect, *per se*, on soil biology and function, a no-till system within which it is used may result in large shifts in microbial communities compared to a soil that was previously cultivated on a routine basis.

6.5. Toxicity of herbicides versus other agricultural inputs

Although their use is increasing, herbicides represent only a part of the overall chemical inputs into most agricultural systems. It is therefore also of interest to compare the impacts of herbicides with those of other crop protection chemicals and fertilizers.

A number of studies directly comparing the non-target effects of herbicide with insecticides and fungicides have found that fungicides generally have more adverse impacts on soil microbiota. For example, Kumar et al. (2012) showed that whereas the herbicide pretilachlor increased MBC and respiration, the insecticides chlorpyrifos and cartap and the fungicide carbendazim inhibited respiration. An *in vitro* study found that fungicides (especially captan and mancozeb) were generally more toxic to rhizobial isolates than the herbicides atrazine, glyphosate, MCPA, paraquat, imazethapyr, linuron or metolachlor, as well as a number of insecticides (Drouin et al., 2010). Compared with the herbicide linuron, a fungicide mixture of mancozeb plus dimethomorph dramatically reduced counts of fungi and N-cycling bacteria

(Cycoń and Piotrowska-Seget, 2007). In another toxicity study, increasing concentrations up to 500 mg kg^{-1} showed no effect of chlorsulfuron or MCPA on respiration, whilst the fungicide propiconazole reduced respiration by 50% when applied at 500 mg kg^{-1} (Ahtiainen et al., 2003). Itoh et al. (2003) also found that a herbicide mixture (daimuron plus bensulfuron methyl) did not affect community level physiological profiles (Biolog) at 1, 10 and 50 times the conventional application rate, but a fungicide mixture of isoprothiolane plus flutolanil at 50 times the conventional application rate altered the microbial community for up to 1 month before recovery. Fungicides have also exhibited higher toxicity to soil mesofauna than herbicides. In one study, the fungicides benomyl and carbendazim were shown to induce avoidance behaviour in *Enchytraeus albidus* at lower concentrations than observed for the herbicide phenmedipham (Amorim, 2005).

Few studies have directly attempted to assess the effects of biocides, including herbicides, against the potential impacts of fertilizer inputs. Seghers et al. (2005) observed that fertilizer (organic vs mineral) had a much greater impact than herbicides (mixture of nicrosulfuron, atrazine and dimethenamide) on the abundance of methanotrophs and methane oxidation rates. However, although larger changes were apparent, it is difficult to assess these changes as being a positive or negative with respect to agricultural productivity or environmental sustainability. Muñoz-Leoz et al. (2012) found that the herbicide ethofumesate had a lower effect compared with an insecticide and fungicide at the same rate, and also compared with fertilizers (NPK and compost). According to their overall soil quality measure, these authors concluded that NPK fertilizer caused the biggest decline in soil quality.

7. Conclusion and Future Research Needs

It is clear that the impact of herbicides on soil biology and functionality is a complex issue. Although herbicides can be grouped according to chemical structure and mode of action, this does not guarantee that they will have similar impacts on soil organisms. Overall, the majority of papers reported negligible impacts of herbicides on soil microbial communities and beneficial soil functions when applied at recommended field application rates. This is in contrast to more frequent reports of altered population dynamics and microbial activities in soils receiving herbicide

inputs of 5-100 × recommended rates. Even still, in the majority of cases where negative effects were observed such effects usually only lasted for periods of less than 1-2 months, demonstrating structural and functional resilience to herbicide-induced disturbance. Furthermore, we found a large amount of variability and inconsistency between different studies on the same herbicide, implying that analytical methodologies and site-specific variables, such as soil type, climate, and soil biology, strongly influence the findings of each study.

Nevertheless, some exceptions to these general trends were apparent and these require further attention from scientists and farmers alike:

- There is some evidence that glyphosate and atrazine may disrupt the feeding behaviour and ecology of certain groups of earthworms, but it is unclear as to the relevance of these laboratory-based findings to field situations where their mobility would allow them to avoid undesirable herbicide concentrations;
- There is also evidence that some sulfonylurea herbicides can inhibit processes involved in N-cycling, and may thereby reduce plant-available N. This appears more likely to occur in alkaline soils where sulfonylurea degradation is slower or in instances where sulfonylurea herbicides are repeatedly applied, resulting in a build-up of residues. Considering that the imidazolinone herbicides act in the same manner, by inhibiting ALS synthase, attention should also given to monitoring the potential impacts of this herbicide class;
- A number of reports show that certain herbicides (e.g. glyphosate, propazine, sulfonylureas) can increase the incidence of disease, but such interactions tend to be site-specific and may not be widespread. More research needs to be done to elucidate the mechanisms by which these events occur. However, in the meantime, it is advisable that farmers maintain vigilance as to potential herbicide-disease interactions.

Aside from qualitatively characterising the hazards posed by herbicides to soil organisms and functions, this review also identified a number of knowledge gaps and issues regarding the framework by which herbicide risks are assessed and the relevance of laboratory-generated knowledge to agronomy and soil science in the field. One of the primary issues is that, unlike most terrestrial or aquatic

ecotoxicological studies involving higher plant and animals, traditional dose-response analyses for describing herbicide impacts on soil microbial communities may not be appropriate or sufficient for describing the environmental hazard. This is for a number of reasons.

