Interactions between arbuscular mycorrhizal fungi and soil greenhouse gas fluxes

Kate Elizabeth Storer

PhD

The University of York

Biology

September 2013

Abstract

Arbuscular mycorrhizal fungi (AMF) can form a mutualistic symbiosis with over two-thirds of all land plants, providing phosphorus and/or nitrogen in exchange for carbon. They can have a significant effect on the surrounding soil, altering pH, water content, structure, and drainage. Important greenhouse gases (GHG) including carbon dioxide (CO_2), methane (CH_4) and nitrous oxide (N_2O) can be influenced by these factors, yet to date the interactions between AMF and soil GHG fluxes are surprisingly understudied.

A microcosm system was developed to study GHG fluxes in the presence and absence of AMF hyphae. A central compartment contained an AMF host plant (*Zea mays* L.), with two outer compartments, that either allowed (AMA) or prevented (NAMA) AMF hyphal access. Organic matter patches of dried, milled, *Z. mays* leaves mixed with soil were added to the outer compartments to encourage proliferation of AMF hyphae and GHG production. Soil-atmosphere fluxes of N_2O, CO_2 and CH_4 from the outer compartments were quantified, and gas probes were developed to measure N_2O concentrations within the organic matter patches.

Data from a series of microcosm experiments provide evidence for AMF interactions with soil fluxes of N_2O and CO_2, but not CH_4. Soil CO_2 fluxes were found to be a useful non-invasive method for determining the presence of AMF in hyphal compartments. The N_2O concentrations in organic patches decreased in AMA treatments, and a subsequent experiment demonstrated that N_2O production by nitrifiers may be limited in the presence of AMF hyphae. In contrast, following harvesting, N_2O fluxes from organic matter patches were higher in the AMA treatment; possibly because carbon release from severed AMF hyphae fuelled denitrification. These interactions have important implications for N cycling and sustainable agriculture. The evidence presented in this thesis suggests that AMF may play a previously unappreciated role in reducing soil-atmosphere losses of N_2O.

Table of Contents

List of Figures

List of Tables

List of Equations

Acknowledgements

I would like to thank my supervisors Phil Ineson and Angela Hodge for their invaluable help and advice throughout my PhD. They have made my PhD enjoyable while helping me to develop as an independent researcher, and I am very grateful for the time, effort and support that they have provided over the last four years. I would also like to thank my training panel members, Alastair Fitter and James Chong who have helped to direct my research and provided excellent discussions. I would like to thank Aisha Coggan for her time, enthusiasm, effort and support. I would also like to thank the Biotechnology and Biological Sciences Research Council for providing the funding to support my PhD studies.

I have been very lucky to be part of the knowledgeable, fun and supporting D0 lab group. The extended lab members both past and present have all been very supportive, and have provided excellent advice and discussions. I'd like to thank Thorunn Helgason, Sue Hartley, Kelly Redeker, Sylvia Toet, Andreas Heinemeyer, Joanna Banasiak, Anne Cotton, David Sherlock, Naomi Voke, Ruth Wade, Debbie Coldwell, James Stockdale, Ben Keane, Claudia Harflett, Tom Thirkell, Vicky Chadfield, Rhys Walden and Sheila Davitt-Betts for their friendship and advice throughout my PhD. Thank you to Anna Riach, who has been there to help me see the bright side, and to Nicola, Emma and Rachel for their friendship and support. I'd also like to thank the horticultural staff, particularly Alison Fenwick, Paul Scott, and Chris Lancaster, and Mark Bentley in mechanics.

I especially want to thank my parents, who have always supported and encouraged me without reservation and have always been there to help when I needed them. Above all, I thank Matt, for his unending support, reassurance and patience.

Authors declaration

I, Kate Elizabeth Storer declare that all the material contained within this thesis except for the work outlined below is a result of my own work and has been written solely by myself.

The microcosm experiment outlined in Chapter 3 was designed by Kate Storer but carried out with help from a summer student (Aisha Coggan). Practical work was both taught and overseen by Kate Storer. Maintenance of the plants (watering/feeding) was planned by Kate Storer, and carried out by both Kate and Aisha. Gas sampling was predominantly carried out by Aisha with one sample by Kate. Harvesting was carried out by Kate and Aisha, and root staining and hyphal extractions were carried out by Aisha but taught and overseen by Kate. Post-harvest analyses including ERM length, colonisation counts and C:N measurements were carried out by Kate. All calculations, statistical analysis and writing up was carried out by Kate.

Kate Storer

University of York

September 2013

Chapter 1. General Introduction

1.1 The gases that contribute to global climate change

It is now widely accepted among the scientific community that global climate change is caused by anthropogenic driven increases in greenhouse gases (GHG; Forster *et al.*, 2007) and that climate change will have major implications ecologically (Parmesan, 2006), socially (Godfray *et al.*, 2010; Patz *et al.*, 2005) and economically (Stern, 2007). Long-lived GHG including carbon dioxide (CO_2), nitrous oxide (N_2O) and methane (CH_4), are significant contributors to global climate change (Forster *et al.*, 2007), and nitrous oxide is also a significant cause of ozone depletion (Ravishankara *et al.*, 2009).

As a result of anthropogenic influences, the atmospheric concentration of CO_2, which had previously been stable at around 270 ppm for ca. 10, 000 years, has increased from ca. 280 ppm in 1750, to ca. 392 ppm in 2011 (Blasing, 2013; Forster *et al.*, 2007). Similarly, the atmospheric concentration of CH_4 is currently higher than it has been in the past 650,000 years (Spahni *et al.*, 2005), and CO_2, CH_4 and N_2O have all been increasing rapidly over the past 200 years (MacFarling Meure *et al.*, 2006). The atmospheric concentrations of CH_4 and N_2O in 2011 were ca. 1.8 ppm and ca. 0.32 ppm respectively (Blasing, 2013). Much of the research to date has focused on the effects of increased CO_2 levels in the atmosphere (e.g. Schimel, 1995; Vargas *et al.*, 2010), largely because CO_2 currently dominates in terms of GHG warming equivalents. However, although N_2O and CH_4 are present at lower concentrations than CO_2, they both have a high mean residence time in the atmosphere of 114 and 12 years, respectively, and also produce higher levels of radiative forcing in relation to their concentrations than CO_2 (Forster *et al.*, 2007). For example, over 100 years, CH_4 has a global warming potential that is 25 times greater than that of CO_2 (Forster *et al.*, 2007). Consequently, CH_4 and N_2O are increasingly being seen as immediate targets to achieve trace gas reductions (Wuebbles & Hayhoe, 2002; Reay *et al.*, 2012), requiring an increased understanding of the sources and sinks of these gases.

One of the major producers and consumers of CO_2, CH_4 and N_2O are soils (Figure 1.1a-c; Ojanen *et al.*, 2010; Schaufler *et al.*, 2010; Anderson-Teixeira & DeLucia, 2011). While the production and consumption pathways of CH_4 from soils are relatively well understood (Chan & Parkin, 2001; Le Mer & Roger, 2001), the production and consumption of N_2O by soils is more complex than previously realised (Baggs & Philippot, 2010; Butterbach-Bahl *et al.*, 2013), and the interactions of CH_4 and N_2O pathways with other soil organisms are not fully understood (Singh *et al.,* 2010).

1.1.1 Methane (CH_4)

Methane is produced from both anthropogenic (e.g. accessing and burning natural gas) and natural (e.g. wetlands) sources, but biogenic sources (i.e. biologically mediated, regardless of anthropogenic influence) account for up to 69% of global CH_4 emissions (Forster *et al.*, 2007; Conrad, 2009). The main biogenic sources of CH_4 are wetlands, ruminant animal production and rice paddies (Wuebbles & Hayhoe, 2002; Forster *et al.*, 2007). The major sink for CH_4 is the chemical reaction with hydroxyl (OH) radicals in the troposphere (the lowest portion of the Earth's atmosphere), forming water and CO_2, but CH_4 can also be consumed by soils via CH_4 oxidation (Wuebbles & Hayhoe, 2002). Although the global atmospheric concentration of CH_4 is currently increasing, if there are any changes in these fluxes (from sources or sinks), it could result in a major change in the net balance of CH_4 world-wide (Nazaries *et al.*, 2013a).

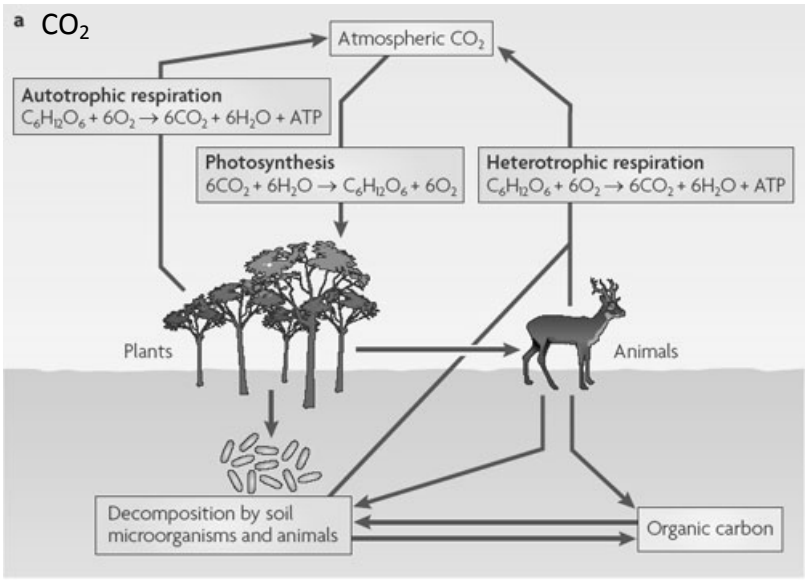
Methane can be both produced and consumed by soils during methanogenesis and methane oxidation, respectively (Figure 1.1b). Both processes tend to occur in most soils, and the dominant process determines if the soil is a net source or sink of CH_4 (Le Mer & Roger, 2001). In methanogenesis, the organisms involved are methanogens, which produce CH_4 and belong to the archaea (Angel *et al*. 2012). Methanogenesis produces both CO_2 and CH_4 as organic material is completely mineralised in the absence of oxygen (O_2). This commonly occurs in waterlogged soils such as rice paddies, but can also occur in anaerobic patches of aerobic soils (Chan & Parkin, 2001;

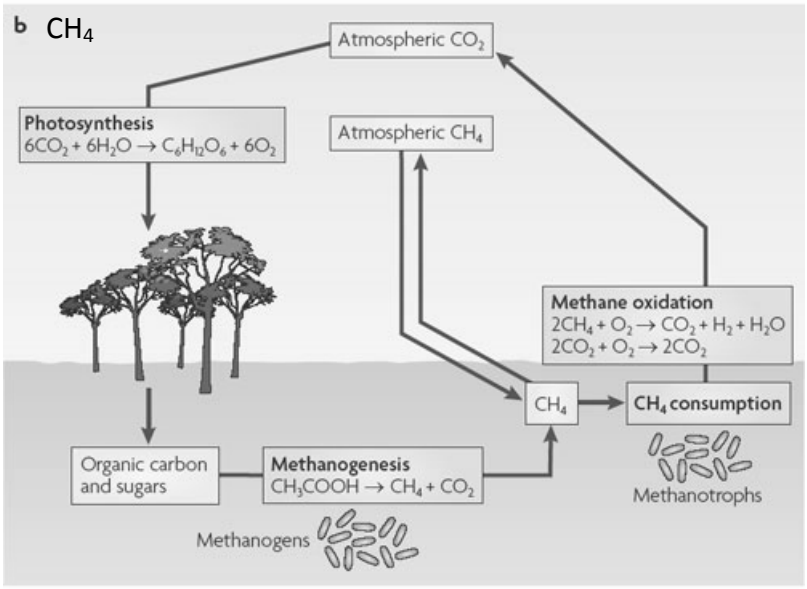
Le Mer & Roger, 2001). In contrast, the organisms responsible for CH_4 oxidation in soils are methanotrophic bacteria and archaea, and nitrifying bacteria (Prosser, 2007; Singh *et al.*, 2010). There are two groups of methanotrophic bacteria; high affinity methanotrophs working at low CH_4 concentrations of < 12 ppm (commonly from the class *Alphaproteobacteria*) and low affinity methanotrophs functioning at higher CH_4 concentrations > 40 ppm (mainly members of the class *Gammaproteobacteria*) (Bender & Conrad, 1992; Singh *et al.*, 2010).

Low affinity CH_4 oxidation tends to occur in soils where methanogenesis is also occurring, with CH_4 concentrations which are high but for short periods (for example in rice paddies). A large proportion of CH_4 produced in soil is consumed by low affinity CH_4 oxidation (Whalen *et al.*, 1990; Conrad & Rothfuss, 1991), but in soils that are exposed to CH_4 concentrations which are closer to the lower atmospheric levels, high affinity CH_4 oxidation is more likely (Bull *et al.*, 2000). However, more is known about the low affinity, than the high affinity CH_4 oxidisers, since the majority of studies on methanotrophs to date have been carried out on wetland soils or cultivated rice paddies (Le Mer & Roger, 2001; Conrad, 2009). There is also some evidence for anaerobic methane oxidation in peatland systems (Gupta *et al.*, 2013), and it has also been revealed that denitrifiers are capable of oxidising CH_4 (Ettwig *et al.*, 2010). The denitrifiers were found to bypass the production of N_2O during denitrification, and subsequently oxidise CH_4 using the oxygen produced (Ettwig *et al.*, 2010).

A range of factors determine whether a soil is a net producer or consumer of CH_4 but, as a result of anthropogenic activity, the CH_4 oxidation capacity of managed land may have been substantially decreased, possibly as a result of increased nitrogen (N) deposition (Hütsch, 1996; Reay & Nedwell, 2004; Aronson & Helliker, 2010). Other factors that can influence soil CH_4 oxidation or production include water content (Bender & Conrad, 1995), organic matter content, soil texture and drainage (Smith *et al.*, 2003a), chemical properties (Reay & Nedwell, 2004) and any factors that may influence microbial activity, such as temperature and pH (Segers, 1998; Le Mer &

Roger, 2001). However, the dominant factor controlling the activity of most CH_4 oxidising and producing bacteria is the concentration of O_2 (Chan & Parkin 2001; Angel *et al*. 2012).





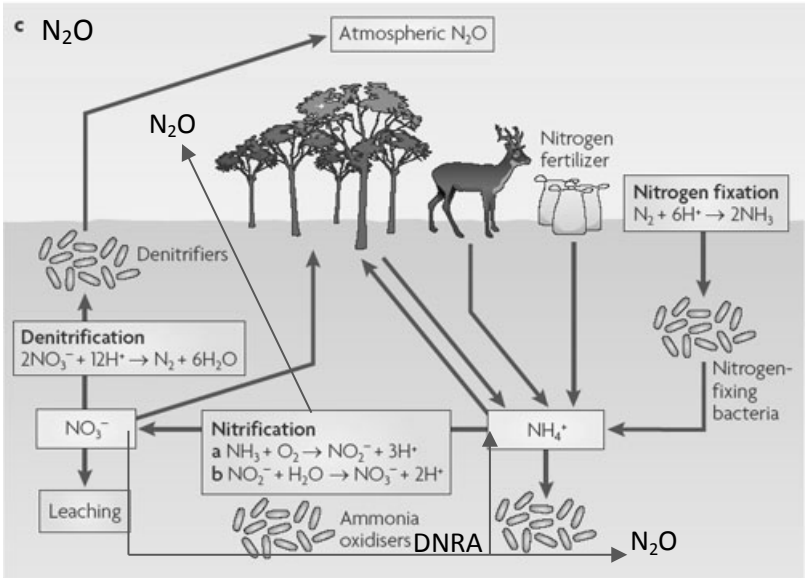


Figure 1.1. The pathways of a. CO_2, b. CH_4 and c. N_2O production and consumption in soils. Adapted with permission from Macmillan Publishers Ltd: [Nature Reviews Microbiology] Singh *et al*. copyright (2010). In Figure 1.1a, $C_6H_{12}O_6$ = glucose; in Figure 1.1b, CH_3COOH = acetic acid; in Figure 1.1c, NO_3 = nitrate, NH_4 = ammonium, NH_3 = ammonia. Figure 1.1c has also been modified to include N_2O sources from nitrifier nitrification and dissimilatory nitrate reduction to ammonium (DNRA).

1.1.2 Nitrous oxide (N_2O)

Nitrous oxide is a gaseous product of the N cycle in soil systems (Figure 1.1c; Singh *et al.*, 2010), and is often produced in agricultural systems (Forster *et al.*, 2007) with the marked global N_2O atmospheric concentration increases between 1940 and 2005 being predominantly a result of increased use of N-based fertilisers (Park *et al.*, 2012). As the world population is expected to increase significantly over the next 40 years (Godfray *et al.*, 2010), the pressure on agricultural systems to increase yields will continue to rise, together with increased global use of nitrogenous fertilisers. Consequently, in order to reduce anthropogenic driven N_2O emissions, there is a need to better understand the factors controlling production of N_2O in agricultural systems (Reay *et al.*, 2012). There are a number of other important natural sources of N_2O, mainly oceans and tropical wet soil, with anthropogenic sources making up about 1/3 of all N_2O sources world-wide. The major sink for N_2O is destruction in the stratosphere (Forster *et al.*, 2007; Schlesinger & Bernhardt, 2013), but N_2O can also be consumed by denitrifiers via the nitrous oxide reductase enzyme during the final stage of denitrification (Spiro, 2012). Since N_2O has a long mean residence time of 114 years compared to only 14 years for CH_4 (Forster *et al.*, 2007), it is essential that we understand the soil derived fluxes of N_2O as, unlike CH_4, any changes in the atmospheric concentration of N_2O will have long term consequences.