First, standardised response variables or endpoints are lacking and single response variables are unlikely to capture all potential hazards. In the context of soil health and ecology, such variables can include measures of biological (community) structure or measures of function. Because numerous methodologies exist for both structural and functional characterisation of soil biological communities, there is a major lack of consistency between studies, which makes interpretation and generalisation of results difficult. Moreover, because the role and ecology of the majority of microbial taxa remain unexplored, we currently lack the means to extrapolate measures of microbial community structure to specific ecosystem and agronomic functions; and vice versa.

Second, even if significant shifts in a microbial community occur in response to a herbicide input, functional redundancy within the community means that such shifts may not translate to a loss of function (that is, the soil exhibits a functional resistance). Compounding this fact is that the pace of physiological and evolutionary adaptation of microorganisms to disturbance is much faster than in higher ecological systems, so that an ecological disturbance caused by repeated herbicide inputs may be overcome or circumvented (that is, the soil exhibits a functional resilience). On the other hand, there is a possibility that non-significant community or functional shifts may still decrease the *capacity* for further resistance or resilience against other stressors, as has been observed for higher organisms (e.g. Bandow et al., 2014). To our knowledge, this issue has not been directly assessed with reference to herbicides and requires attention.

Third, what is the relevance of changes to structural and functional response measurements? Certain response measures, particularly those which are general descriptors of microbial diversity or microbial activity, such as respiration, dehydrogenase activity or hydrolase activity, provide ambiguous evidence for ‘negative’ impacts to soil health. In many cases it cannot be determined whether increases or decreases in these measures are a consequence of stress towards

microbial cells; increased turnover of microbial cells due to cell death and lysis; specific degradation of the herbicide input; or potential priming and increased degradation of indigenous organic matter. Depending on the agronomic situation, a reduction in a particular function may be beneficial or detrimental. A prime example is that of nitrification: under high nitrogen loading, inhibition of nitrification is sometimes desirable in order to reduce subsequent leaching or denitrification loss of N. In comparison, inhibition of nitrification under low soil nitrate availability can reduce crop N uptake and productivity.

An additional constraint to our understanding of the impact of herbicides on soil functions contributing to crop/pasture productivity is that it is difficult to translate the results of short-term, spatially-confined laboratory experiments into a longer-term, field perspective. Many of the response indicators used to assess soil health are 'snapshot' measures, such as enzyme activities, that are only measured periodically at (usually) arbitrary time points. Since microbial dynamics vary on timescales of hours to days, it is difficult to assess the impact of a short-term stimulation or inhibition over a cropping season or longer. For example, conclusions from short experiments on the impact of a herbicide thoroughly mixed through soil and maintained at constant water content cannot be directly applied to field conditions where the same herbicide is applied to the soil surface, which has a stratified organic matter profile and soil moisture is highly variable both spatially and temporally. If negative impacts only occur in the zone where the herbicide is present in damaging concentrations (which may only be in the top few mm of soil for some herbicides) then does this really influence the capacity of the soil as whole to support plant productivity? Alternatively, is the impact at a time critical to growth and nutrient supply? Hazards identified in dose-response studies therefore need to be translated to risks in the field, via modelling that incorporates temporal and spatial aspects, such as herbicide redistribution and dissipation, microbial population evolution and adaptation, and physico-chemical changes within the soil profile.

There is therefore a strong need for a consistent framework for assessing meaningful endpoints to agricultural production systems. Such a framework needs to integrate the extent and duration of disruption to critical processes. We recommend that future studies in this area of research should:

- Report the concentration of herbicide applied to soil as both a field rate (i.e. kg herbicide ha^{-1}) and a mass concentration (i.e. mg herbicide kg^{-1} soil);
- Utilize a range of techniques to give a comprehensive picture given the inherent bias in some techniques for determining changes in soil biological community structure and function;
- Aim to better understand the effect of commercial formulations relative to active ingredients;
- Attempt to link the findings of laboratory incubations to field situations through additional semi-field or field studies, or complementary modelling to extrapolate impacts to realistic field scenarios.

It is our intention that this review, although not fully exhaustive, provides a rigorous starting point from which future studies can improve our understanding of the potential impacts of herbicides on soil biology and function.

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Tables

Table 1. Major herbicide classes, model compounds and their application rates. Explanations for the modes of action are given in the text.

Herbicide Class	Mode of action	Example herbicides	Typical label application rate (kg ha^{-1})	Predicted concentration in soil (mg kg^{-1}) [a]	Sorption coefficient (K_{oc}) [b]	Persistence (half-life in days) [b]
Glycine	Inhibition of enolpyruvlshikimate-3-phosphate (EPSP) synthase	Glyphosate	2.2	2.9	1435	12
Chloroacetimide	Inhibition of very long chain fatty acid synthesis (cell division)	Metolachlor	2.9	3.9	120	90
		Acetochlor	5.8	7.7	156	14
Sulfonylurea	Inhibition of acetolactate synthase (branched chain amino acid synthesis)	Chlorosulfuron	0.02	0.027	36.3	160
		Metsulfuron	0.005	0.007	92	66
Triazine	Inhibition of photosynthesis at photosystem II	Atrazine	3	4	100	75
		Simazine	2	2.7	130	60
Phenoxy-carboxylic acids	Synthetic auxins	2,4-D	1.1	1.5	4.4	39.3
		MCPA	1.1	1.5	74	24
Ureas, Amides	Inhibition of photosynthesis at photosystem II	Diuron	1	1.33	813	75.5
		Propanil	6	8	152	0.4
Dinitroaniline	Inhibition of microtubule assembly	Trifluralin	1	1.33	15800	181
		Pendimethalin	1.5	2	17581	90
Imidazolinone	Inhibition of acetolactate synthase (branched chain amino acid synthesis)	Imazethapyr	0.1	0.133	52	90
		Imazamox	0.035	0.047	67	25

[a] Assuming a bulk density of 1.5 g cm^{-3} and a herbicide distribution in the top 50 mm of soil (European Economic Community, 2007).

[b] Sorption and degradation data sourced from PPDB (2015)

Table 2. Culture-independent methods for assessing soil microbial community biomass and diversity (adapted from Rincon-Florez et al. 2013). Methods include Chloroform Fumigation-Extraction (CFE); Phospholipid Fatty Acid Analysis (PLFA); Quantitative PCR (Q-PCR); Denaturing Gradient Gel Electrophoresis (DGGE); Temperature Gradient Gel Electrophoresis (TGGE); Single-Strand Conformation Polymorphism (SSCP); Terminal Restriction Fragment Length Polymorphism Fingerprinting (T-RFLP); Automated Ribosomal Intergenic Spacer Analysis (ARISA/RISA); Length-Heterogeneity PCR (LH-PCR); Random Amplified Polymorphic DNA (RAPD); Amplified Ribosomal DNA Restriction Analysis (ARDRA); Fluorescence In Situ hybridization (FISH).

Endpoint	Method	Advantages	Disadvantages
BIOMASS	**CFE**	- Measurements of microbial biomass can be done in recently added and freshly decomposed substrates	-Clay soils may need to be corrected for the amount of chloroform C added to assess the concentration of biomass C
	PLFA	-Sensitive detection and accurate quantification of different microbial groups -Rapid and efficient -Useful information on the dynamics of viable bacteria -Reproducible	-Time consuming -Low number of samples can be treated at the same time
	Q-PCR	-Quick, accurate and highly sensitive method for sequence quantification that can also be used to quantify microbial groups -Relatively cheap and easy to implement -Specific amplification can be confirmed by melting curve analysis.	-Can only be used for targeting of known sequences. -DNA impurities and artefacts may create false-positives or inhibit amplification.
COMMUNITY STRUCTURE AND DIVERSTIY	**DGGE/ TGGE**	-Sensitive to variation in DNA sequences -Bands can be excised, cloned and sequenced for identification	-Time consuming -Multiple bands for a single species can be generated due to micro-heterogeneity -Can be used only for short fragments - Requires optimisation to obtain good separation of DNA from complex communities - Limited to dominant community members
	SSCP	-Community members can be identified	-Short fragments -Lack of reproducibility

		-Screening of potential variations in sequences -Helps to identify new mutations	-Several factors like mutation and size of fragments can affect the sensitivity of the method
	T-RFLP	-Enables analyses of a wide array of microbes -Highly reproducible	-Artefacts might appear as false peaks -Distinct sequences sharing a restriction site will result in one peak. -Unable to retrieve sequences
	RISA/ ARISA	-High resolution when detecting microbial diversity -Quick and sensitive	-More than one peak could be generated for a single organisms -Similar spacer length in unrelated organisms may lead to underestimations of community diversity
	LH-PCR	-Results are reproducible -Easy and rapid -Efficient and reliable	-Limited by the bacterial species known in public databases -Not enough information is available for fragment length on databases to compare LH-PCR lengths with environmental microorganisms.
	RAPD	-Suitable for unknown genomes -Requires low quantities of DNA. -Efficient, fast and low cost	-Low reproducibility -Sensitive to reaction conditions
	ANDRA	-Highly useful for detection of structural changes in simple microbial communities -No special equipment required	-More applicable to environments with low complexity -Several restrictions are needed for adequate resolution -Labour- and time-intensive -Different bands can belong to the same group
	FISH	-Allows detection and spatial distribution of more than one samples at the same time	-Autofluorescence of microorganisms -Accuracy and reliability is highly dependent on specificity of probe(s)
	DNA ARRAY	-Analyzes a vast amount of genetic information simultaneously -Requires the construction of an array and access to a scanner	-Issues with specificity/cross hybridization -Requires normalization -Sensitivity and reproducibility can be problematic -Limited to known gene sequence probes and presence on the array
	Next Generation	-Rapid method to assess biodiversity and abundance of many species/organizational taxonomic units simultaneously	-Relatively expensive -Replication and statistical analysis are

	Sequencing (16S rRNA amplicon sequencing)	and at a considerable depth compared to the methods that have been available so far	essential -Computational intensive -Challenging in terms of data analysis
	Next Generation Sequencing (meta-genomics)	-Biodiversity can be studied in more detail -Captures polymorphism in microbial communities -Reveals the presence of thousands of microbial genomes simultaneously -Provides information about the functions of microbial communities in a given environment	-High cost -Data analysis is challenging and time consuming -Difficult to use for low-abundance communities. -High biodiversity in soil leads to many incomplete genomes -Current sequencing methods and computing power still in its infancy relative to the high biodiversity in soil

Table 3. Methods for assessing soil microbial community function (adapted from Rincon-Florez et al. 2013). Abbreviations are fluoroscein diacetate (FDA), dehydrogenase (DHA), Quantitative polymerase chain reaction (qPCR) and Ribonucleic acid (RNA).