In recent years, our understanding of N_2O production in soil systems has significantly improved, mostly as a result of the development of isotopic methods for tracing the sources of N_2O (Baggs, 2008; Kool *et al.*, 2011; Ostrom & Ostrom, 2011). There is now evidence for a wide range of pathways and organisms in soils that are capable of producing N_2O, including nitrification, denitrification, nitrifier denitrification, dissimilatory nitrate reduction to ammonium, co-denitrification, and chemo-denitrification (Baggs, 2011; Butterbach-Bahl *et al.*, 2013), which are discussed in more detail in Chapter 4 (Section 4.1). The production of N_2O and CH_4 in soils is also being increasingly linked as some organisms that control the production and consumption of N_2O and CH_4 are capable of carrying out more than one function. Denitrification rates

can be positively related to the rate of CH_4 oxidation (Khahil & Baggs, 2005), possibly as a result of methanotroph-dependent denitrification (Islas-Lima *et al.*, 2004). Methanotroph-dependant denitrification is not well understood, but an increase in denitrification may be driven by a provision of carbon (C) to denitrifiers during methane oxidation (Knowles, 2005). Alternatively, the close-coupling of CH_4 oxidisers and denitirifers may be a route for CH_4 oxidisers to obtain oxygen under anaerobic conditions (Raghoebarsing *et al.*, 2006). Ammonia oxidising bacteria (AOB) are capable of oxidising CH_4 (Prosser, 2007), and methanotrophs can produce N_2O (Lee *et al.*, 2009). However, because the AOB have higher oxidation rates for NH_4 than for CH_4, and the opposite is true for methane oxidisers, it is thought that the role of CH_4 and NH_4 oxidisers is only minor in the production of N_2O and CH_4 respectively (Prosser, 2007).

Although there are now a wide range of known pathways which produce N_2O, the main factors that influence N_2O production rates via these pathways in soils are relatively well understood. These include O_2 availability (which might be limited by diffusion through the soil structure or water content; Bollmann & Conrad, 1998), availability of the required N source (NO_3^- or NH_4^+; Hino *et al.*, 2010) and factors that generally influence the activity of microorganisms (e.g. temperature, C availability and/or pH; Bollmann & Conrad, 1998; Prosser, 2007; Thomson *et al.*, 2012). Wallenstein *et al*. (2006) defined proximal and distal controls on denitrifiers and denitrification. Proximal controls include factors that affect the rate of N_2O production by denitrifiers that are already present (e.g. the short term availability of C), whereas distal controls such as long term availably of O_2 or C will affect the community composition of denitrifiers (Wallenstein *et al.*, 2006). These terms could also be used to describe the controls on nitrification rates. Thus, any change in N_2O production could be a result of either proximal or distal controls, or both. Whilst nitrification pathways are generally aerobic and autotrophic (Wrage *et al.*, 2001), and denitrification pathways are generally anaerobic and heterotrophic (Giles *et al.*, 2013), their interactions with other soil organisms are likely to also vary.

Although requiring varying conditions, many of the N_2O producing pathways can occur in parallel in soils. Anaerobic pathways (e.g. denitrification) can occur in microsites at the centre of soil aggregates or between soil particles that trap water (Singh *et al.*, 2010), even in the same soils as aerobic pathways such as nitrification. Despite understanding some of the major controls on these pathways, there is still a gap in knowledge necessary for prediction of which soils are most likely to produce N_2O (Butterbach-Bahl *et al.*, 2013). This is partly because, to date, it has been difficult to link the populations of N_2O producers with functional roles and their controls; the recent advancement of molecular methods is now making this a very real possibility for certain organisms. For example, Whitby *et al*. (2001) demonstrated, using ^{13}C-CO_2 enrichment cultures of AOB in fresh water sediment, that *Nitrosomonads* out-competed *Nitrosospiras* in their laboratory incubation system. Nonetheless, these methods are still under development (Butterbach-Bahl *et al.*, 2013), and there are still many unknowns with respect to the controls on N_2O producing microorganisms (Singh *et al.*, 2010).

1.2 Arbuscular mycorrhizal fungi (AMF)

Arbuscular mycorrhizal fungi (AMF) form a symbiosis with over 2/3 of all land plants (Smith & Read, 2008) which goes back as far as the first colonization of land by plants (Remy *et al.*, 1994; Redecker *et al.*, 2000), and is found across all major terrestrial biomes (Treseder & Cross, 2006). The symbiosis is formed by AMF penetrating the cell walls of plant roots and forming intraradical structures including intraradical mycelium (IRM), arbuscules and vesicles (Parniske, 2008). Arbuscules are 'tree-like' fungal structures that are formed inside plant cells, but do not penetrate the host cell membrane. Instead, the AMF branches are surrounded by a peri-arbuscular membrane produced by the plant which separates the fungus from the plant cytoplasm (Parniske, 2008). Arbuscules have a large surface area and are thought to be sites of nutrient exchange between the AMF and plant host (Parniske, 2008).

Vesicles are also formed by many AMF species (although not all) and these 'balloon-like' structures are thought to be storage organs (Smith & Read, 2008). Arbuscular mycorrhizal fungi also produce an extraradical mycelium (ERM), which are hyphae that extend out into the soil and beyond the plant root system. The ERM is involved in nutrient uptake from the soil and in searching for new plant hosts (Friese & Allen, 1991; Olsson *et al.*, 2003). The ERM length densities produced by AMF can far exceed root length densities, with a recent field study on tropical soils finding AMF hyphal densities (10.4 m g^{-1} soil) to be ca. 13 times higher than root lengths (Camenzind & Rillig, 2013). Therefore, AMF hyphae can significantly extend the volume of soil available for nutrient uptake (Smith & Smith, 2011b).

1.2.1 AMF modifying the soil environment

Arbuscular mycorrhizal fungi can affect soil systems both in the mycorrhizosphere, the volume of soil influenced by an AMF colonised root and AMF hyphae, and in the hyphosphere, the volume of soil influenced by AMF hyphae away from the plant roots (Figure 1.2; Johansson *et al.*, 2004). These terms are referred to throughout this thesis and the experiments detailed in the following Chapters focus on interactions between AMF hyphae and microorganisms in the hyphosphere.

The main role of the arbuscular mycorrhizal (AM) symbiosis is thought to be in aiding plant phosphorus (P) nutrition, as the AMF can access P that is outside the depletion zone that builds up around the root surface (Fitter *et al.*, 2011). However, there are a range of AMF benefits to host plants that have proven the symbiosis to be more complex. Arbuscular mycorrhizal fungi can improve soil structure (Rillig *et al.*, 2002) and water status of their host plants (Augé, 2001; Ruiz-Lozano *et al.*, 2001), host plant disease and pest resistance (Fritz *et al.*, 2006; Jung *et al.*, 2012), protection from heavy metal contamination (Guo *et al.*, 1996; Göhre & Paszkowski, 2006), reduction in nutrient leaching (Asghari & Cavagnaro, 2011; Asghari & Cavagnaro, 2012), and uptake of additional nutrients including copper (Liu *et al.*, 2000a), zinc (Thompson, 1996; Cavagnaro, 2008) and nitrogen (Leigh *et al.*, 2009). Three main influences of AMF on

the soil environment will be considered in more detail here, including AMF mediated changes in pH, glomalin production, and changes in nutrient availability (Section 1.2.2) as these could all mediate interactions between AMF and soil GHG production.

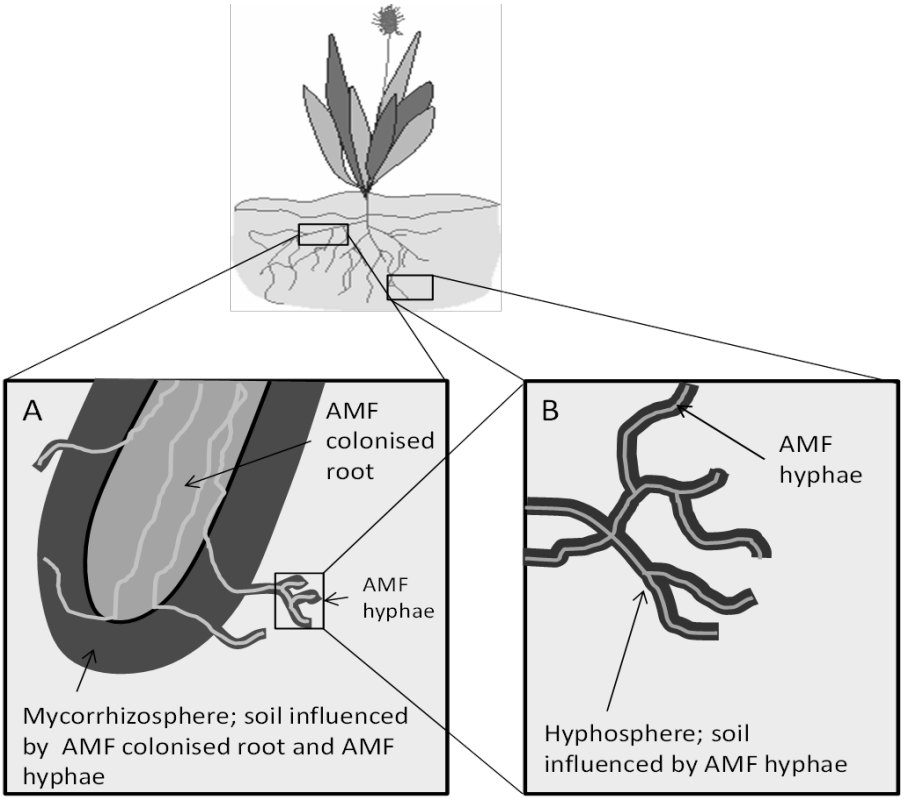


Figure 1.2. Schematic diagram of the areas of soil influenced by the arbuscular mycorrhizal (AM) symbiosis (not to scale). The soil influenced by a root colonised by AMF and AMF hyphae (in A this is denoted by the dark shading around the AMF colonised root and AMF hyphae) is known as the 'mycorrhizosphere'. The soil influenced by the AMF hyphae only (in B this is the dark shading around the AMF hyphae) away from the AM root is termed the 'hyphosphere'. Adapted from Johansson *et al*. (2004) with permission from John Wiley & Sons.

In a compartmentalised pot system Li *et al.* (1991a) found that AMF can reduce the pH of both mycorrhizosphere and hyphosphere soils by up to 1 pH unit. Villegas & Fortin (2001) also found that AMF hyphae reduced the pH of growth media in a Petri plate system using transformed carrot roots when grown with NH_4 as a N source, and this

has also been found in the mycorrhizosphere of onion roots grown in soil with added NH_4 (Bago & Azcón-Aguilar, 1997). It has been proposed that the observed decreases in pH were a result of H^+ release during NH_4 uptake (Li *et al.*, 1991a; Villegas & Fortin, 2001). In contrast, Bago *et al.* (1996) reported an increase in the pH of growth media when AMF hyphae were grown in hairy root cultures and provided with nitrate (NO_3). Bago *et al*. (1996) proposed that the AMF were actively taking up NO_3, which subsequently could release OH^- into or remove H^+ from the surrounding media during NO_3 uptake (Smith & Smith, 2011b), in a similar way to plants (Marschner *et al.*, 1986). A recent study using compartmentalised boxes found that hyphae of *Glomus intraradices* L. reduced the pH of the hyphosphere when supplied with phytin (an organic P supply) and NH_4, but not when supplied with phytin and NO_3 (Wang *et al.*, 2013). Consequently, because phosphatase activity can be increased at lower pH (Ding *et al.*, 2011), in the NH_4 and phytin treatment, shoot P content was increased under the lower pH conditions (Wang *et al.*, 2013). Thus, NH_4 uptake by AMF hyphae may also increase P availability (also see Section 1.2.2 on nutrient cycling).

As well as modifying soil pH, AMF hyphae can produce a glycoprotein called glomalin, which is operationally described as a glomalin related soil protein (GRSP) in soil studies (owing to the extraction and purification methods not being specific; Purin & Rillig, 2007). Nonetheless, GRSP has been linked to benefits for soils including stabilising soil structure (Rillig & Mummey, 2006), C storage (Rillig *et al.*, 2001; Wilson *et al.*, 2009) and water retention under drought conditions (Augé, 2001). It is N-rich (Lovelock *et al.*, 2004) and may act as an N source for microorganisms in N limited soils (Purin & Rillig, 2007). The concentration of GRSP in soil frequently correlates with the soil aggregate stability (Wright & Upadhyaya, 1998; Rillig *et al.*, 2002), leading to the suggestion that this is the major role of glomalin in soils (Wright & Upadhyaya, 1998). However, Gadkar and Rillig (2006) discovered that glomalin is in fact a homolog of Heat Shock Protein 60 and Driver *et al*. (2005), using *in vitro* cultures, found that ca. 80% of the glomalin produced by *G. intraradices* was located within hyphae and spores; consequently the majority of glomalin may only enter the soil once the hyphae senesce. As a result, Purin and Rillig (2007) proposed the primary role of glomalin was

physiological within living AMF hyphae, perhaps as a chaperonin. Secondly, because of the location of glomalin in cell walls, Purin & Rillig (2007) proposed that glomalin may make the hyphae less palatable to fungal grazers. The hyphae of AMF have been found to be less palatable to microarthropods (mites and collembola) than other saprobic soil fungi (Klironomos & Kendrick, 1996). Therefore, glomalin's role in improving soil aggregate stability may only be a simple coincidental by-product of these two more recently discovered functions, because it is relatively persistent in soils (Purin & Rillig, 2007).

Through increasing the aggregate stability of soils (Rillig & Mummey, 2006), AMF may improve soil structure and aid plant water uptake and diffusion of gases through soils (Bronick & Lal, 2005; Horn & Smucker, 2005). Thus, AMF-mediated changes in pH and improvements in soil structure via hyphae and glomalin production will impact on the soil environment for soil microorganisms, which will undoubtedly include GHG producers and/or consumers that are sensitive to edaphic conditions.

1.2.2 Roles of AMF in soil nutrient cycling

The main role of AMF in most plant-soil systems is believed to be improved P uptake for the host plant in exchange for C (Fitter *et al.*, 2011). However, AMF can also take up N (Ames *et al.*, 1983; Tanaka & Yano, 2005) and promote decomposition of organic material (Hodge *et al.*, 2001), and, consequently AMF are closely related to P, N and C cycling in soils, these interactions are outlined in more detail below (Sections 1.2.2.1, 1.2.2.2 and 1.2.2.3 respectively).

1.2.2.1 Phosphorus acquisition by AMF

The available form of P in soils (predominantly orthophosphate) can rapidly become adsorbed by clay mineral surfaces (Plante, 2007), thereby reducing the availability of phosphate in soils, and diffusion is insufficient to provide the P required by plants for

growth. Consequently, P depletion zones develop around plant roots, and plants can easily become limited by the availability of P (Jackson & Caldwell, 1993; Tinker & Nye, 2000). Arbuscular mycorrhizal fungi are thought to aid in plant P uptake by extending their fine hyphae and exploring a larger soil volume than is possible by the plants root system alone, rather than producing enzymes to mineralise organic P (Li *et al.*, 1991b; Smith & Read, 2008). Despite Toljander *et al*. 2007 demonstrating that AMF hyphal exudates can include organic acids, there is no evidence to date that these are used by AMF to aid in accessing P. However, there is also some evidence of AMF mediated decreases in pH increasing the mineralisation of phytate (an organic P source) and therefore the availability of phosphate for AMF uptake and transfer to their host plant (Wang *et al.*, 2013). Consequently, AMF play a significant role in P modifications and availability in soils.

The uptake of inorganic phosphate (Pi) by AMF is via high-affinity transporters in the ERM (Lei *et al.*, 1991; Harrison & van Buuren, 1995), and this P is then transferred to the IRM in the form of polyphosphate (polyP; Hijikata *et al.*, 2010). Once released from the arbuscules, Pi is transported into the plant roots via mycorrhizal-specific plant Pi transporters in the peri-arbuscular membrane (Maeda *et al.*, 2006; Javot *et al.*, 2007), although the transporters that release Pi from the IRM are still unknown (Smith & Smith, 2011b). The contribution of AMF to plant P nutrition can be significant and although AMF may not necessarily increase the total P content of their host plant they can, in some cases, be responsible for all P supply for their host (Smith *et al.*, 2004).

1.2.2.2 Nitrogen cycling

The published interactions of AMF with soil microbial N cycling are summarised in Figure 1.3. Nitrogen mineralisation involves the breakdown of organic matter into readily available inorganic forms, predominantly NH_4 and NO_3 by bacteria and saprotrophic fungi (Booth *et al.*, 2005). However, as AMF have no known saprotrophic capability (Smith & Read, 2008), it is unlikely that AMF play a direct role in

mineralisation of organic N forms. Nonetheless, there is evidence for enhanced decomposition of organic matter in the presence of AMF (Hodge *et al*., 2001; Atul-Nayyar *et al*. 2009) possibly because the presence of AMF can improve the ability of decomposers to break down organic N forms into inorganic forms, for example, via C exudation in the hyphosphere (Hodge *et al.*, 2001; Toljander *et al.*, 2007). Atul-Nayyar *et al.* (2009) showed that N mineralization in added organic matter increased by, on average, 228% in the AM treatments and this occurred in the three different AMF species screened (*Glomus intraradices*, *Glomus claroideum*, and *Glomus clarum*). There is also evidence to show that AMF hyphae proliferate in organic matter patches (usually containing dried shoot material) in soils (St John *et al.*, 1983; Hodge & Fitter, 2010; Leigh *et al.*, 2011) and can acquire N from these sources (Leigh *et al.*, 2009; Hodge & Fitter, 2010).