Endpoint	Method	Advantages	Disadvantages
FUNCION	**Enzyme activity assays (FDA, DHA)**	-Low-cost, easy and fast method to measure microbial activity for soil samples -High sensitivity to changes in the soil environment	-Enzyme activities can be contaminated by external sources, e.g. plant matter -Limited substrate availability with a bias towards hydrolytic enzymes
	qPCR	-Quick, accurate and highly sensitive method for quantification of functional genes -Relatively cheap and easy to implement	-Can only be used for targeting of known sequences. -DNA impurities and artefacts may create false-positives or inhibit amplification.
	Functional Gene Arrays (RNA-based)	-Analyzes a vast amount of genetic information simultaneously	-Requires the construction of an array and access to a scanner -Issues with specificity/cross hybridization -Requires normalization -Insufficient sensitivity and reproducibility can be problematic -Limited by the presence of probes on the array -Issues with RNA extraction from soil
	Next Generation Sequencing (Metatranscriptomics)	-Allows rRNA and/or mRNA profiling and quantification without prior knowledge of sequence -Provides a snapshot of microbial transcripts at the time of sampling that may allow deduction of microbial ecosystem function -Helps to understand the response of microbial communities to changes in their environment	-Many issues with isolation of RNA from soil -mRNA isolation and often amplification are required for gene expression analyses -Current sequencing methods, data bases and computing power are not sufficient yet to cover the high biodiversity in soil.

Table 4. Summary of studies investigating the effect of glyphosate on soil microbial biomass. NR indicates not reported. Arrows indicate a statistically significant (P<0.05) increase (↑) or a decrease (↓) in biomass relative to control (no herbicide) treatments at a specific time point (d = days) after application. Treatments causing an effect of extended duration are reported as having an effect *until* a specific time point after application.

Application rate (*cf.* average agricultural rate of 2.2 kg ha^{-1})	**Soil organic matter (%)**	**Soil pH**	**History of glyphosate application**	**Total Microbial Biomass**	**Bacteria**	**Fungi**	**Actinomycetes**	**Reference**
> 2.2 kg ha^{-1}	7	6.5	NR	NR	↑, *until* 30 d	No effect	No effect	Wardle and Parkinson (1990)
	3.5	7	NR	NR	↑, *until* 108 d	NR	NR	Sihtmäe et al. (2013)
	1.9	6.2	NR	No effect	NR	NR	NR	Liphadzi et al. (2005)
	2.79	6.9	Yes	↓, 4 d; No effect, 45 d	NR	NR	NR	Gomez et al. (2009)
< 2.2 kg ha^{-1}	7	6.5	NR	NR	No effect	No effect	No effect	Wardle and Parkinson (1990)
	3.5	7	NR	NR	↑, *until* 21 d	NR	NR	Sihtmäe et al. (2013)
	2.3	5.7	No	NR	No effect	No effect	↑, 32 d	Araújo et al. (2003)
	2.3	5.9	Yes	NR	↑, 32 d	No effect	No effect	Araújo et al. (2003)
	2.6	5.6	No	NR	No effect	No effect	No effect	Araújo et al. (2003)
	2	5.2	Yes	NR	No effect	No effect	No effect	Araújo et al. (2003)
	1.9	6.2	NR	No effect	NR	NR	NR	Liphadzi et al. (2005)
	2.79	6.9	Yes	No effect, 4 d; ↑, 45 d	NR	NR	NR	Gomez et al. (2009)
	2.4	3.8	NR	No effect	No effect	No effect	No effect	Stratton and Stewart (1992)
	35.6	5.2	NR	No effect	NR	NR	NR	Houston et al. (1998)
	3.4	4.8	NR	No effect	NR	NR	NR	Houston et al. (1998)
	NR	NR	NR	NR	↓, 180 d	↓, 60 d	NR	Chakravarty and Chatarpaul (1990)

Table 5. Studies investigating the effect of sulfonylurea herbicides on soil microbial activity. NS = no statistically significant effect (P>0.05) relative to control (no herbicide) treatments.

Level with respect to conventional application rate	Herbicide	Application rate (µg kg^{-1})	Function	Effect	Duration	Soil Organic Carbon (%)	Soil pH	Reference
1×	Bensulfuron-methyl	16	Respiration	NS	-	2.3	7.7	Gigliotti et al. (1998)
1×	Bensulfuron-methyl	16	Respiration	NS	-	0.7	5.5	Gigliotti et al. (1998)
1×	Metsulfuron-methyl	10	Respiration	NS	-	1.53	6.06	Zabaloy and Gómez (2008)
1×	Metsulfuron-methyl	10	Respiration	NS	-	2.13	7.44	Zabaloy and Gómez (2008)
1×	Cinosulfuron	137	Respiration	NS	-	1.29	7.25	Sofo et al. (2012)
1×	Prosulfuron	21	Respiration	Decrease	30 d	1.29	7.25	Sofo et al. (2012)
1×	Thifensulfuron-methyl	4	Respiration	Increase	30 d	1.29	7.25	Sofo et al. (2012)
1×	Triasulfuron	14	Respiration	Decrease	30 d	1.29	7.25	Sofo et al. (2012)
1×	Nicosulfuron	300	Respiration, dehydrogenase	NS	-	1.9 [a]	7.1	Radivojević et al. (2012)
5×	Nicosulfuron	1500	Respiration, dehydrogenase	NS	-	1.9 [a]	7.1	Radivojević et al. (2012)
10×	Bensulfuron-methyl	160	Respiration	NS	-	2.3	7.7	Gigliotti et al. (1998)
10×	Bensulfuron-methyl	160	Respiration	NS	-	0.7	5.5	Gigliotti et al. (1998)
10×	Metsulfuron-methyl	100	Respiration	NS	-	1.53	6.06	Zabaloy and Gómez (2008)
10×	Metsulfuron-methyl	100	Respiration	Decrease	> 40 d	2.13	7.44	Zabaloy and Gómez (2008)
10×	Cinosulfuron	1370	Respiration	Increase	30 d	1.29	7.25	Sofo et al. (2012)
10×	Prosulfuron	210	Respiration	Increase	30 d	1.29	7.25	Sofo et al. (2012)
10×	Thifensulfuron-methyl	40	Respiration	Increase	30 d	1.29	7.25	Sofo et al. (2012)
10×	Triasulfuron	140	Respiration	Increase	30 d	1.29	7.25	Sofo et al. (2012)
10×	Nicosulfuron	3000	Respiration, dehydrogenase	Increase, decrease	10 d	1.9 [a]	7.1	Radivojević et al. (2012)
10×	Triasulfuron	200	Respiration, dehydrogenase	NS	-	1.3	6.5	Dinelli et al. (1998)
10×	Primisulfuron-methyl	200	Respiration, dehydrogenase	NS	-	1.3	6.5	Dinelli et al. (1998)
10×	Rimsulfuron	200	Respiration, dehydrogenase	NS	-	1.3	6.5	Dinelli et al. (1998)