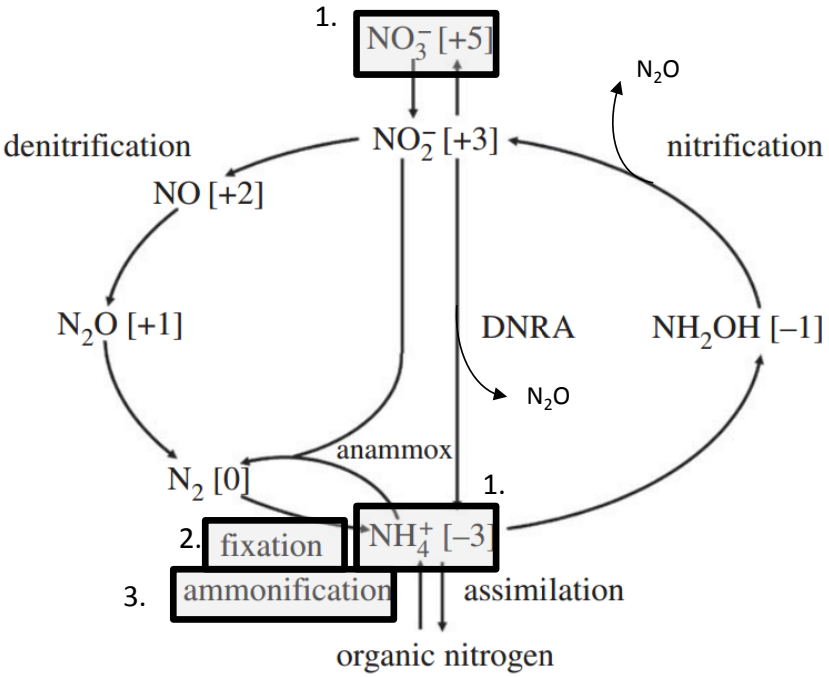


Figure 1.3. The soil microbial nitrogen (N) cycle. Adapted from Thomson *et al.* copyright (2012) with permission from The Royal Society. The known interactions of arbuscular mycorrhizal fungi (AMF) with soil N cycling are numbered and outlined. 1. Assimilation: AMF can take up both NH_4 and NO_3 in soils, 2. Fixation: AMF can improve nitrogen fixation in plant roots and 3. Ammonification: AMF can increase mineralisation rates in organic matter patches. These interactions may subsequently affect rates of denitrification, nitrification and dissimilatory reduction of nitrate to ammonium (DNRA). Numbers in brackets are the oxidation states of the N forms.

It has been proposed that AMF have a significant role in soil N cycling (Hodge & Fitter, 2010) and it has been demonstrated, firstly in laboratory systems, that AMF can take up and transfer N to their host plant (Ames *et al.*, 1983; Tanaka & Yano, 2005) and secondly, in field systems, that AMF can improve the N status of their host plants (Blanke *et al.*, 2011; Cavagnaro *et al.*, 2012). The range of N forms that AMF hyphae can take up is varied, with evidence for AMF uptake of inorganic N as NH_4 (Tanaka & Yano, 2005) or NO_3 (Bago *et al.*, 1996) and as amino acids (Hawkins *et al.*, 2000). In a field study using quantum dots to trace the uptake of organic N, Whiteside *et al.*

(2012) found that both labile (glycine) and recalcitrant (chitosan) organic N forms can be taken up by AMF hyphae. However, the specific uptake rates (per unit biovolume) of labile organic N were reduced under conditions of higher inorganic N availability (Whiteside *et al.*, 2012). Hodge *et al*. (2001) also demonstrated that AMF can uptake N from an organic source, although this was probably following mineralisation by other microorganisms (Hodge & Fitter, 2010). In an experiment using microcosm units in which AMF hyphae were allowed access to ^{15}N and ^{13}C labelled organic matter, Hodge and Fitter (2010) found that whilst nearly a third of total N in the AMF hyphae was derived from the organic matter, there was no ^{13}C enrichment of fungal or plant material. Therefore, the AMF were not taking up N in simple organic forms, and mineralisation must have occurred prior to AMF uptake of N. Furthermore, using hairy root cultures under gnotobiotic conditions, Leigh *et al*. (2011) found that AMF hyphae did not improve root N acquisition from a range of organic matter types, supporting the idea that they compete with microorganisms for decomposition products.

The reported amounts of N transferred from AMF to the host plant are varied, values of 30-50% for root N derived from AMF hyphae are reported from root organ culture studies (Govindarajulu *et al.*, 2005; Jin *et al.*, 2005). Some studies report no effect of AMF on plant N content (Reynolds *et al.*, 2005) or concentration (Cui & Caldwell, 1996) but in a microcosm based study using *Glomus aggregatum* (Schenk & Smith) and *Zea mays* L., Tanyaka and Yano (2005) found that when slow-release NH_4 was added to a hyphal-only compartment, up to 74% of total shoot N was derived from that NH_4 source, whereas in the NO_3 treatment, this value was only 2.9%. Furthermore, Leigh *et al*. (2009) found that by adding ^{15}N labelled organic matter patches to a hyphal-only compartment up to ca. 13% or 22% of plant N may have been patch derived when either *Glomus hoi* or *G. intraradices* were the AMF partner respectively, even though total plant N capture did not change. While there is evidence of AMF hyphae taking up both NH_4 (Tanaka & Yano, 2005) and NO_3 (Bago *et al.*, 1996), it is thought that AMF preferentially take up inorganic N in the form of NH_4 (Hawkins *et al.*, 2000; Tanaka & Yano, 2005), although, in water stressed conditions, NO_3 uptake can increase (Tobar *et al.*, 1994). In the ERM of *Glomus intraradices*, two high affinity NH_4 transporters have

been characterised (López-Pedrosa *et al.*, 2006; Pérez-Tienda *et al.*, 2011), and there is also some evidence for the existence of a low-affinity NH_4 transporter (Pérez-Tienda *et al.*, 2012). Ammonium is probably assimilated via the glutamate synthase or glutamine synthase/glutamine oxoglutarate aminotransferase (GS/GOGAT) cycle (Govindarajulu *et al.*, 2005). Transport of nutrients between the ERM and IRM is usually in a 'packaged form' as diffusion is too slow (Parniske, 2008) and the form of N transferred to the IRM is likely to be arginine (Govindarajulu *et al.*, 2005; Cruz *et al.*, 2007). The uptake of N (as NH_4) by the ERM before transfer to the host plant may require less energy than uptake of NO_3, as it would avoid the step of converting NO_3 to NH_4 via nitrate reductases, which is thought to be required before the N can be incorporated into amino acids for transfer to the IRM (Govindarajulu *et al.*, 2005).

There have been suggestions that transfer of N from the AMF to the plant host may be linked to the uptake of P. Once in the ERM, orthophosphate can form polyphosphate (polyP) which is transported along hyphae but also acts to buffer cytoplasmic Pi and store P (Hijikata *et al.*, 2010; Smith & Smith, 2011b). However, because both orthophosphate and polyP have a negative charge, cations are necessary to balance this, and arginine (Arg^+; the form of N thought to transport N from the ERM to the IRM (Govindarajulu *et al.*, 2005) may fulfil this role (reviewed by Smith & Smith, 2011b). However, AMF can pass N on to their host plants (Hawkins *et al.*, 2000; Tanaka & Yano, 2005) and in systems where N is limiting, but P is not, AMF can aid in plant N nutrition in the field (Blanke *et al.*, 2011). This supports the model described by Treseder and Allen (2002) whereby if a plant is limited by N or P it will allocate C to its associated AMF, and only when both nutrients are not limiting, will the plant reduce this allocation.

This model, proposed by Treseder and Allen (2002), complements the model proposed by Fitter (2006) describing C and P fluxes in AMs. Fitter (2006) proposed that plants may respond to AMF nutrient provision by directing C supply to the areas of high P (and/or N) availability in the roots (extended to include N by Hodge *et al*. (2010)).

Fitter (2006) suggested that the roots are unaware whether the increased P and/or N is derived from AMF or epidermal uptake and, even if the AMF were not present, the root would direct C to the location of higher P and/or N availability, and would result in root proliferation. Kiers *et al*. (2011) demonstrated using *in vitro* Ri T-DNA-transformed carrot root ('hairy root') cultures and stable isotope probing in pot cultures, that AMF hosts can re-direct C to the 'most co-operative' AMF partner (based on P provision, plant growth responses, C costs or hoarding strategy). Furthermore, the AMF provided more ^{33}P to the roots with the highest sucrose available to them (Kiers *et al.*, 2011). Fellbaum *et al*. (2012) recently published similar findings for N, and proposed that root C flux acts as a trigger for AMF N uptake.

However, while all these studies provide evidence of AMF P and/or N allocation to plant hosts depending upon root C provision, they are based on conditions of artificially high C availability, with no plant shoots, and the hormonal balance of the transformed roots can differ to that of normal roots (Hu & Du, 2006). In contrast, shading of the host plant (to reduce C supply to the AMF) had no effect on the AMF uptake and transfer of N to the host plant in a microcosm based study by Hodge and Fitter (2010), where the AMF hyphae had access to an organic matter patch. Therefore, the mechanisms of AMF changing P and/or N provision for the plant host in response to the host plant C supply still remain to be demonstrated under more realistic conditions. Nonetheless, it is probable that AMF are in competition for N with other soil microorganisms both in the mycorrhizosphere and the hyphosphere, and subsequently, the implications of the presence of AMF for N cycling may be significant (Figure 1.3).

There is limited and variable evidence for interactions between AMF and N cycling organisms in field studies (Cavagnaro *et al.*, 2007; Veresoglou *et al.*, 2011a). *Glomus mosseae* has been shown to increase free-living bacterial nitrogenase activity in a study using a mixed crop of clover (*Trifolium alexandrinum* L., B and *Trifolium resupinatum* L., P; Zarea *et al.*, 2009). In the same study, the activity of bacterial

nitrogenase was further increased upon the addition of earthworms, and shoot N uptake also followed the same pattern (Zarea *et al.*, 2009). Furthermore, nitrification rates can decrease in the mycorrhizosphere of AM, when compared to low- or non-AM plants (Veresoglou *et al.*, 2011a). However, the number of ammonia oxidisers (AO) was not different in the mycorrhizosphere of an AM tomato compared to the rhizosphere of an AM defective tomato mutant (Cavagnaro *et al.*, 2007) yet, in a pot-based study, the number of AO increased in the AM *Z. mays* treatments compared to the non-AM treatments (Amora-Lazcano *et al.*, 1998).

There is also some evidence for the number (Amora-Lazcano *et al.*, 1998) and community structure (Veresoglou *et al.*, 2012a) of denitrifiers changing in the mycorrhizosphere of AM plants, possibly driven by an AMF mediated distal control. Although limited in number, these studies indicate that AMF are influencing communities involved in larger scale nutrient cycling. As yet, only one study has researched the effect of AMF presence on N_2O fluxes from field soils. Using an AM defective mutant of *Solanum lycopersicum* L. (tomato) as a non-AM control, and the AM wildtype progenitor as the AM treatment, Cavagnaro *et al*. (2012) found that while root presence decreased $^{15}N_2O$ fluxes from soils, the presence of AM roots had no significant effect on soil N_2O fluxes, even though the uptake of $^{15}NO_3$ was higher in the AM treatments than in the non-AM treatments (Cavagnaro *et al.*, 2012), but the NO_3 addition may have masked any AMF-mediated effects under conditions of lower N availability.

Another major N input into soils comes from nitrogen fixation, and this can also be significantly affected by AMF. Nitrogen fixation involves the conversion of atmospheric nitrogen (N_2) to ammonium (NH_4) and is carried out by N-fixing bacteria and archaea (Figure 1.3; Canfield *et al.*, 2010). These microbes inhabit the rhizosphere alongside AMF and therefore are likely to interact. There is both glasshouse and field based evidence that suggests the presence of compatible AMF and N-fixing bacteria increases the availability of N to plants (Toro *et al.*, 1998; Xavier & Germida, 2003; Tajini *et al.*,

2011; Wang *et al.*, 2011). This effect is generally more pronounced under low P conditions, with little difference observed when N and P are not limiting. Generally, it is thought that the AMF provide P for the N-fixers under low P conditions which will release the N-fixers from P limitation and thus increase N availability for the plant (Artursson *et al.*, 2006; Tajini *et al.*, 2011; Wang *et al.*, 2011). In a field study using soybeans, the deep rooting genotype of the soybean used had greater nodulation of roots with a higher P supply (Wang *et al.*, 2011), demonstrating that P availability was limiting soybean nodulation. Under nutrient limited conditions, the benefits of dual symbiosis for host plant growth may be significant compared to a single symbioses with either AMF or rhizobia (Mortimer *et al.*, 2013), although this is not always the case (Kaschuk *et al.*, 2010). Additionally, N-fixing genes have also been discovered in the endosymbionts of certain species of AMF (Minerdi *et al.*, 2001), but the role of this is not yet understood.

1.2.2.3 Carbon cycling

As AMF rely on their plant host for C, they are also closely linked to soil C transfers. The AM symbiosis involves a rapid (Johnson *et al.*, 2002b) and significant transfer of atmospheric C to the below-ground system, with between 1 and 20% of plant photosynthate being transferred to the AMF (Paul & Kucey, 1981; Jakobsen & Rosendahl, 1990; Johnson *et al.*, 2002b), although around 5-10% is more typical (Bryla & Eissenstat, 2005). Furthermore, the net C gain, and therefore, uptake of CO_2 may be increased when a plant is AM (Miller *et al.*, 2002). Using a meta-analysis Kaschuk *et al*. (2010) demonstrated that the photosynthetic rates of a legume were increased by 14% when it was AM compared to non-AM, possibly as a result of C sink stimulation, which may help to ensure that the host plants do not become C limited when colonised by AMF (Kaschuk *et al.*, 2010).

Arbuscular mycorrhizal fungi may have an important role in soil C sequestration (Treseder & Allen, 2000; Wilson *et al.*, 2009); predominantly for two main reasons,

firstly AMF hyphae can improve the aggregate stability of soils, protecting C sources from decomposition inside macro-aggregates (Rillig & Mummey, 2006). Secondly, AMF can produce glomalin, a glycoprotein that also has a role in improving soil structure (Rillig *et al.*, 2002; but see Section 1.2.1). Furthermore, glomalin has a long residence time in soils (Steinberg & Rillig, 2003), and therefore may act as a further form of C storage. However, a recent study by Cheng *et al*. (2012), found that under increased atmospheric CO_2 concentrations there was a greater loss of C from organic matter that was colonised by AMF hyphae. Cheng *et al.* (2012) also provided evidence to suggest that increasing plant demand for NH_4 under elevated CO_2 could lead to an increase in AMF mediated priming of decomposition by soil saprotrophs. Whether or not soils become a sink or source of C seems to depend on the relative interactions between soil N and C cycling (Kowalchuk, 2012), and may also be different over the short and long term (Verbruggen *et al.*, 2013), with short term losses as a result of increased decomposition (Cheng *et al.*, 2012), but long term C storage as a result of AMF mediated improved aggregate stability and production of recalcitrant C forms such as glomalin (Steinberg & Rillig, 2003; Rillig & Mummey, 2006).

In non-AM roots, C exudation from plant roots constitutes a significant C input into the rhizosphere (Phillips *et al.*, 2006; Jones *et al.*, 2009), which may in turn fuel mineralisation of soil organic matter (Jones *et al.*, 2004). In contrast, when a plant is AM, the release of C is reduced in the mycorrhizosphere, as it is instead passed directly on to the AMF (Jones *et al.*, 2004). However, AMF hyphae themselves can exude low molecular weight C compounds in the hyphosphere which can modify the bacterial community composition (Toljander *et al.*, 2007). Furthermore, Staddon *et al*. (2003) demonstrated, using ^{14}C labelling, that some AMF hyphae can turnover in only 5-6 days. However, more than one type of hyphal structure is produced by AMF (Friese & Allen, 1991) and it is thought that the finer hyphae turn over rapidly (days) whereas the thicker 'runner' hyphae persist for weeks (Steinberg & Rillig, 2003; Zhu & Miller, 2003). Additionally, the CO_2 flux from field soils surrounding AM tomato plants was increased compared to those surrounding soils of AM defective tomato plants. Similarly in a microcosm-based study, the same authors demonstrated that the CO_2

flux per unit root-length can be higher in AM roots (Cavagnaro *et al.*, 2008) and, in grassland systems, the contribution of AMF hyphae to soil CO_2 fluxes can be significant (Heinemeyer *et al.*, 2012b). Thus the presence of AMF may result in both C loss, as well as C storage, in soils.