100×	Metsulfuron-methyl	1000	Respiration	NS	-	1.53	6.06	Zabaloy and Gómez (2008)
100×	Metsulfuron-methyl	1000	Respiration, dehydrogenase, hydrolase	NS	-	2.05	6.06	Zabaloy et al. (2008)
>100×	Bensulfuron-methyl	5000	Respiration	Decrease	1-7 d	2.49	6.64	Hou et al. (2009)
>100×	Bensulfuron-methyl	5000	Respiration	Decrease	1-7 d	2.2	4.96	Hou et al. (2009)
>100×	Triasulfuron	5000	Respiration, dehydrogenase	Increase	50 d	1.3	6.5	Dinelli et al. (1998)
>100×	Primisulfuron-methyl	5000	Respiration, dehydrogenase	Increase	40 d	1.3	6.5	Dinelli et al. (1998)
>100×	Rimsulfuron	5000	Respiration, dehydrogenase	Increase	15 d	1.3	6.5	Dinelli et al. (1998)

[a] converted from reported organic matter %, using a factor of organic matter/organic carbon = 1.75.

Table 6. Impact of herbicides on phosphatase activity in soils from published laboratory incubation studies

Reference	Soil type	Herbicide	Application rate (mg kg^{-1})	Time frame	Impact on phosphatase activity	Comments
Perucci et al. 2000	Vertic Aquic Ustorthent	rimsulfuron	5	30 d	Significant decline in acid and alkaline phosphatase activity at 7d (10-15%) but no differences at 14 and 30 d	
			50	30 d	No effect on acid phosphatase activity but caused a significant increase in alkaline phosphatase activity, but only at 7d (5-10%)	
		imazethapyr	16.7	30 d	Acid phosphatase activity declined significantly at 7 d and 30 d (around 30%) but not at 14 d, while alkaline phosphatase activity declined significantly at 14 d and 30 d (5-10%).	
			167	30 d	Significant increase in acid and alkaline phosphatase activity at 7 d, 14 d ad 30 d (20-60%)	
Cycoń et al. 2013	Orthic Luvisol	napropamide	2.25	28 d	Significantly reduced acid and alkaline phosphatase activity by around 5% and 10%, respectively, at 1 d after application but no difference by 14 d after application	
			22.5	28 d	Significantly reduced acid and alkaline phosphatase activity by around 20% and 40%, respectively, at 1 d after application, and these differences were sustained at 14 d and 28 d after application	
Sofo et al. 2012	Vertic Ustorthens	cinosulfuron	137	30 d	No reduction in alkaline or acid phosphatase activity at 30 d	Rates in paper were given as 350, 55, 10 and 37 g ha^{-1} for cinosulfuron, prosulfuron, thifensulfuron methyl and triasulfuron, respectively. The authors did not state the actual amounts applied to the incubated soils but stated that the rates were calculated assuming an even distribution of the herbicides in the 0–20 cm layer (bulk density of 1.28 g cm^{-3}). We have calculated rates in µg/g soil based on
			1370	30 d	Significant (around 20%) reduction in both alkaline and acid phosphatase activity at 30 d	
		prosulfuron	55	30 d	No reduction in alkaline or acid phosphatase activity at 30 d	
			550	30 d	No reduction in acid phosphatase activity, but significant increase in alkaline phosphatase activity (5%) at 30 d	
		thifensulfuron methyl	10	30 d	Significant increase in alkaline (around 7%) and acid (around 12%) phosphatase activity at 30 d	
			100	30 d	Significant increase in alkaline (around 7%) and acid (around 5%) phosphatase activity at 30 d	
		triasulfuron	37	30 d	Significant increase in alkaline phosphatase activity (around 2%) but no effect on acid phosphatase activity at 30 d	
			370	30 d	Significant increase in alkaline (around 5%) and acid (around	

					10%) phosphatase activity at 30 d	these numbers and have assumed that the rates referred to g of active ingredient.
Wang et al., 2007	phaeozem	butachlor	5	28 d	No significant effect on acid phosphatase activity	We have assumed concentrations given referred to active ingredient.
			10	28 d	Significant reduction (around 20%) in acid phosphatase activity	
			50	28 d	Significant reduction (around 60%) in acid phosphatase activity	
Wang et al., 2009	Not stated	butachlor	50	28 d	No significant effect on acid phosphatase activity	We have assumed concentrations given referred to active ingredient.
			100	28 d	Significant reduction (around 30%) in acid phosphatase activity	
Xia et al., 2011	Not stated	butachlor	5	21 d	Reduction in acid phosphatase activity of about 5% after 1 d to about 8 % after 21 d, but no significance was tested.	We have assumed concentrations given referred to active ingredient.
			10	21 d	Reduction in acid phosphatase activity of about 7% after 1 d to about 20 % after 21 d, but no significance was tested.	
			50	21 d	Reduction in acid phosphatase activity of about 10% after 1 d to about 30 % after 21 d, but no significance was tested.	
			100	21 d	Reduction in acid phosphatase activity of about 25% after 1 d to about 35 % after 21 d, but no significance was tested.	
Rasool et al., 2014	Not stated	butachlor	23	35 d	Under flooded conditions alkaline phosphatase activity was significantly reduced at 28 d but was not different to the control at 14, 21 and 35 d. Under aerobic conditions alkaline phosphatase activity was significantly increased at 14, 21 and 28 d, but no effect by 35 d.	Herbicide rates were applied at 23 µg AI/g soil, 230 µg AI/g soil and 23 mg AI/g soil representing 1x, 10x and 100x field rates, respectively where 1x is the equivalent 1.5 kg active ingredient/ha assuming a uniform distribution of the chemical in the top 0-10 cm of soil (bulk density of 1.3 g cm^{-3}). However, the rate of 25mg AI/g soil is actually 1000x field rate and we presume that 25 mg AI/g soil was actually added rather than the true 100x rate.
			230	35 d	Under flooded conditions alkaline phosphatase activity was significantly higher at 14 d, significantly lower and 21 and 28d, and not different to the control at 35 d. Under aerobic conditions alkaline phosphatase activity was significantly higher than control at 14 d and 28 d and not significantly different from control soil at 21 and 35d.	
			2300	35 d	Under flooded conditions alkaline phosphatase activity was significantly higher at 14 d and 21d, significantly lower and 28 and not different to control soil at 35 d. Under aerobic conditions alkaline phosphatase activity was not significantly higher than control at 14 d and 35 d and but was significantly higher than control soil at 21 and 28d.	

Figure Legends

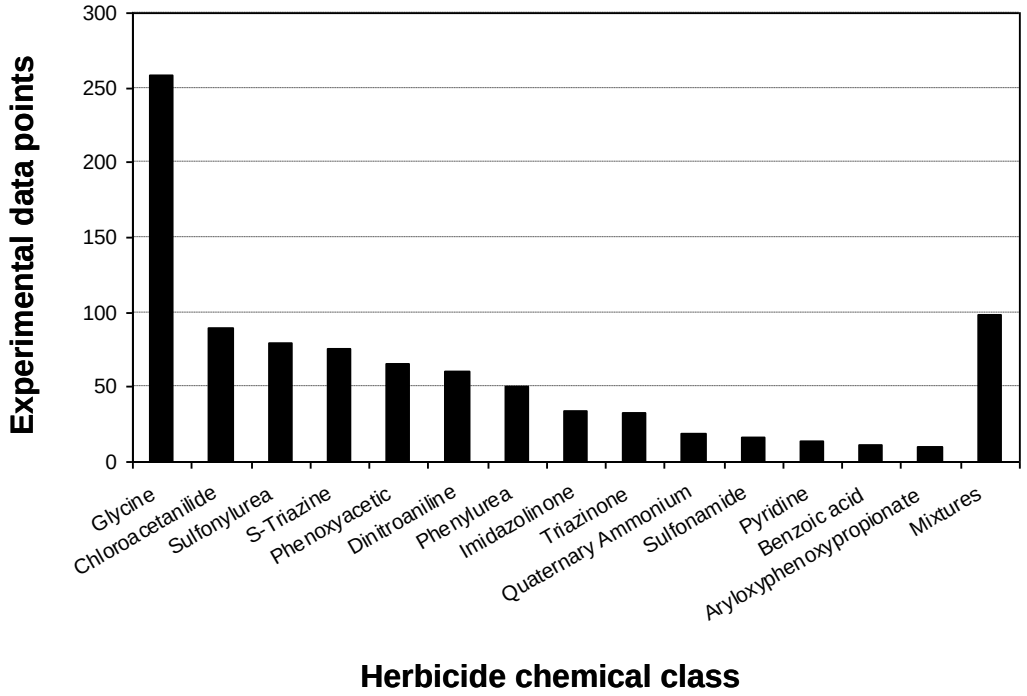


Figure 1.Frequency of experimental studies on different herbicide classes returned from a search of the database Scopus, using the search terms *herbicide* AND *soil* AND (*microb** OR *function**)Figure 1. Resistance mechanisms by which microorganisms may avoid or overcome herbicide toxicities caused by enzyme inhibition (as represented by the conversion of substrates to products). Red hexagons indicate active herbicide, whilst green shapes represent inactive herbicides, herbicide metabolites or bypass systems.