1.2.3 AMF and global change

In light of current climate change concerns, there has been a substantial effort in attempting to model future GHG production, which has mainly focused on CO_2 fluxes (e.g. Cox *et al.*, 2000) but, more recently, there has been an increased realisation of the importance and need to model both N_2O (Chirinda *et al.*, 2011) and CH_4 (Riley *et al.*, 2011) fluxes in terrestrial systems. Microbial interactions are often considered as a 'black box' when modelling biogeochemical cycling (Nazaries *et al.*, 2013b) but there is increasing evidence, for CH_4 at least, the need to incorporate microbial regulation of biogeochemical cycling (Nazaries *et al.*, 2013b). Changes in microbial community following tree growth on bog, grassland and moorland sites were strongly correlated to the subsequent increase in CH_4 oxidation rates (Nazaries *et al.*, 2013b) but this relationship may partly be explained by the narrow and well characterised range of CH_4 oxidisers. Any change in environmental conditions is likely to affect the interactions among, and therefore community of, the CH_4 oxidisers present. This may not be the case for functions which have a larger associated microbial diversity and therefore increased potential for functional redundancy (Nazaries *et al.*, 2013b). Nonetheless, the results presented by Nazaries *et al*. (2013b) demonstrate that by understanding the interactions between the microbes controlling N_2O and CH_4 fluxes from soils, we can get a better grasp on the future changes of these gases (Butterbach-Bahl *et al.*, 2013).

There have been a number of studies on the response of AMF to global change, usually in terms of their responses to abiotic factors including temperature (Hawkes *et al.*, 2008), ozone (Duckmanton & Widden, 1994; Yoshida *et al.*, 2001), drought (Augé,

2001; Ruiz-Lozano *et al.*, 2001), nutrient deposition (Treseder, 2004) and CO_2 increases (Staddon & Fitter, 1998; Rillig *et al.*, 2000; Staddon *et al.*, 2004). It has been demonstrated using stable isotope probing (SIP) that there is a high flux of C from plants to AMF and other active soil organisms, with indications that some AMF may receive more C than others (Vandenkoornhuyse *et al*., 2007). Furthermore, under elevated CO_2, rather than changes in soil C availability simply increasing microbial activity, there can be shifts in active AMF species as well as changes in the active rhizosphere bacterial and fungal communities (Drigo *et al*., 2009; Drigo *et al*., 2010). Thus, AMF may be important mediators of soil C dynamics under global change. However, other than considering the potential effects of AMF on soil C storage under predicted future conditions (e.g. Cheng *et al.*, 2012) very few studies have considered to what extent AMF contribute to, or could buffer the effects of global change. Our ignorance is largely a consequence of the difficulty of working with AMF, but techniques have been developed (e.g. microcosms and hairy root cultures) which now enable the study of AMF as individual organisms (e.g. Fitter *et al.*, 2000; Fortin *et al.*, 2002).

There has been recent debate over the nomenclature used for AMF, particularly with reference to *Glomus intraradices* which is commonly used throughout this thesis. The new nomenclature system suggests that *Glomus intraradices* is actually *Rhizophagus irregular* (Krüger *et al.*, 2012), but, in order to remain consistent with the referenced literature, the old nomenclature of *Glomus intraradices* is used throughout this thesis.

1.2.4 The potential for interactions between AMF and soil greenhouse gas fluxes

There have been very few studies that have looked at the involvement of mycorrhizas in GHG fluxes from soils, but recent studies on soil CO_2 fluxes suggest that both ectomycorrhizal (ECM) fungi (Heinemeyer *et al.*, 2007) and AMF (Vargas *et al.*, 2010) may be an important component in determining these fluxes at a large scale. To date, there have been no studies on the interactions between AMF and soil CH_4 fluxes, and

only one study that has measured N_2O fluxes in the presence of AMF, and then only in the mycorrhizosphere (Cavagnaro *et al.*, 2012). As outlined in Sections 1.2.1 and 1.2.2, AMF can alter aggregate stability (Rillig *et al.*, 2002), pH (Li *et al.*, 1991a), C, N and P status of soils (Hodge *et al.*, 2010) which are all important controls on the net fluxes of CH_4 and N_2O (Section 1.1; Singh *et al.*, 2010). When one considers that AMF hyphae and soil GHG producers and consumers will inhabit similar environments, alongside the numerous overlaps between the effects of AMF on soils, and the controls on soil GHG fluxes, there is significant potential for interactions between AMFs and GHG fluxes. As a result of the ubiquitous nature of AMF (Smith & Read, 2008) and the critical importance of atmospheric concentrations of CH_4 and N_2O (Forster *et al.*, 2007), any interactions between AMF and GHG fluxes could have implications at a global scale.

The aims of this thesis were to determine the following:

- A suitable methodology for studying the effects of the presence of AMF hyphae on soil GHG fluxes
- If there were any effects of AMF hyphae on the net flux of CH_4 or N_2O from soil and organic matter patches (dried, milled *Z. mays* leaves)
- The use of *in situ* CO_2 flux as a non-invasive measure of AMF hyphal growth into microcosm compartments, when compared to those that contained neither roots nor AMF hyphae
- If the N_2O and CO_2 fluxes in an AMF hyphal-only compartment systems changed when the connections between AMF hyphae and their plant host were severed
- The process controlling production of N_2O in the presence or absence of AMF hyphae and to determine if the cause for any observed changes in N_2O production were a consequence of a change in N and/or P availability

Chapter 2. The interactions between arbuscular mycorrhizal fungi (AMF) and soil greenhouse gas fluxes

2.1 Introduction

In recent years, studies have demonstrated that arbuscular mycorrhizal fungi (AMF) can take up substantial amounts of N from organic matter patches (Hodge *et al.*, 2001; Leigh *et al.*, 2009), and are therefore likely to have a significant, previously unappreciated, role in soil N cycling (Hodge & Fitter, 2010). AMF can also modify the C available in the mycorrhizosphere and hyphosphere, thus there are close links between AMF and soil C cycling (Jones *et al.*, 2009). As outlined in the General Introduction, greenhouse gases (GHG) including carbon dioxide (CO_2), methane (CH_4) and nitrous oxide (N_2O) are also closely linked to C and N cycling in soils (Schlesinger, 1997), thus it is highly likely that there are interactions between AMF and soil GHG fluxes.

However, it is difficult to predict the effect of AMF on soil GHG fluxes as there are various, potentially opposing interactions that could occur, and thus it is unknown if CH_4 and N_2O fluxes will increase or decrease in the presence of AMF. It is unlikely that AMF can produce or consume CH_4 or N_2O themselves, they have no known saprotrophic capability (Langley & Hungate, 2003), and although fungi, including ectomycorrhizal fungi (ECM; Prendergast-Miller *et al.*, 2011) have a role in denitrification (Herold *et al.*, 2012), and saprotrophic fungi can produce CH_4 (Lenhart *et al.*, 2012); to date, there is no evidence of AMF producing or consuming either CH_4 or N_2O. However, AMF can host endobacteria (Bianciotto *et al.*, 2000; Bianciotto & Bonfante, 2002) forming tripartite interactions, which although not yet fully understood (Bonfante & Anca, 2009; Ghignone *et al.*, 2012), may have a role in nutrient cycling, possibly involving N_2 fixation (Minerdi *et al.*, 2001; Cruz & Ishii, 2012). It is therefore considerably more likely that AMF influence the production or consumption of GHG such as CH_4 and N_2O from soils indirectly.

The uptake of N by AMF is probably in an inorganic form, most likely as NH_4 (Govindarajulu *et al.*, 2005; Jin *et al.*, 2005; Tanaka & Yano, 2005), and this N is partly used by the N rich hyphae (Hodge & Fitter, 2010). However, when AMF hyphae are allowed access to organic matter patches, some patch derived N can also be transferred to the host plant and this N is also likely to be taken up by AMF in an inorganic form (Leigh *et al.*, 2009). Hyphal uptake therefore has the potential to reduce the availability of NH_4 and/or NO_3. In turn, this could reduce the rates of N_2O production because N_2O producers are reliant on supplies of NH_4 and/or NO_3 (Hino *et al.*, 2010). Conversely, AMF hyphal presence can increase mineralisation rates in soils (Hodge *et al.*, 2001; Atul-Nayyar *et al.*, 2009), and N mineralisation rates are closely linked to N_2O production as they increase the available NH_4 and NO_3 (Vinther *et al.*, 2004; McLain & Martens, 2005); thus N_2O fluxes may increase in the presence of AMF hyphae.

Alternatively, as nitrifiers can be limited by the availability of P (Purchase, 1974), a reduction in P availability in the presence of AMF hyphae may limit the production of N_2O. Arbuscular mycorrhizal (AM) mediated decreases in pH (Li *et al.*, 1991a) could also result in lower N_2O fluxes as N_2O production can be limited at lower pH (Baggs *et al.*, 2010). Arbuscular mycorrhizal fungi are also known to alter the soil structure and water retention by producing glomalin (a glycoprotein that improves soil structure; Rillig & Mummey, 2006). They are aerobic organisms, thus will also reduce the availability of O_2 as the hyphae respire (Smith & Read, 2008). By increasing the frequency of anaerobic microsites, this may move the balance from methanotrophy (aerobic, consuming CH_4) to methanogenesis (anaerobic, producing CH_4; Le Mer & Roger, 2001; Yu *et al.*, 2001), potentially turning soils from net sinks to net sources of CH_4. Additionally, any anaerobic process such as denitrification (Zumft, 1997) could increase if O_2 availability is lowered as AMF presence creates more anaerobic patches, and therefore the net N_2O fluxes may also increase.

Arbuscular mycorrhizal fungi also alter C inputs into the soil, AM colonisation usually reduces the amount of C released from roots into the soil in the mycorrhizosphere as the AMF hyphae act as a sink for excess C (Smith & Read, 2008). In contrast, there may be a C flow into the soil from the AMF hyphae in the hyphosphere via hyphal exudation (Toljander *et al.*, 2007) or turnover which may be rapid; Staddon *et al*. (2003) found that fine AMF hyphae may turnover in only 5-6 days. Toljander *et al*. (2007) demonstrated, using root organ cultures, that AM mycelia can also produce hyphal exudates of low molecular weight sugars and organic acids, as well as other unidentified high molecular weight compounds, another available C source in the hyphosphere. It is not clear how much C is added to the soil by AMF hyphae or its importance as a C source in natural systems, but in the hyphosphere this could act as an additional substrate for heterotrophic CH_4 oxidising or for N_2O producing organisms, whereas the opposite may be true in the mycorrhizosphere.

There is increasing evidence for AMF hyphae interacting with both bacteria (Albertsen *et al.*, 2006; Welc *et al.*, 2010; Herman *et al.*, 2012) and archaea (Nuccio *et al.*, 2013) and this can be significant; in one microcosm based study, AMF hyphae modified the soil bacterial community of an organic patch to a similar extent as an active root growing through soil (Nuccio *et al.*, 2013). Potential nitrification rates have also been found to be decreased in the presence of AM plants (Veresoglou *et al.*, 2011a), but the impacts of AMF presence on soil N_2O fluxes in the field are unclear (Cavagnaro *et al.*, 2012). Thus, there are many contrasting ways in which AMF may potentially affect soil CH_4 and N_2O fluxes, yet to date these interactions are surprisingly understudied.

There is considerably more information on the impact of AMF hyphae to soil CO_2 fluxes. In a grassland study, Johnson *et al*. (2002a) demonstrated that between 3.9-6.2% of fixed C could pass through AMF hyphae and into the atmosphere as $^{13}CO_2$ within 21 h of pulse-labelling the host plants with ^{13}C. The rapidity of this transfer suggested that hyphal respiration was predominantly controlled by the provision of recent photosynthates from the host plant, which has also been demonstrated under

laboratory conditions (Johnson *et al.*, 2002b; Heinemeyer *et al.*, 2006). The presence of AMF hyphae may also increase heterotrophic activity through the release of low molecular weight (LMW) compounds during hyphal exudation (Toljander *et al.*, 2007). In forest systems, LMW compounds such as sugars and amino acids represent a small but high turnover pool of C that is rapidly used and respired by soil microorganisms (van Hees *et al.*, 2005). The effect of AMF hyphae on soil CO_2 flux is likely to be driven at least in part by hyphal exudation, but the extent of this is not well understood (reviewed by Finlay, 2008). If it can be demonstrated in microcosm studies that hyphal compartment CO_2 fluxes were directly related to AMF extraradical mycelium (ERM) lengths, it may be possible to use the CO_2 flux as an alternative indicator of AMF hyphal presence, removing the need for the destructive, time consuming harvests that are usually required to quantify ERM length densities (e.g. Drew *et al.*, 2003; Barrett *et al.*, 2011); this possibility was planned to be assessed.

It has been suggested that the whole soil environment may be classed as the mycorrhizosphere as AMF are so ubiquitous (Johansson *et al.*, 2004). However, when considering the size of microorganisms such as bacteria, fungi and archaea, which are the predominant producers of CH_4 and N_2O (Singh *et al.*, 2010; Lenhart *et al.*, 2012), the mycorrhizosphere and hyphosphere could be very different environments; this study focused on the interactions between AMF hyphae and GHG fluxes in the hyphosphere. Microcosm systems, in which AMF hyphae are allowed, or prevented, access to a non-root compartment have been essential for understanding the role of AMF hyphae in nutrient uptake and transfer to host plants (e.g. Hodge *et al.*, 2001; Leigh *et al.*, 2009; Hodge & Fitter, 2010). AMF hyphae both colonise and proliferate in organic matter patches placed in these hyphal-only compartments which are usually high in N with a low C:N ratio (e.g. Leigh *et al.*, 2009; Hodge & Fitter, 2010). The C:N ratios of organic material can be related to mineralisation rates (Janssen, 1996; Antil *et al.*, 2013) and have been used to model C and N mineralisation rates (Nicolardot *et al.*, 2001). As C:N ratios decrease, both decomposition rates (Cotrufo *et al.*, 1994) and N_2O fluxes (Huang *et al.*, 2004) can increase. Organic material mixed with a natural

microbial inoculum should therefore provide a suitable system for studying interactions between AMF hyphal presence and GHG production.

In order to measure GHG fluxes from the hyphal compartments, a microcosm similar in design to those used by Barrett *et al*. (2011) and Leigh *et al*. (2009) was modified to incorporate a cover-box lid (described in Section 2.2.3.2). Cover-boxing techniques are commonly employed in the field (Liang *et al.*, 2004; Heinemeyer & McNamara, 2011) and have been used to assess the contribution of ECM to forest CO_2 fluxes (Heinemeyer *et al.*, 2007). A soil core is inserted into the ground with a small section remaining above the soil surface. A cover-box lid is attached above this core, enclosing a known headspace above the soil, creating either a static chamber (closed static chamber, CSC) or a dynamic chamber (closed dynamic chamber, CDC) depending on the attachments fitted (Heinemeyer & McNamara, 2011; Kutsch *et al*. 2010; Rochette *et al*. 1997). The CSC has no air flow over the soil surface, any gases produced from the soil build up in concentration over time inside the headspace, and a gas sample is removed at regular intervals (10-30 min), usually over an hour period. These samples are analysed and used to calculate the change in concentration over time (flux). In contrast, the CDC usually has an attached gas analyser (e.g. infrared gas analyser to measure CO_2). The analyser is attached to the cover-box by inlet and outlet tubing through which there is a constant flow of air in a closed loop. The gas is passed through the analyser which is frequently measuring the gas concentration over time (Heinemeyer & McNamara, 2011). This method usually results in more accurate flux calculations as the higher rate of sampling means that there is reduced error. More traditional soil incubations were also used to assess the organic matter patch and soil GHG fluxes independently during destructive harvests. This was to assess spatial variability in N_2O and CH_4 production. By adding CH_4 to the atmosphere in these incubations, the potential CH_4 oxidation rates could be assessed (as used by Wang & Ineson, 2003). Addition of NO_3 and glucose to the soil incubations also assisted in understanding if any AMF effects on N_2O were a result of C and/or NO_3 limitation (Gillam *et al.*, 2008).

In order to test the hypothesis that AMF hyphae affected CO_2, N_2O or CH_4 fluxes, a number of technical issues initially needed to be addressed. Firstly, a host plant species that had agricultural relevance, which could be grown in a container with limited space/nutrients and would produce significant quantities of AMF hyphae in an adjoined root-free compartment needed to be identified. As AMF are often proposed for use in sustainable agriculture (reviewed by Fester & Sawers, 2011), and agricultural soils are some of the largest soil GHG producers (Reay *et al.*, 2012), the suitability of two crops (*Zea mays* L. (maize) and *Linum usitatissimum* L. (flax)) were compared as AMF host plants in a microcosm experiment. Both of these crops are grown world-wide (Kim *et al.*, 2009; FAO, 2012), and are known to form a symbiosis with AMF (Olsson *et al.*, 1999; Boomsma & Vyn, 2008). The ERM length densities produced for each host species were compared to those from *Plantago lanceolata* L. (ribwort plantain) as it is commonly used as a host plant in AMF studies because it supports high levels of AMF colonisation (e.g. Gange & West, 1994; Hodge *et al.*, 2001; Veresoglou *et al.*, 2011b). The hypothesis was that there would be no significant difference in the ERM length produced in the organic matter patches that the AMF hyphae had access to for the three AMF host plant species. Secondly, preliminary measurements of the rate of CO_2 diffusion across the mesh membrane barriers that are normally used to prevent (0.45 μm) or allow (20.0 μm) AMF hyphal access in the microcosm were taken to ensure that the microcosm design did not affect the flux of GHG across the membranes.

The microcosm system developed as a result of these preliminary studies, alongside more traditional soil and organic patch incubations, were used to assess four main hypotheses. Firstly, it was expected that there would be a lower flux of N_2O from microcosm units that contained an organic matter patch when AMF hyphae were present, as the N available to nitrifiers and/or denitrifiers would be decreased. This difference was predicted to be maintained in both the soils and patches following a destructive harvest. In contrast, the CO_2 flux was expected to increase in the presence of AMF hyphae as a result of hyphal respiration, and CH_4 oxidation rates to decrease when AMF hyphae were present as a result of decreased O_2 availability. Finally, after

separating the patches and soils in a destructive harvest, the N_2O fluxes from soils, with or without AMF hyphae present, were predicted to increase following the additions of glucose or KNO_3, thus indicating that availability of N or C may be limiting.