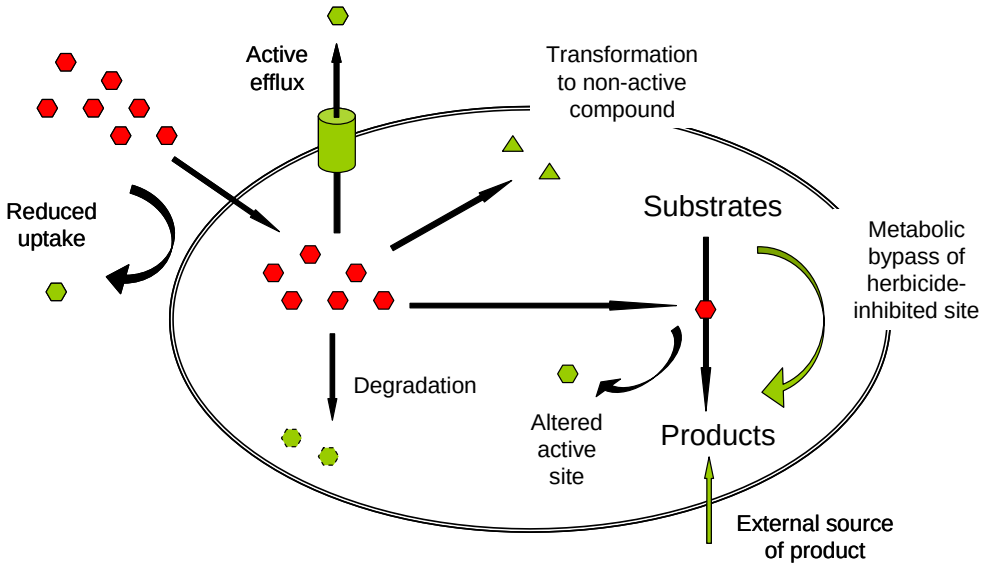


Figure 2. Resistance mechanisms by which microorganisms may avoid or overcome herbicide toxicities caused by enzyme inhibition (as represented by the conversion of substrates to products). Red hexagons indicate active herbicide, whilst green shapes represent inactive herbicides, herbicide metabolites or bypass systems.

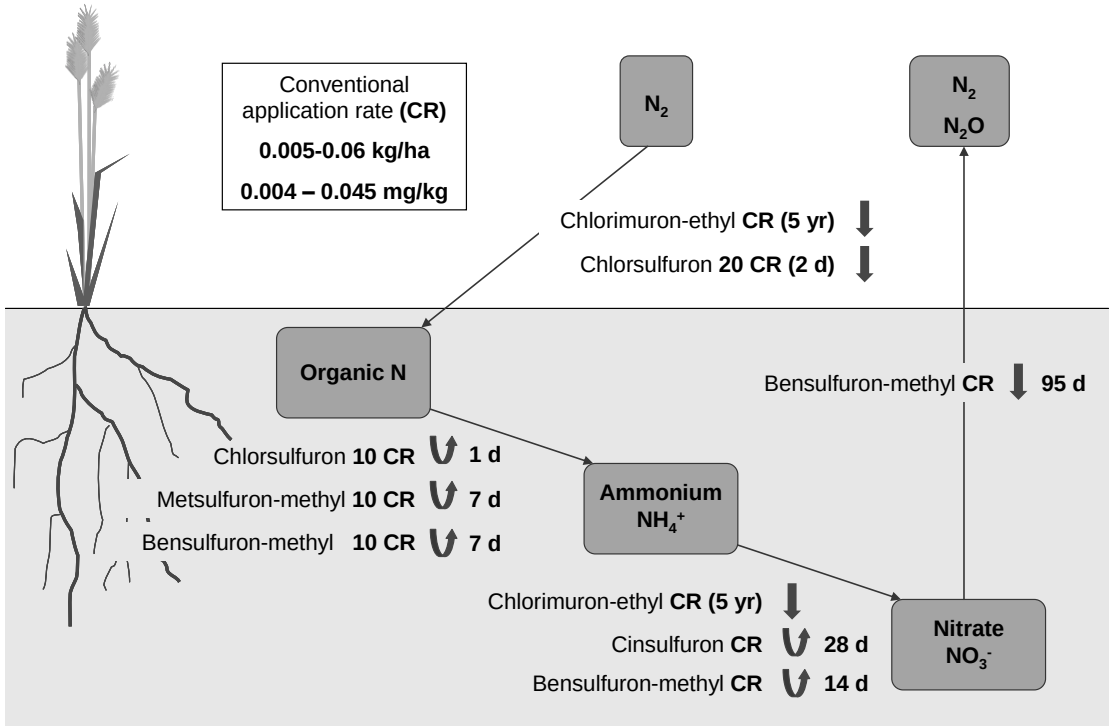


Figure 3. Documented effects of different sulfonylurea herbicides on processes involved in soil nitrogen cycling. Numbers in brackets after the herbicide name and application rate indicate repeated applications over that time frame. Down arrows indicate decreases in function with no return to control levels within the experimental timeframe, whereas convex arrows indicate a temporary decrease in function over the time period specified after the arrow.