2.2 Materials and Methods

2.2.1 Selecting a suitable host plant to encourage proliferation of AMF hyphae in organic matter patches

2.2.1.1 Experimental design

Microcosm units were used to compare the ERM length densities produced by AMF when in symbiosis with three different plant species, *L. usitatissimum*, *P. lanceolata* and *Z. mays*. There were a total of 15 microcosms, with 5 replicates of each plant species.

2.2.1.2 Microcosm design

The microcosms were made from two 1L plastic boxes (145 mm x 145 mm x 70 mm), screwed together on the largest surface with a 6 mm thick perspex sheet in the middle. This perspex sheet had a 70 x 70 mm window in the centre covered by a double layer of 20.0 µm mesh membrane (John Stanier & Co., Whitefield, Manchester, UK) glued to the perspex using industrial strength superglue (Everbuild Building Products Ltd. Leeds, UK). The two boxes were joined together using four equally spaced screws with aquarium sealant between the joining surfaces (Aquamate, Everbuild Building Products Ltd. Leeds, UK) to prevent hyphal breakthrough. The aquarium sealant was guaranteed free from fungicides, and therefore should not adversely affect the AMF inoculum added. Before use, all microcosm parts were soaked in a 50% (v/v) solution of sodium hypochlorite for a minimum of 30 min then thoroughly rinsed in water to prevent any contamination from use in previous experiments.

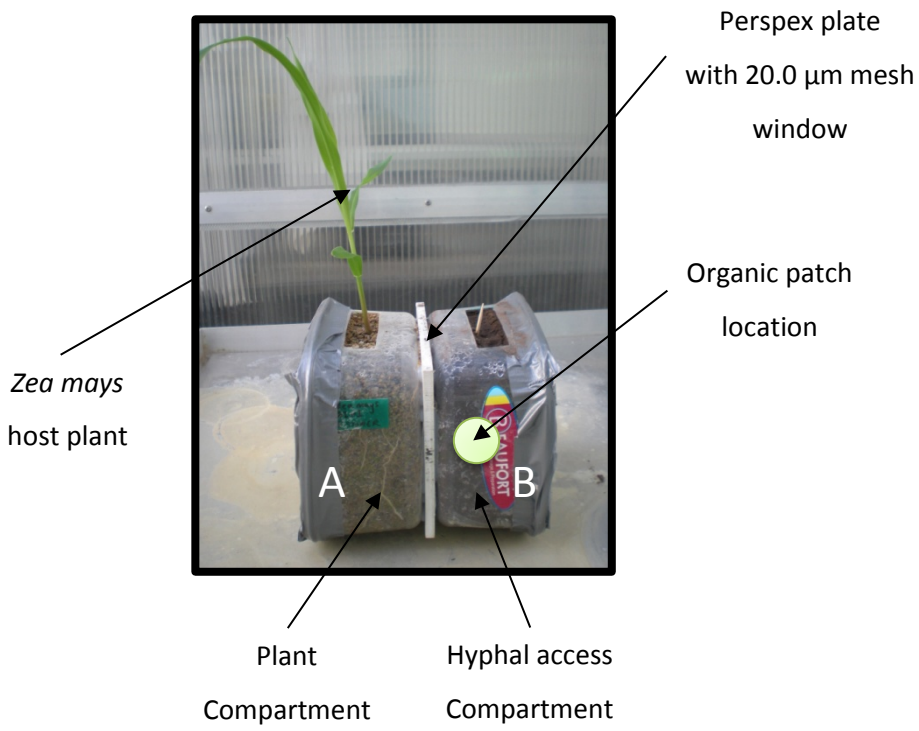


Figure 2.1. Microcosm design used in experiment 2.2.1. Compartment A contained the host plant in a sand and Terra-Green® medium (50:50 mix, v:v) with a *Glomus intraradices* inoculum and plant roots present. The white perspex plate had a window through to compartment B which was covered with a 20.0 µm mesh membrane, allowing AMF hyphal access to compartment B, but preventing root access. Compartment B was filled with sieved agricultural soil and contained an organic matter patch in a 20.0 µm mesh membrane bag represented in the photograph by the green circle. The microcosm unit was covered in aluminium foil throughout to prevent photosynthetic growth inside the compartments, but was removed for the purpose of this image.

2.2.1.3 Microcosm planting and growth media

One side of the microcosm was filled with washed and autoclaved sand and Terra-Green® (a calcined attapulgite clay soil conditioner, Oil-Dri(UK)Ltd, Cambridgeshire) mix (plant compartment, 50:50, v:v), with the other side filled with a locally sourced soil (53˚92'N, -1˚00'E). In each plant compartment, the sand and Terra-Green® were

further well mixed with 0.25 g L^{-1} of bonemeal (a complex N and P source; 3.5% N, 8.7% P; Vitax, Leicestershire, UK) and 85 g fresh weight (FW) of *Glomus intraradices* (Schenck & Smith; isolate BB-E; Biorize, Dijon, France) live inoculum and root mix (*P. lanceolata/Trifolium repens* L.) that had been growing for at least 6 months. Three pre-germinated seeds were added to each of the plant compartments on 18th March 2011; either, *Z. mays* (F1 incredible variety; Mr Fothergill's, Suffolk, UK), *P. lanceolata* (Emorsgate seeds, Norfolk, UK), or *L. usitatissimum*, (Chiltern seeds, Wallingford, UK) for each treatment, respectively. All seeds were sterilised in 3% sodium hypochlorite for 5 min, washed in deionised water then placed on moist filter paper in Petri dishes for 5 d before planting. The soil was locally sourced from an agricultural site that had previously had a *Z. mays* crop on it (53˚92'N, -1˚00'E). It was sieved through a 6 mm sieve and added to the soil compartment 10 d post-planting to allow the AMF time to colonise the plants. The soil used had a pH of 6.6 in 0.01M $CaCl_2$ (following Allen (1974)). The units were wrapped in three layers of aluminium foil to prevent any photosynthetic growth inside the units.

2.2.1.4 Organic matter patches

The organic patches consisted of a 20.0 µm mesh bag (John Stanier & Co., Whitefield, Manchester, UK). A 120 x 60 mm piece of 20.0 µm mesh was folded in half and sealed with aquarium sealant. Each bag was filled with 1 g dry weight (DW) of milled *Z. mays* shoots (F1 incredible variety) well mixed with 3.25 g (FW, equivalent to 3 g DW) of the same soil as used in the outer compartment to provide a natural microbial community within the patch material (see Section 2.2.1.3). The plant material used in the patches contained 4.5% nitrogen (N), whereas the soil contained 0.1% N, thus providing at total of 48.3 mg N per patch. The patches were added 23 d after planting to the soil compartment, 2 cm from the mesh membrane window and at a depth of 7 cm.

2.2.1.5 Growth conditions and nutrient solution

The microcosm units were placed in a temperature controlled glasshouse in a randomised block design and were rotated within blocks every two weeks. The mean daily temperature was 18.58 ± 0.04^{o}C and overhead lights (400 W, Son-T Agro) were used to ensure a 16 h day. Photosynthetically available radiation (PAR) readings were measured at plant level in each block, weekly at midday. There was no significant difference in PAR between blocks and the mean midday PAR level was 110 ± 4 μmol m^{-2} s^{-1}. The plant and soil compartments were watered daily as required with deionised water. The plant compartments also received 50 cm^{3} of a nutrient solution containing 1/10th of N and P (modified from Thornton & Bausenwein (2000); Appendix 1) once a week.

2.2.1.6 Harvest data collection

All microcosms were harvested on 11th May 2011, 54 d post-planting, 31 d post-patch addition. Upon harvesting the following data were collected; fresh and dry weights of shoots, roots, sand/Terra-Green®, soil and patches. AMF colonisation was assessed by staining a sub-sample of fresh roots in 0.01% acid fuchsin (following Koske & Gemma, 1989; Grace & Stribley, 1991) in order to collect information on the percentage root length colonised (% RLC) measured at x200 magnification (following McGonigle *et al*. (1990)) using both bright light and epifluorescence (Merryweather & Fitter, 1991). The length of external AMF hyphae (ERM) was assessed using the modified membrane filter technique described by Staddon *et al*., (1998), and hyphal length estimation as by Miller and Jastrow (1992) in the plant compartment, patch and soil compartment (at least 5 cm away from the mesh membrane). As the patches were too small to sample for ERM length densities alone, a 5 g (FW) soil sample was taken from between the patch and the 20.0 μm mesh window and used to increase the patch sample to generate an ERM estimate (as 10 g was needed and the patch fresh weight at harvest was ca. 5 g).

2.2.1.7 Data analysis

All data were tested for normality and equality of variance assumptions using Kolmogorov-Smirnov and Levene's Homogeneity of Variance tests respectively. Percentage colonisation data were arcsine transformed before analysis in SAS (v9.3 SAS institute Inc., North Carolina, USA), and both colonisation and extraradical mycelium (ERM) length data were analysed using a two-way ANOVA including block, with Duncans tests used for *post-hoc* analysis. Untransformed data are shown in all figures.

2.2.2 The effect of fine mesh presence on CO_2 diffusion rates in microcosm systems used to study AMF

This preliminary study investigated the flux of CO_2 across the different meshes commonly used in AMF studies. This involved the development of a new microcosm that could be used to take gas flux measurements using a cover-box technique yet still fulfilled the needs of a standard microcosm, being large enough to accommodate a grown plant with associated soil, and preventing hyphal access to a second compartment as required. The microcosm also had to prevent gas from entering or exiting the unit unless required, whilst also being cost effective, reusable, and adaptable for any future applications (e.g. soil gas flux monitoring and addition of nutrients). As such, the new systems also required removable lids that were air tight and meshes which could be easily replaced between experiments. CO_2 was used as a test gas as it is a large molecule (molecular weight = 44) and concentration changes could be measured easily over seconds rather than hours using an infra-red gas analyser (IRGA, LI-COR® Biosciences, Lincoln, USA).

2.2.2.1 Microcosm unit design

A microcosm unit was designed to facilitate GHG measurements, using air-tight food containers (140 x 140 x 160 mm; Lock & Lock, Australia PTY Ltd., Austraila; see Figure

2.2). Two containers were joined by a metal, externally threaded, tunnel (90 mm long x 40 mm diameter) and sealed with aquarium sealant free of anti-microbial chemicals (Aqua Mate, Everbuild Building Products Ltd., Leeds, UK.). To create the mesh membrane treatments, in turn, each different membrane was screwed onto the end of the tunnel (with care being taken not to split it) using plastic bulkhead nuts. As the 0.45 µm membrane was easily damaged, instead of screwing it into place, it was stuck onto the flat surface of a bulkhead nut using industrial strength superglue (Everbuild building products Ltd. Leeds, UK) and the bulkhead was then screwed onto the end of the threaded tunnel (Figure 2.3). By screwing the meshes into place, and sealing all other routes between the two units, this ensured that the only route for gas transfer between the two units was through the mesh.

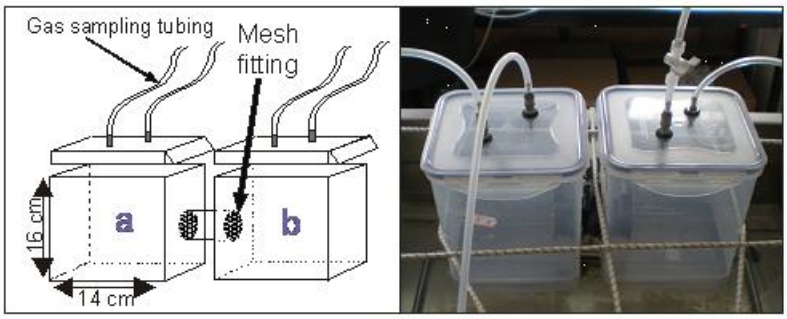


Figure 2.2. Microcosm unit with mesh fittings shown in a diagram and photograph. Photograph shows the whole microcosm unit in a water bath with lids and gas tubing connected. When testing the unit for an air-tight seal it was submerged and held under the water with the elastic cords. When comparing the diffusion of CO_2 across the different meshes, the mesh fittings were alternated between 0.45 µm, 20.0 µm or fully blocked. A known concentration of CO_2 was added to compartment 'a' and the concentration of CO_2 in compartment 'b' was then measured over time, the rate of change in CO_2 in compartment 'b' following CO_2 addition to compartment 'a' was compared between the three mesh treatments.

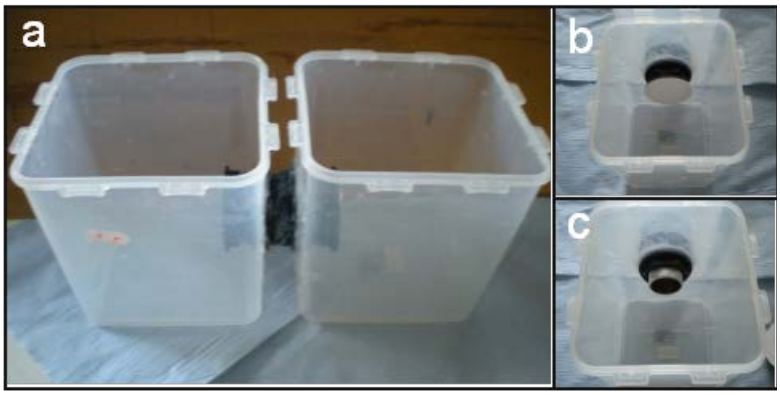


Figure 2.3. Microcosm unit with 0.45 µm mesh fitted to the 'back-nut' (a,b) and with no mesh fitted (c).

2.2.2.2 Experimental design

There were four treatments; no mesh, 0.45 µm mesh (GE Water & Process Technologies, Belgium), 20.0 µm mesh (Section 2.2.1.2), and fully blocked (solid plastic - to check that the mesh was the only route of air flow between or into the units). The unit was placed in a water bath to maintain a constant temperature of between 18-19.5^{o}C whilst gas testing (Figure 2.2).

For each mesh treatment, a known concentration of CO_2 was added to side 'a' and the subsequent increase in CO_2 concentration over time in side 'b' was measured using an infra red gas analyser (IRGA; LI-COR® Biosciences, Lincoln, USA; Figure 2.2). Preceding each new measurement, the unit was flushed with N_2 for 20-30 min; until very low levels of CO_2 were present (< 9.29 ppm). One microcosm unit was constructed and the length of time taken for the CO_2 concentration in the second compartment (side 'b', Figure 2.2) to reach half of its maximum was calculated three times for each mesh treatment (totalling three replicates). The mesh type was alternated between each measurement in a random order and fresh mesh was used in each of the replicates. Following the flushing with N_2, CO_2 was added to side 'a' (Figure 2.2), and an equal volume of gas was removed at the same rate. The volumes of CO_2 added to the unit

were calculated to deliver a target concentration in the 5.2 L unit, post diffusion, of ca. 500 ppm (915 mg m^{-3}). The CO_2 concentration in side 'b' was measured every 30 seconds for up to 200 min. Concentration vs time was then plotted and the time taken to reach half of the absolute CO_2 concentration in the unit (ca. 250 ppm) was calculated using Lineweaver-Burk (double-reciprocal) plots. The time taken to reach half of the maximum could not be calculated for the solid plate experiments as there was no transfer to the second compartment, therefore these data are not included.

2.2.2.3 Data analysis

Data were assessed for normality and equality of variance assumptions using Kolmogorov-Smirnov and Levene's Homogeneity of Variance tests respectively. As these assumptions were fulfilled, differences between treatments were evaluated using a one-way ANOVA in SAS v. 9.3 (SAS institute Inc., North Carolina, USA).

2.2.3 The interactions between AMF and soil CO_2, N_2O and CH_4 fluxes

2.2.3.1 Experimental design

Microcosms were designed as outlined in Section 2.2.3.2 below measure interactions between AMF hyphae and soil GHG fluxes. Each microcosm unit contained one side that allowed AMF hyphal access (AMA), and one that did not (NAMA), creating a paired design. There were three destructive harvests to allow the quantification of ERM length densities over time. The patch and soil N_2O fluxes were also measured independently following each destructive harvest, allowing identification of the main site of N_2O production at different stages of the experiment. There were 18 units in total, with six replicates per harvest. The plants were grown for 8 weeks (56 d) until the first harvest, 12 weeks (84 d) until the second harvest and 15 weeks (105 d) until the third harvest (from planting), equivalent to 30 d, 58 d and 79 d post-patch addition. Pre-germinated seeds were added to the plant compartments on 8th June

2011 and soil was added to the outer compartments 12 d later, with patches inserted 26 d after planting (described in Section 2.2.3.4).

2.2.3.2 Microcosm design

Plants were grown in microcosm units each with three compartments separated by double layered mesh membranes (see Figure 2.4 and Figure 2.5). The units were developed following the testing of CO_2 fluxes across the mesh membranes outlined in Section 2.2.2. Each microcosm consisted of a central 'plant' compartment (volume: 2L, dimensions: 150 x 150 x 150 mm; Thumbs Up Ltd., Bury, UK), containing a single *Z. mays* plant inoculated with *G. intraradices* (see Section 2.2.3.3), and on either side of the central plant compartment, two soil compartments (volume: 2.6L, dimensions: 140 x 140 x 160 mm; Lock & Lock, Australia PTY Ltd., Australia). The central compartment had windows cut out of either side (65 x 65 mm) to which the outer soil compartments were attached.

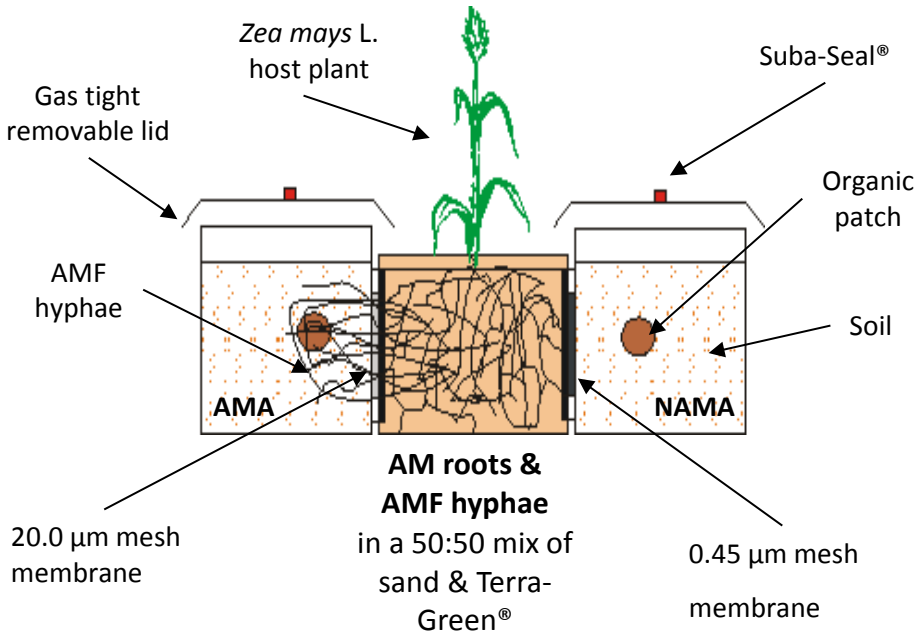


Figure 2.4. Diagram of the three compartment microcosm design. The two outer compartments both contained soil; AMF hyphae had access to one side (AMA; 20.0 µm mesh) but not the other (NAMA; 0.45 µm mesh). The central plant compartment contained AMF hyphae and plant roots, grown in a sand and Terra-Green® medium (50:50 mix v:v) with no soil. The gas sampling lids were removable and when not in used were replaced by aluminium foil to reduce photosynthetic growth in and moisture loss from the outer compartments.

There was a 5 mm thick Perspex plate (95 mm tall x 12 mm wide) separating the central and outer compartments which also had a 65 x 65 mm window cut out of the centre. The window in the Perspex plate was covered on either side by mesh that was stuck on using industrial strength superglue (Everbuild building products Ltd. Leeds, UK) creating a double layered mesh membrane which, when the unit was filled with media, were pushed together preventing any air gaps. The mesh window either allowed AMF hyphal access (AMA; 20.0 µm mesh; Section 2.2.1.2) or prevented AMF hyphal access (NAMA; 0.45 µm mesh; Section 2.2.2.2) from the central plant to the

outer soil compartment. Each unit had one AMA outer compartment and one NAMA outer compartment, creating a paired design.

There was a supporting stainless steel mesh (0.25 mm aperture; Mesh Direct, Hanscan Ltd., Burslem, UK) inside the plant compartment to reduce root pressure on the fine meshes (0.45 µm and 20.0 µm) which was held in place using a thinner PVC plate (2 mm thick, 90 mm tall x 120 mm wide, with a 65 x 65 mm window in the centre). The mesh covered Perspex plates were fastened in-between the central and outer compartment using a series of eight screws and nuts positioned at regular intervals, these also held the stainless mesh and supporting PVC plate in position. Aquarium sealent (Aquamate, Everbuild Building Products Ltd. Leeds, UK) was applied between all adjoining surfaces and around the screws to prevent the AMF hyphae accessing the outer compartments through any route other than the mesh membrane. Drainage holes (6 mm diameter) were drilled into the base of each box within the units to allow drainage of water. All seals were coated in petroleum jelly (Vaseline®, Surrey, UK) to minimise the risk of gas leaks when the gas tight lids were attached. The microcosms were wrapped in double layered aluminium foil to prevent any photosynthetic growth on the walls of the units, and the soil compartments were covered with a foil layer when the lids were not attached to prevent them from drying out. These fittings are outlined in Figure 2.5.

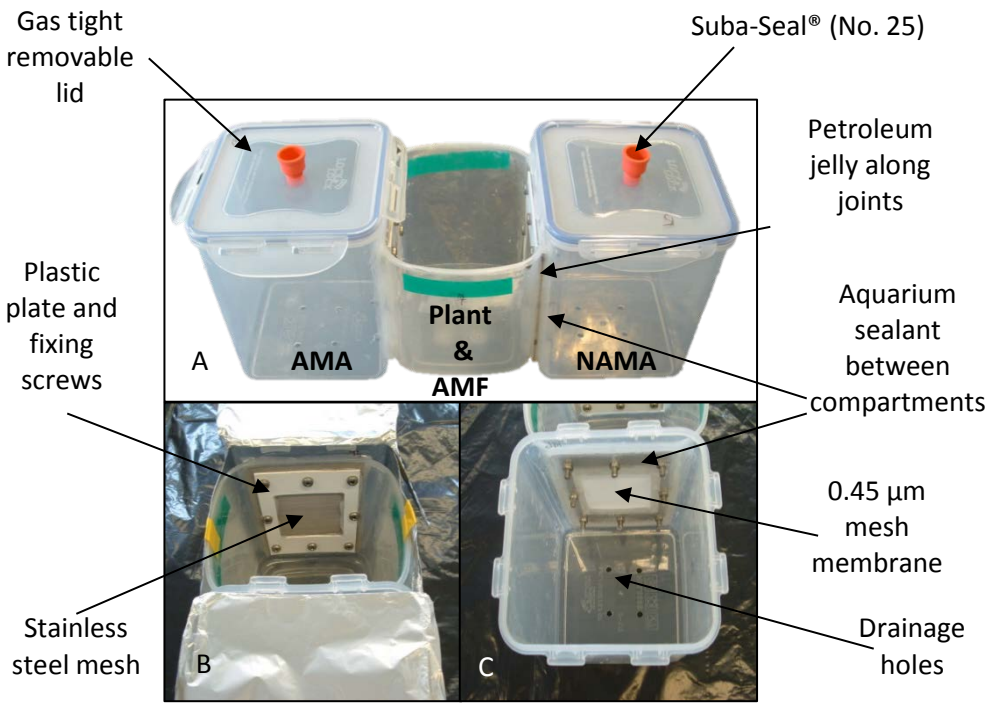


Figure 2.5. Photographs of the three compartment microcosm design showing the unplanted unit with cover-boxing lids (A), a stainless steel covered mesh window inside the central plant compartment (B) and the fine mesh membrane (0.45 µm) as viewed from the outer compartment (C).The AMF access (AMA) and no AMF access (NAMA) compartments were on either side of the planted compartment (Plant & AMF) (A) and fittings including drainage holes (C), screws (B), sealant (A,C), and petroleum jelly (A) used to drain, attach and seal the compartments have been identified.

2.2.3.3 Growth media

The plant compartments contained a 50:50 (v:v) mix of sand and Terra-Green® (Section 2.2.1.3). The sand and Terra-Green® mix had been washed in deionised water and autoclaved twice with a week gap between autoclave events, then mixed with 0.25 g L^{-1} of bonemeal (Section 2.2.1.3) and 150 g (FW) of live *Glomus intraradices* inoculum (see Section 2.2.1.3), totalling 2 L per pot. *Z. mays* seeds (Section 2.2.1.3) were sterilised in 3% sodium hypochlorite and pre-germinated for 24 hours on filter paper before planting three seedlings per unit. The seedlings were thinned to one per unit two weeks after sowing. The soil was sieved through a 2 mm sieve and well mixed

before 2 L was added to each of the outer compartments, 12 d after the plant compartments were planted.

2.2.3.4 Organic matter patches

All outer soil compartments contained a discrete zone or 'patch' of organic matter comprised of 2 g (DW) of dried, milled *Z. mays* shoots and 14.8 g (FW, equivalent to 13 g DW) of soil and made as in Section 2.2.1.4. This patch was added to each outer compartment to encourage hyphal growth and create potential N_2O 'hot-spots'. Sterile centrifuge tubes (50 cm^3), were placed in the soil to create a space for the patches which were added two weeks after soil addition (26 d after planting). The *Z. mays* shoots were purposely grown for patch material to have a high N content. *Zea mays* seedlings (Incredible F1, Mr Fothergills, Newmarket, UK) were grown in F2 + S compost (Scotts Levington, UK) for between 4 and 6 weeks and fed as required with 80 cm^3 of a full nutrient solution (Thornton & Bausenwein, 2000) modified to include 3 x Iron (Fe) as some shoots were showing Fe deficiency. The leaves were then harvested, dried at 70^oC until they reached constant mass before milling for use in the patches.

The composition of the patches is outlined in Table 2.1. Patches were added to the soil compartments 3 cm from the mesh and 8 cm deep by removing the centrifuge tubes and placing the patch bags into the remaining hole. Each patch received 1 cm^3 of deionised water to bring the patch water to approximately the same water content as the surrounding soil. Any remaining gap was filled with soil of the same type with the same water content.

Table 2.1. Carbon (C) and nitrogen (N) content of the mixed organic patch material (13 g DW equivalent soil mixed with 2 g DW milled *Z. mays* leaves) and *Z. mays* leaves that were used in the mixed organic matter patches before addition to the microcosms. Total values are for 15 g (DW equivalent) mixed organic patch and 2 g DW of milled *Z. mays* leaves.

	Total C (mg)	% C	Total N (mg)	% N	C:N Ratio
Mixed patch	1845	12.3	145.5	0.97	13:1
Plant Material	876.2	43.81	79	3.95	11:1

2.2.3.5 Experiment growth conditions

The microcosms were placed in a temperature controlled glasshouse. There were temperature probes in the centre of each block and a PAR sensor rotated between blocks weekly, all of which were logging every 30 min. A hand-held PAR sensor was used to measure PAR readings from each block three times a week and there were no significant differences in PAR between blocks. The mean PAR level measured daily at midday was 187 ± 7 µmol m^{-2} s^{-1} and the mean daily temperature over the experimental period was 20.1 ± 0.02°C.

Water contents of the soil compartments were maintained by measuring soil water content weekly using a SM200 Water Sensor (Delta-T Devices, Cambridge, England) attached to a Multimeter (Meterman, UK) calibrated to the soil used. Deionised water was added once a week to these compartments to equal approximately 19% moisture (volumetric) content, thus counteracting any effects of AMF hyphal presence on water availability. This level of water content was based on the original field water content at soil collection. Additionally, one unit in each block had moisture probes in both sides of the unit to determine if the water content of the AMA vs. NAMA compartments drifted apart throughout the week as a result of the presence of AMF hyphae. Once a week the plant compartments received 50 cm^3 of a nutrient solution modified from

Thornton & Bausenwein (2000) ($1/10^{th}$ N and P; see Appendix 1). This addition was increased to twice a week at 44 d post-planting as the plants were showing signs of nutrient deficiency. At 58 d the nutrient additions were increased to 3 times per week, two at $1/10^{th}$ N and P and one at full N, $1/10^{th}$ P as the plants were showing signs of N deficiency following the first harvest.

2.2.3.6 Microcosm based gas sampling

The two outer (soil) compartments had a gas tight lid that could be fitted during gas sampling *in situ* (as used in Section 2.2.1.1), enclosing a 600 cm^3 headspace volume above the soil surface. These lids were used to enable gas sampling by one of two methods (see Figure 2.6; as described in Section 2.1); firstly manual samples were taken using a cover-box technique (forming a closed static chamber (CSC)) followed by the second method of measurements taken using a continuous flow loop (similar to the closed-dynamic chamber, CDC method). Following the cover-box sampling (CSC), a modified lid was fastened onto each soil compartment in turn which was attached to a flow through system (CDC). This system was attached to a continuous flow loop with a Los Gatos bench top CH_4 recorder (LGR) which measured the concentration of CH_4 once a second (Los Gatos Research, Inc. California, USA), and an infra-red gas analyser (IRGA) that measured the CO_2 concentration once a second (LI-COR® Biosciences, Lincoln, USA). The lid was attached to each of the 36 soil compartment fluxes in sequence for a minimum of 5 min, with 2 min of flushing the system with lab air between each compartment measurement and a circulating air flow rate of approximately 1.5 L min^{-1}.

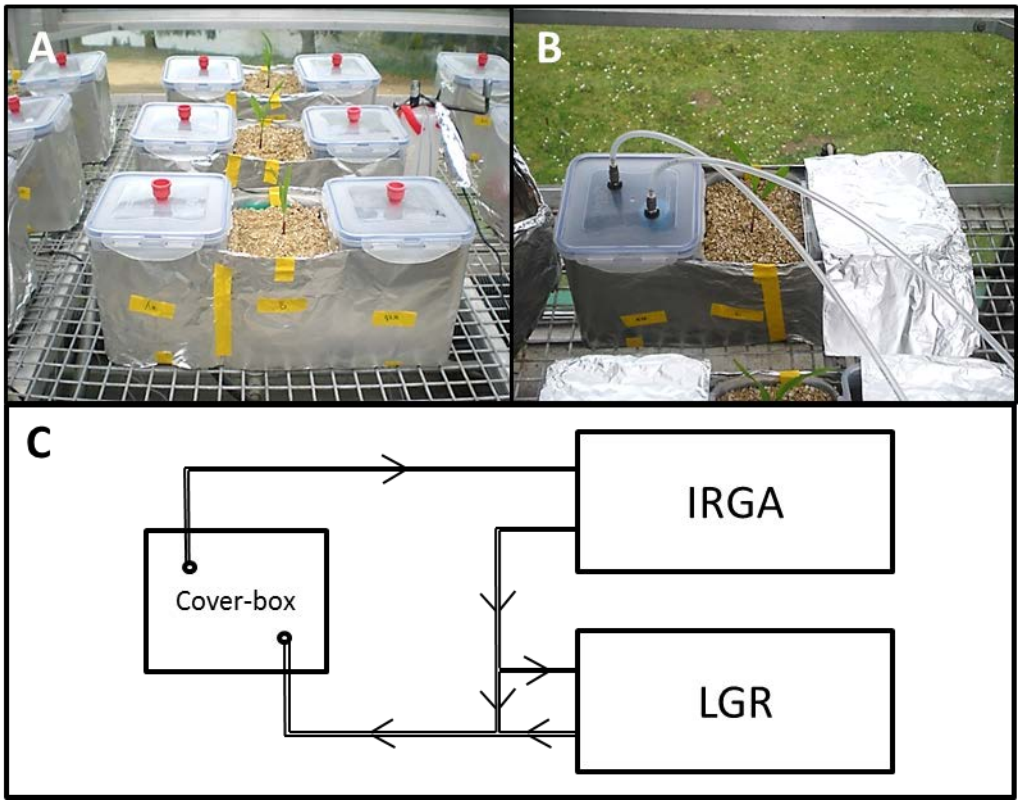


Figure 2.6. Photographs (A,B) and diagram (C) showing the two microcosm based pre-harvest (*in situ*) gas sampling methods, firstly forming a closed static chamber (CSC) using the cover-box technique (A) and secondly forming a flow-through system (B,C) with a closed loop of air flowing in a loop through the attached IRGA and LGR (CDC; not to scale).

Background gas fluxes were measured 24 h after soil addition (13 d after planting). Gas sampling was carried out at 13, 27, 41, 55, 69, 83 and 104 d post planting (pre-harvest; equivalent to 1, 15, 28, 42, 56, 70, and 91 d post-soil addition). During the first week of gas sampling, the drainage holes in the soil compartments were sealed with tape to reduce potential gas leaks but because of concerns for disturbance of growing hyphae, this discontinued for future measurements with the drainage holes being left open. For the cover-box samples, the lids were attached for 2.5 h with 7 cm^3 gas samples being removed through a Suba-Seal® (No. 25, Sigma-Aldrich, UK) in each lid every 50 min. All manual gas samples were stored in pre-evacuated 3 cm^3 Exetainers® (Labco Ltd., Buckinghamshire, England) which were over pressured to 7 cm^3. The Exetainers were

pre-evacuated for a minimum of 15 s using a vacuum pump (KNF Neuberger, Inc. Oxfordshire, Great Britain).

2.2.3.7 Harvest data collection

At each harvest the following data were collected; fresh and dry weights of shoots, roots, stalk, flowers, ear, soil, sand/Terra-Green® and patches, soil and patch gravimetric water content (g g DW $^{-1}$). The shoot material was cut off and separated into leaves, dying leaves (> 50% of the leaf dried up), stalk, ear and tassel and the roots were picked out of the growth medium using forceps for 5 min before washing to clear away any attached growth medium, patting dry and weighing. Two 5 g (FW) samples of sand/Terra-Green®, soil (from AMA and NAMA compartments) and AMA and NAMA organic patches (post-gas sampling, as outlined in Section 2.2.3.8) were taken to determine the length of the AMF ERM. This was measured following the modified membrane filter technique (as in Section 2.2.1.6). At least 50 grid squares were counted at x 100 magnification on a Nikon Eclipse 50i microscope (Nikon Instruments Europe B.V.) and ERM lengths were converted to densities (m hyphae g^{-1} DW soil). The soil samples were taken from all four sides, the top and bottom of each soil compartment, well mixed and stored in a plastic bag at 4^{o}C in the dark until ERM measurements were taken (no more than 96 h post-harvesting). Additional, 20 g (fresh weight), soil samples were taken from the same sample and stored in Wheaton bottles for gas flux analysis in the absence of the patches post-harvest (explained in detail in Section 2.2.3.8).