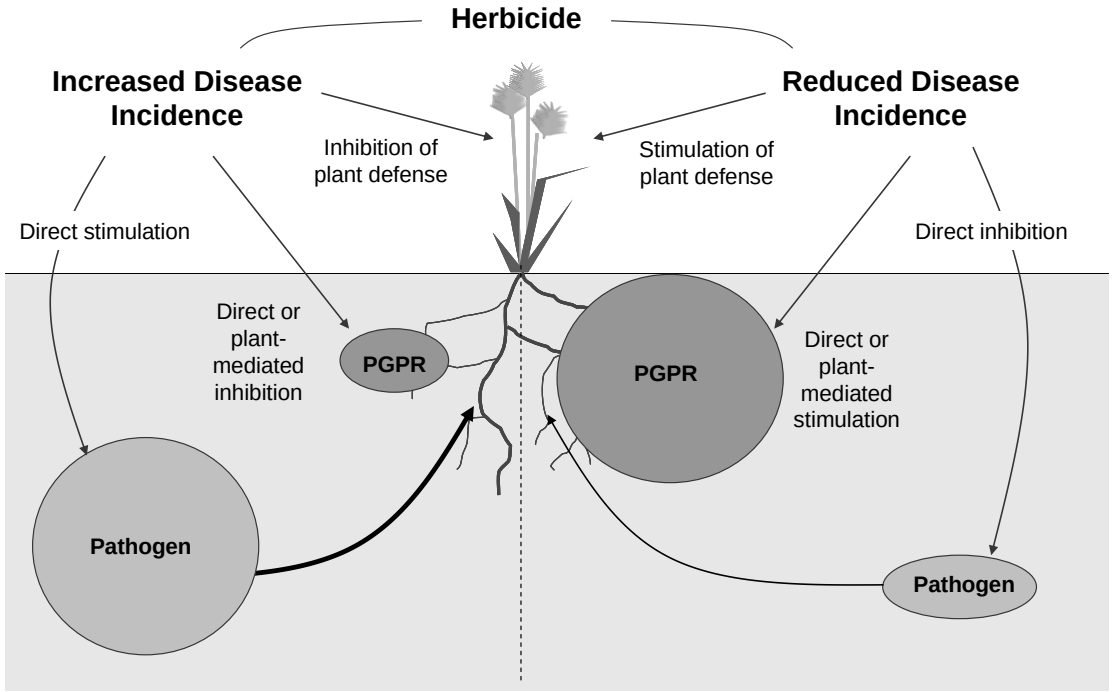
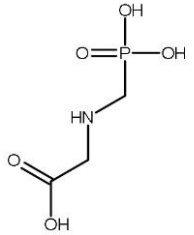
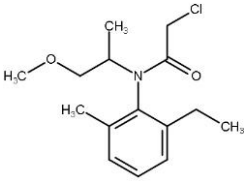
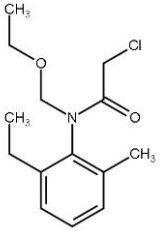
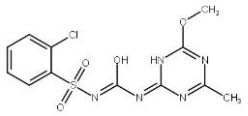
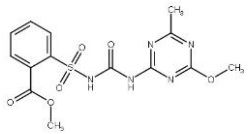
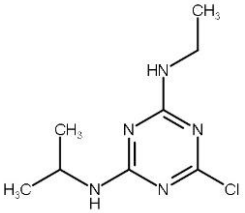
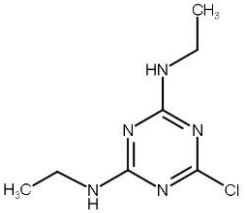
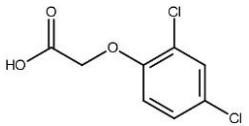
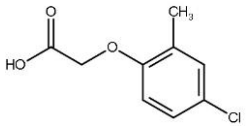
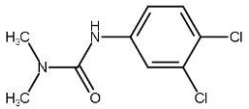
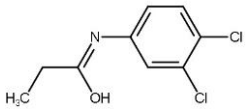


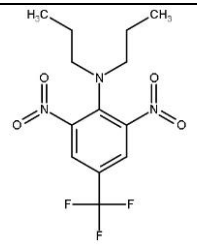
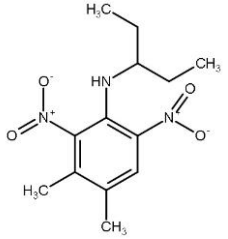
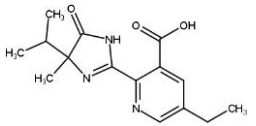
Figure 4. Potential mechanisms by which herbicides can impact on the incidence of disease in cropping systems. PGPR = plant growth promoting rhizobacteria.

Appendix 1 – Chemical structures and IUPAC names of commonly used herbicides.

Herbicide Class	Example herbicides	IUPAC name	Chemical structure
Glycine	Glyphosate	2-[(phosphonomethyl)amino]acetic acid	
Chloroacetimide	Metolachlor	2-chloro-N-(2-ethyl-6-methylphenyl)-N-(1-methoxypropan-2-yl)acetamide	
	Acetochlor	2-chloro-N-(ethoxymethyl)-N-(2-ethyl-6-methylphenyl)acetamide	

Sulfonylurea	Chlorsulfuron	N'-(2-chlorobenzenesulfonyl)-N-(6-methoxy-4-methyl-1,2-dihydro-1,3,5-triazin-2-ylidene)carbamimidic acid	
	Metsulfuron-methyl	methyl 2-({[(4-methoxy-6-methyl-1,3,5-triazin-2-yl)carbamoyl]amino}sulfonyl) benzoate	
Triazine	Atrazine	6-chloro-N2-ethyl-N4-(propan-2-yl)-1,3,5-triazine-2,4-diamine	
	Simazine	6-chloro-N2,N4-diethyl-1,3,5-triazine-2,4-diamine	

Phenoxy-carboxylic acids	2,4-D	2-(2,4-dichlorophenoxy)acetic acid	
	MCPA	2-(4-chloro-2-methylphenoxy)acetic acid	
Ureas, Amides	Diuron	1-(3,4-dichlorophenyl)-3,3-dimethylurea	
	Propanil	N-(3,4-dichlorophenyl)propanimidic acid	

Dinitroaniline	Trifluralin	2,6-dinitro-N,N-dipropyl-4-(trifluoromethyl)aniline	
	Pendimethalin	3,4-dimethyl-2,6-dinitro-N-(pentan-3-yl)aniline	
Imidazolinone	Imazethapyr	5-ethyl-2-[4-methyl-5-oxo-4-(propan-2-yl)-4,5-dihydro-1H-imidazol-2-yl]pyridine-3-carboxylic acid	
	Imazamox	5-(methoxymethyl)-2-[4-methyl-5-oxo-4-(propan-2-yl)-4,5-dihydro-1H-imidazol-2-yl]pyridine-3-carboxylic acid