The percentage total root length colonised (RLC), % arbuscules, and % vesicles for the *Z. mays* roots were assessed following the method of McGonigle *et al*. (1990). Briefly, the roots were picked out of the sand/Terra-Green® mix, washed in deionised water and then split into sub-samples for dry weight and staining. The sub-samples for staining were placed into 20% KOH for 3 d and then placed into 1% HCl to acidify the roots for 1 hour before being moved into a 0.01% acid fuchsin stain (lactic acid,

glycerol, deionised water 14:1:1 and 0.1 g L^{-1} acid fuchsin) for a further 3 d. The roots were then stored in destain (lactic acid, glycerol, deionised water 14:1:1) for at least 2 d before being mounted onto microscope slides (after Grace & Stribley, 1991; Koske & Gemma, 1989; Walker & Vestberg, 1994). The number of intersects with a gridline at 200 x magnification were counted for each of the hyphae, arbuscules and vesicles, with at least 100 root intersections taken for each replicate (Mcgonigle *et al.*, 1990). The percentage of the root length colonised was then calculated from these values. Patch C:N ratios were determined by analysing dried, milled patch samples in an elemental combustion system (Costech Analytical Technologies Inc., California, USA). Patch N and C contents were calculated using the % N and C values from elemental combustion system and converted to masses using the dried weights of the patches following each harvest.

2.2.3.8 Post-harvest gas flux measurements

Patches

Following the first harvest (56 d), the organic patches were removed intact (in their bags), surface debris removed and weighed. The patches were then placed into 50 cm^3 syringes and left in the dark for 24 hours at 20^oC and then assessed for gas production rates. This involved creating a syringe based gas sampling system, as shown in Figure 2.7. The patch was placed in the 50 cm^3 syringe; a second 10 cm^3 syringe was attached to the 50 cm^3 syringe at the inlet via a 3-way tap with a luer lock fitting to which a needle was connected. The 50 cm^3 syringe was filled with 57 cm^3 of air and the 10 cm^3 syringe was empty, with the whole system closed (no ambient air access). A known concentration of CH_4 was added to produce a final volume of 60 cm^3, with an initial CH_4 concentration of 20 ppm (see methane dilutions Section below).

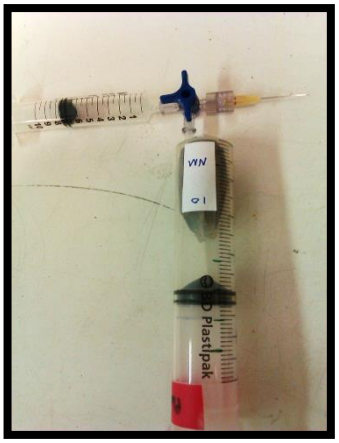


Figure 2.7. Syringe-based system for measurement of gas fluxes from nutrient patch mesh bags following destructive harvests. The patch bag was placed inside the 50 cm^3 syringe which was then closed to outside air. Gas samples (7.5 cm^3) were removed from the 50 cm^3 syringe at regular intervals using the 10 cm^3 syringe attached by the 3-way tap. This allowed any change in N_2O production to be quantified over time.

At each gas sampling event, the 3-way tap was opened to the connected 10 cm^3 and 50 cm^3 syringes, allowing air surrounding the patch (in the 50 cm^3 syringe) to be sampled. This was well mixed by pumping the 10 cm^3 syringe, with a final 7.5 cm^3 sample collected in the 10 cm^3 syringe. The tap was then moved to connect the 10 cm^3 syringe to the needle and slight pressure was applied to the 10 cm^3 syringe plunger (releasing 0.5 cm^3 into the atmosphere) and the remaining 7 cm^3 sample was transferred into a pre-evacuated 3 cm^3 Exetainer through the needle. At the first harvest (56 d), four 7.5 cm^3 samples were taken at 50 min intervals over 2.5 hours. This was found to yield very high N_2O concentrations and consequently, during the subsequent harvests it was decided to take four 7.5 cm^3 samples every 20 min over one hour. The N_2O fluxes were found to be linear during this time. The syringes were kept in the dark at 20^oC when not being sampled. Following gas sampling, the patches were destructively harvested for ERM analysis and dry weight collection within 96 h of the start of the harvest (as described in Section 2.2.3.7).

Soils

At each harvest 20 g (FW) samples of soil were sampled from both soil compartments of each unit. Each time a soil sample was collected it was well-mixed sample from the front, sides, top and bottom of the compartment. Each sample was then placed into a Wheaton bottle (detailed in Section 2.2.3.7) which was then sealed with Parafilm (Pechiney Plastic Packaging Company; Chicago, Illinois, USA), to allow gas flow but limit water loss, and then stored in the dark at 20^{o}C for 24 hours before gas sampling.

During each gas sampling event, the Wheaton bottles were initially left outside for a minimum of 30 min with the Parafilm removed to allow the mixing of air from within the bottles with outdoor air to reduce any chance of contamination. The bottles were then sealed using rubber septa and aluminium crimp caps and immediately over-pressured (by 25 cm^3) with a mixture of air and a known concentration of methane (CH_4) to make the CH_4 concentration approximately 20 ppm within the bottle (see methane dilutions Section below). Gas was then sampled (7.5 cm^3) every 50 min for 2.5 h. Soil dry weights were estimated from additional parallel soil samples, dried at 105^{o}C to constant mass.

This procedure was carried out once for harvest 1 (56 d post-planting) soils. The soils were sampled again at harvest 2 (84 d post-planting). At harvest 3 (105 d post-planting), two samples were taken from each of the AMA and NAMA compartments, and an average of the two samples for each were used as replicates for the soil gas fluxes, and otherwise treated the same as those taken previously. After the first sampling event, half of the bottles (6 AMA, 6 NAMA) received 7 cm^3 of a 60 mM KNO_3 solution (equivalent to a mid-range to high N addition) (Abbasi & Adams, 1999; Wang & Ineson, 2003), whilst the other half of the bottles received 7 cm^3 of glucose ($C_6H_{12}O_6$) at a concentration equivalent to 200 µg C g^{-1} DW. This concentration was chosen as a mid-range glucose addition (see Koops *et al.*, 1996; Abbasi & Adams, 1999; Murray *et al.*, 2004). These were then left for 24, 48 and 96 h between the 2nd, 3rd and 4th gas

sample events respectively. In between sample events the Wheaton bottles were covered with Parafilm and stored in the dark at 20^{o}C.

Methane dilutions

A known amount of CH_4 was added to allow quantification of potential CH_4 oxidation rates as preceding the experiment, the soil used had been found to oxidise CH_4. In all of the sealed bottles/syringes measured for gas sampling, a known concentration of CH_4 was added. The CH_4 added was diluted from a pure (1000000 ppm) sample of CH_4 in a series of dilution steps using evacuated 12 cm^3 Exetainers (over-pressured to 20 cm^3) and Wheaton bottles. The Wheaton bottles were sealed, and flushed with N_2 for at least 10 minutes using one needle as an inlet and another as an outlet (0.5 mm x 25 mm, BD Microlance 3; Becton, Dickinson and Co., USA). They were then over-pressured using a mix of N_2 and CH_4 to 200 cm^3. A set amount (depending on the required CH_4 concentration/volume) of this CH_4/N_2 mix was then removed from this stock bottle and added to the syringe/Wheaten containing the patch/soil sample respectively to make the CH_4 concentration up to 20 ppm. A CH_4 concentration of 20 ppm was selected as this concentration would be suitable for high affinity methane oxidisers and is similar to the range used in previous studies (e.g. Wang & Ineson, 2003) to quantify potential high affinity CH_4 methane oxidation rates.

2.2.3.9 Gas sample analysis

All manually collected gas samples (i.e. not sampled using the IRGA or Los Gatos) were stored in 3 cm^3 Exetainers (overpressured to 7 cm^3) and analysed using a Perkin Elmer ARNEL Autosystem XL Gas Chromatograph (GC) with a flame ionisation detector (FID) and an electron capture detector (ECD) which measured CH_4, CO_2 and N_2O concentrations. Eight reference samples of known concentrations (certified mixed standard from BOC, UK) of CH_4 (103 ppm), N_2O (9.4 ppm) and CO_2 (523 ppm) were analysed within each GC run as pairs at regular intervals to allow calculation of the concentrations of the unknown samples. These references were also used to correct

for any machine drift, although machine drift was rare. Nitrogen 'blanks' were also included at the beginning and end of each run to check for consistency. Once the samples has been run through the GC, the gas inside the exetainers would be back at atmospheric pressure, leaving 3 cm^3 of gas sample in the 3 cm^3 exetainer. Where N_2O concentrations were out of range of the GC detection (i.e. in the 56 d harvest patch samples and the 105 d harvest soil + glucose samples) the Exetainers were re-pressurised to 7 cm^3 by adding 4 cm^3 of N_2. Parallel standards of known N_2O (9.4 ppm) concentration were also diluted and re-run through the GC. This was repeated until the samples came into the range of the GC again and the original ppm values were calculated using the dilution factors calculated from the concentration of the diluted standards. Post-harvest CO_2 fluxes were not used as the sample period was 50 min between time points, and therefore an accurate flux rate could not be generated for CO_2 from either the patches or the soils.

2.2.3.10 Gas flux calculations

Gas concentrations of unknown samples were calculated from the GC outputs (as area values) using SAS (v9.3 SAS institute Inc., North Carolina, USA) and were always calibrated against gases of known concentrations (outlined in Section 2.2.3.9). To prevent the use of data arising from rare injection or Exetainer failures, two criteria had to be met otherwise standards were discarded. The first was that the area measure on the GC for each gas could not be below a low minimum value (CH_4: 150000, CO_2: 200000, N_2O: 200000). This was to prevent the inclusion of standards that had failed to inject correctly. The second criterion was that the mean of the pair of standards at any point in the run was not greater than 1.96 x their standard deviation away from the mean of all standards in the run. This was to ensure that there were no contaminated standards included in the run. For example, if the standard had become contaminated with CO_2 and had a higher than certified CO_2 concentration, this step would remove that sample from the calculations. Contamination or false injection of any kind was very rare but both were allowed for by using these criteria to ensure that the most accurate data were obtained.

Linear regressions were used to calculate flux values for each of the three gases, N_2O CH_4 and CO_2 by plotting the concentration change against time. For each gas the concentrations (ppm) were converted to mass (mg) using the headspace volumes at standard temperature and pressure. There were four measurement time points for each gas, t_0 (0 min), t_1 (20 or 50 min), t_2 (40 or 100 min), t_3 (60 or 150 min), the different time intervals being for patch (20 min) and soil or cover-box (50 min) sampling respectively. A regression procedure was used to calculate the gradient of the line representing the change in mg over time for each gas. As for the reference standards, there was a small possibility that a sample may have become contaminated or falsely injected into the GC. As the samples were far more variable than the standards, different criteria were used to prevent any values with undue influence from being included. Studentized residuals were calculated for each value in each regression by dividing the residual by the estimated error variance in the absence of that data point. Studentized residuals are considered to indicate an outlier if the absolute value is > 2 (SAS, v9.3 SAS institute Inc., North Carolina, USA). In this case, if the number of time points included in the initial regression was greater than three and the absolute value of the studentized residual for any of the measurements in that regression exceeded 5, then that measurement was not included in the final regression used to calculate the flux value. A studentized residual of 5 was used to ensure that the measurements were only removed rarely and in extreme cases (e.g. false injections/contamination). This method was used for all flux calculations derived from GC gas analyses.

Gas concentration measurements from the flow-through system were calculated in a similar way. Initially, concentration values were converted to masses as above (Section 2.2.3.10); these were then converted to flux values (mg m^{-2} h^{-1}). The length of time that the lid was attached for was recorded. The fluxes were calculated using values measured between 120 to 200 s after the lid was attached for both CO_2 and CH_4. Linear regressions were then used to calculate the change in the concentration of each gas over time, with the time interval between measurements being one second. All regressions were run in SAS (v9.3 SAS institute Inc., North Carolina, USA) to obtain mg

s^{-1} values. The headspace in the microcosm unit (0.6 L), volume of connecting tubing (0.274 L) and internal volume for each instrument (IRGA, 0.019 L; LGR, 0.408 L) along with the surface area of soil sampled (0.024 m^{-2}) were used in the calculation of the flux rates of each of the gases, in mg m^{-2} h^{-1}. As this method did not involve any storage of samples or dilution procedures, there were no analytical outliers.

Cumulative fluxes were calculated by plotting the bi-weekly flux values, integrating the area under the curve for the days specified and then dividing that value by the number of days. At the 56 d post-planting harvest, the 0 and 50 min samples were the only ones used for the patch N_2O flux calculations as the N_2O concentrations were high and may have started to back diffuse past this point. In order to create true replicates for soil N_2O fluxes at the third (105 d post-planting) harvest (when two soil samples were taken from each replicate) the mean of these two samples was used as the true replicate to prevent pseudo-replication. All gas flux calculations were carried out in SAS (v9.3 SAS institute Inc., North Carolina, USA).

It was originally intended to compare the compartment (pre-harvest) and post-harvest gas fluxes to check the methods used. However, the disturbance of the destructive harvests greatly affected the gas fluxes from the patches (particularly for N_2O) therefore these comparisons were not informative and as such have not been included.

2.2.3.11 Data analysis

All data were tested for normality and equality of variance assumptions using Kolmogorov-Smirnov and Levene's Homogeneity of Variance tests in SAS (v9.3 SAS institute Inc., North Carolina, USA) respectively. SAS was used for all data analyses.

Where patch compartment data for AMA or NAMA treatments (e.g. gas fluxes, patch moisture contents, patch C and N content etc.) were analysed over independent time points or among harvests, a two-way analysis of variance (ANOVA) including block with Duncan's multiple range *post hoc* tests was used. Differences between AMA and NAMA treatments were analysed by comparing the AMA – NAMA value to zero using a one-sample t-test at each time point or harvest. Where normality or equality of variance assumptions were not fulfilled, the data were $\log_{10}$ transformed. If transformations failed to produce normality or equal variances, an equivalent Friedman's non-parametric two-way ANOVA with Wilcoxon *post hoc* tests and Bonferroni corrections was used. Similarly, Wilcoxon Signed Ranks tests were used for comparing differences from zero. All proportion or percentage data were arcsine transformed before analysis.

Plant compartment data (e.g. percentage root length colonised by AMF) were also analysed using a two-way ANOVA including block with Duncan's multiple range *post hoc* tests, unless assumptions were not fulfilled in which case a Friedman's non-parametric two-way ANOVA with Wilcoxon *post hoc* tests and an applied Bonferroni correction were used. Where all harvest data were combined to assess relationships between variables either a Pearson or Spearman rank order partial correlation was used to control for the effect of harvest time depending on whether data passed or failed normality assumptions respectively. Where relationships between variables within harvests were tested, unless data failed normality assumptions, data were correlated using Pearson's correlations; if data failed normality assumptions, Spearman's correlations were used.

Measured soil N_2O fluxes from harvest 3 (105 d post-planting) following KNO_3 addition failed normality and equality of variance assumptions; and were $\log_{10}$ transformed before analysis. Soil N_2O fluxes from the same harvest following glucose addition fulfilled all normality assumptions and were not transformed. In both cases, paired t-tests were carried out for each time point. Repeated measures ANOVAs failed to show

any significant effects other than time and, as the number of time points was low, the risk of type one error from individual t-tests was considered very low.

Patch C:N ratios did not fulfil equality of variance or normality assumptions, and were therefore analysed using a Friedman's two-way non-parametric ANOVA. The differences between AMA and NAMA patches were compared using a Wilcoxon Signed Ranks test on the difference compared to zero.

2.3 Results

2.3.1 Selecting a suitable host plant

The percentage total root length colonised by AMF (RLC) and arbuscules was higher in *Z. mays* and *P. lanceolata* roots compared to *L. usitatissimum* roots (RLC: $F_{2,8}$ = 19.79, P = 0.0008; arbuscules: $F_{2,8}$ = 9.17, P = 0.0085; Figure 2.8). However, the percentage of roots containing vesicles was significantly higher in the *Z. mays* roots than both the *L. usitatissimum* and *P. lanceolata* roots ($F_{2,8}$ = 10.99, P = 0.0051; Figure 2.8).

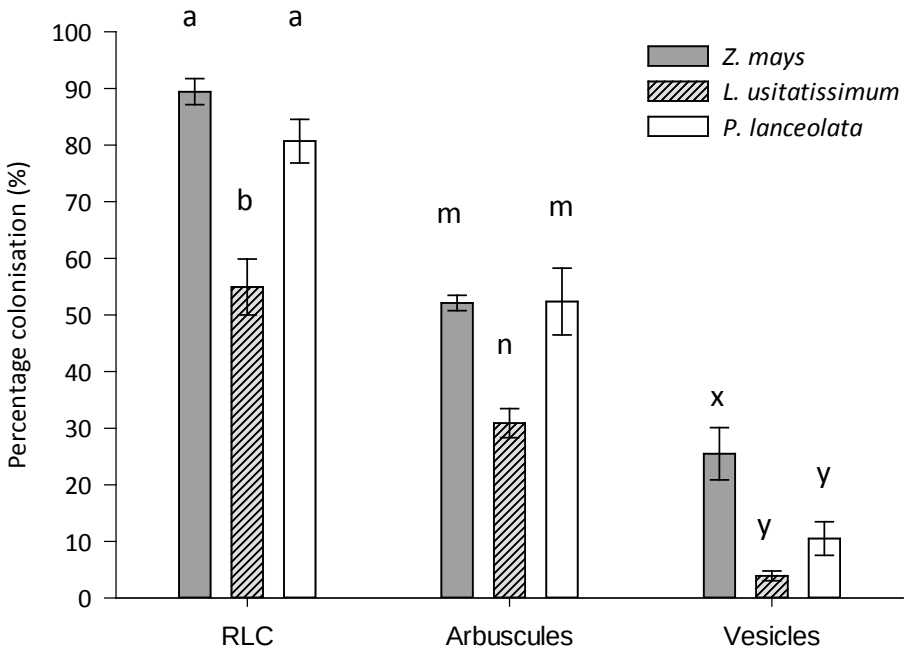


Figure 2.8. Mean percentage root length colonisation (RLC) and root length colonisation by arbuscules and vesicles (%) in *Z. mays* (solid bars), *L. usitatissimum* (hatched bars) and *P. lanceolata* (open bars) plants. Error bars represent ± standard error of the mean (n = 5). Different letters represent significantly different averages within each group identified using Duncans multiple range *post-hoc* tests (at the P = 0.05 level).

The *Z. mays* treatments had significantly higher extraradical mycelium (ERM) length densities in all three locations (Figure 2.9) than either *P. lanceolata* or *L. usitatissimum* (Plant: $F_{2,8}$ = 23.2, P = 0.0005; Patch: $F_{2,8}$ = 41.18, P < 0.0001; Soil: $F_{2,8}$ = 13.88, P = 0.0025), but there was no difference between these latter two species for any location (P > 0.05 in all cases).

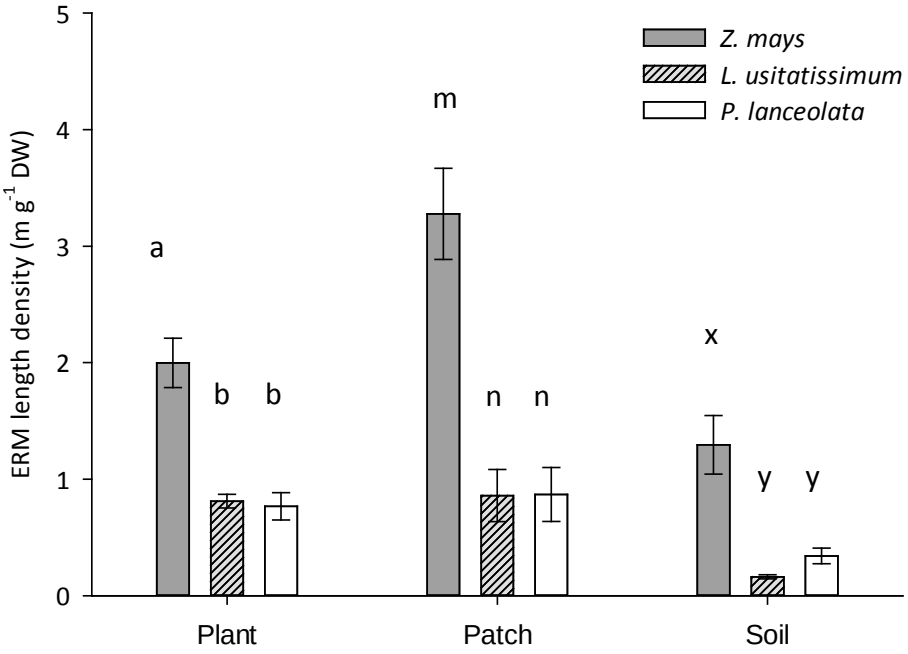


Figure 2.9. Mean extraradical mycelium (ERM) length density (m g^{-1} DW) measured in plant compartment, patch, and soil for *Z. mays* (solid bars), *L. usitatissimum* (hatched bars) and *P. lanceolata* (open bars) plants.Error bars represent ± standard error of the mean (n = 5). Different letters represent significantly different results within each group (plant, patch or soil; at the P = 0.05 level) following *post hoc* analysis using Duncans multiple range tests.

2.3.2 The effect of fine mesh presence on CO_2 diffusion rates in microcosm systems used to study AMF

There was no significant difference in the length of time taken for the CO_2 concentration to reach half of its absolute maximum in the second compartment among the three mesh treatments (Figure 2.10; $F_{2,6} = 0.68$, $P = 0.543$).

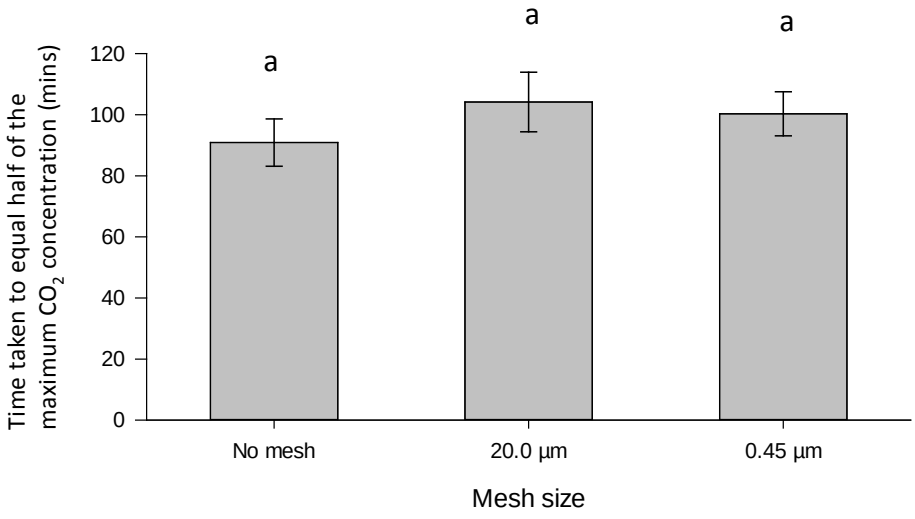


Figure 2.10. The mean length of time taken for the CO_2 concentration in the second half of the microcosm unit to reach half of its maximum for three mesh treatments; no mesh, 20.0 µm mesh and 0.45 µm mesh. There was no significant difference between treatments (one-way ANOVA) as indicated by the lettering. Error bars represent ± standard error of the mean ($n = 3$).

2.3.3 The interactions between AMF and soil CO_2, N_2O and CH_4 fluxes

2.3.3.1 AMF growth and colonisation of *Zea mays*

Colonisation of *Zea mays* roots

The *Z. mays* roots were well colonised by *G. intraradices* at the first harvest (56 d) and remained highly colonised thereafter with no significant differences between subsequent harvests (Table 2.2; Q_2 = 4.95, P = 0.084). The percentage of roots colonised by vesicles did not differ among harvests (Table 2.2; $F_{2,13}$ = 3.02, P = 0.084), but the frequency of arbuscules was higher at harvests 2 (84 d) and 3 (105 d) compared to harvest 1 (56 d; Table 2.2; Q_2 = 9.810, P = 0.0074). All percentage colonisation data were significantly different from zero ($P < 0.05$).

Table 2.2. Mean percentage root length colonised by AMF (%RLC) and percentage of roots colonised by arbuscules and vesicles for *Zea mays* L. plants at each harvest (days since planted) ± standard error of the mean (n = 6).Statistical significance among harvests was determined using Duncans multiple range *post hoc* tests for vesicle data and Bonferroni *post hoc* tests for RLC and arbuscule data. Rows within the same column with the same letters are not significantly different ($P > 0.05$).

	RLC (%)	Arbuscules (%)	Vesicles (%)
Harvest 1 (56 d)	88.2 ± 3.1^{a}	28.9 ± 4.2^{w}	16.1 ± 2.8^{z}
Harvest 2 (84 d)	93.1 ± 1.5^{a}	52.0 ± 6.6^{x}	27.1 ± 3.3^{z}
Harvest 3 (105 d)	94.4 ± 2.2^{a}	43.6 ± 2.9^{x}	28.9 ± 5.3^{z}

Extraradical mycelium (ERM) length densities

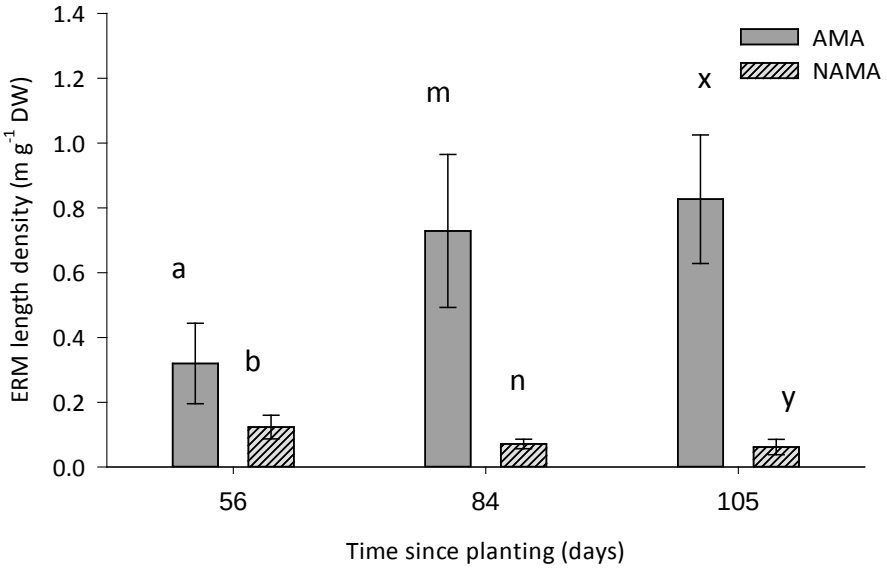
Hyphae were present in the plant compartments at the first (56 d) harvest, and the mean ERM length densities for all harvests were greater than zero ($P < 0.05$ in each case). There was no significant difference between the ERM length densities in the plant compartment at each of the three harvests (Table 2.3; $F_{2,13} = 1.40$, $P = 0.280$).

Table 2.3. Mean extraradical mycelium (ERM) length densities (m g^{-1} DW) in the central planted compartments for each harvest ± standard error of the mean ($n = 6$). There were no significant differences among harvests following a two-way ANOVA as indicated by the lettering ($P > 0.05$).

	ERM length density (m g^{-1} DW)	
Harvest 1 (56 d)	1.08 ± 0.25	a
Harvest 2 (84 d)	1.35 ± 0.12	a
Harvest 3 (105 d)	1.33 ± 0.09	a

There were no significant differences in the ERM length densities measured in the AMF access (AMA) compartments among harvests in either the soil (Figure 2.11a; $Q_{2,13} = 3.43$, $P = 0.180$) or patch (Figure 2.11b; $Q_{2,13} = 3.43$, $P = 0.180$). There were consistently higher ERM length densities in the AMA compartments than NAMA compartments for both soils and patches (Figure 2.11a,b; Table 2.4). There were no significant differences in AMA-NAMA ERM length densities among the three harvests in the soil ($Q_2 = 5.14$, $P = 0.077$) or patch ($Q_2 = 3.43$, $P = 0.180$).

a. Soils



b. Patches

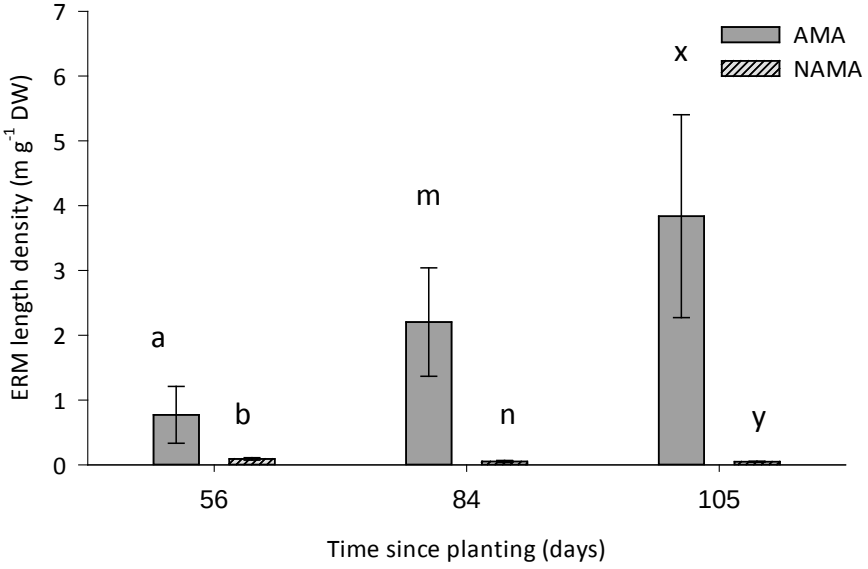


Figure 2.11. Extraradical mycelium (ERM) length densities (m g^{-1} DW) from AMF access (AMA; solid bars) and no AMF access (NAMA; hatched bars) (a) soils and (b) patches for each harvest at 56, 84, and 105 d since planting. Error bars represent ± standard error of the mean (*n* = 6). Different letters within a harvest represent a significant difference between AMA and NAMA treatments ($P < 0.05$) and were determined using a Wilcoxon test to compare the AMA – NAMA values to zero.

Table 2.4. One-way ANOVA or Wilcoxon signed ranks test results for AMF hyphal access (AMA) – no AMF hyphal access (NAMA) extraradical mycelium (ERM) length density data from patches and soils split by harvest (at 56, 84 or 105 d post-planting). The AMA-NAMA values were compared to zero using either a one way ANOVA or Wilcoxon signed ranks test. Significant values are highlighted in bold (*$P < 0.05$).

	Harvest		Test statistic	*P*
Patch	56	S_5	10.5	**0.031***
	84	t_5	2.59	**0.049***
	105	S_5	2.41	**0.031***
Soil	56	S_5	10.5	**0.031***
	84	t_5	2.77	**0.039***
	105	t_5	3.94	**0.011***

2.3.3.2 Pre-harvest trace gas fluxes in the presence of AMF hyphae

Cumulative CO_2 production decreased between harvests for both AMA ($F_{2,13}$ = 11.98, P = 0.0038) and NAMA ($F_{2,13}$ = 27.48, P < 0.0001) treatments. There was no significant difference between AMA and NAMA treatments preceding the first harvest (56 d post-planting; Figure 2.12; t_5 = 0.58, P = 0.587), but there was a significant difference in AMA and NAMA CO_2 fluxes leading up to the 84 d and 105 d harvests (Figure 2.12; 55 to 83 d post-planting: t_5 = 3.77, P = 0.013 and 83 to 104 d post-planting: t_5 = 2.62, P = 0.047). There was however, no difference between the AMA-NAMA CO_2 fluxes among the three time points ($F_{2,13}$ = 2.50, P = 0.120).

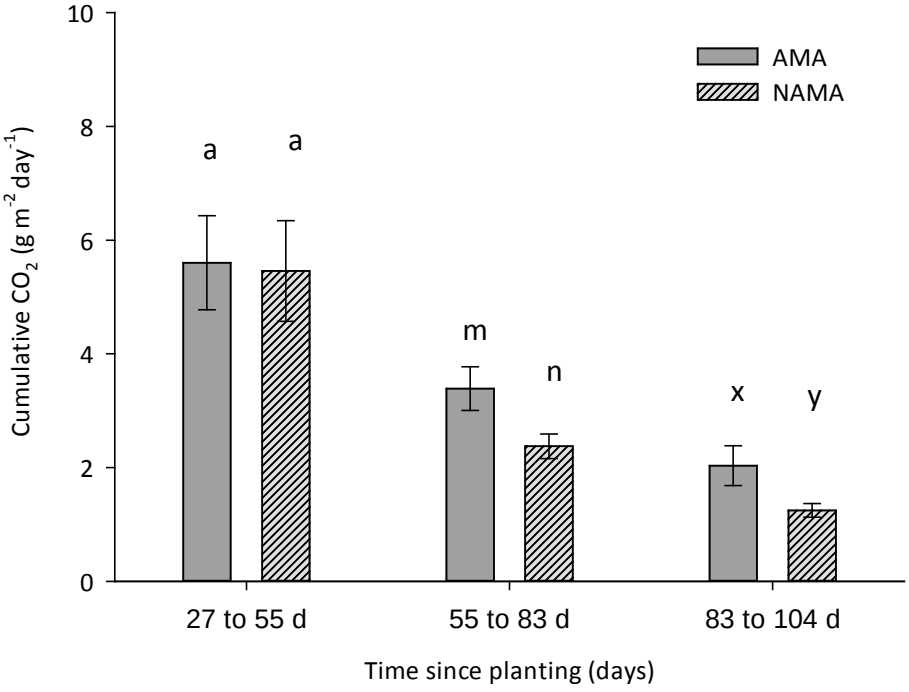


Figure 2.12. Mean cumulative CO_2 production for AMF access (AMA; solid bars) and no AMF access (NAMA; hatched bars) compartments for the time preceding each harvest (n = 6). Error bars represent ± standard error of the mean (n = 6). Bars with different letters indicate significant differences (P < 0.05) within each time period. Significant differences within each time period were determined using a one-sample t-test to compare the AMA – NAMA values to zero.

Both the AMA and NAMA compartments were producing N_2O but the rate of this production did not differ between the three harvest times in the presence of AMF hyphae (AMA; Q_2 = 1.81, P = 0.405) whereas it was lower leading up to the final harvest for the NAMA compartments (Q_2 = 8.86, P = 0.012). The difference between AMA and NAMA cumulative N_2O fluxes did not differ from zero at any time point (Figure 2.13; P > 0.10 in each case) or from each other over all three time points (Q_2 = 2.47, P = 0.291).

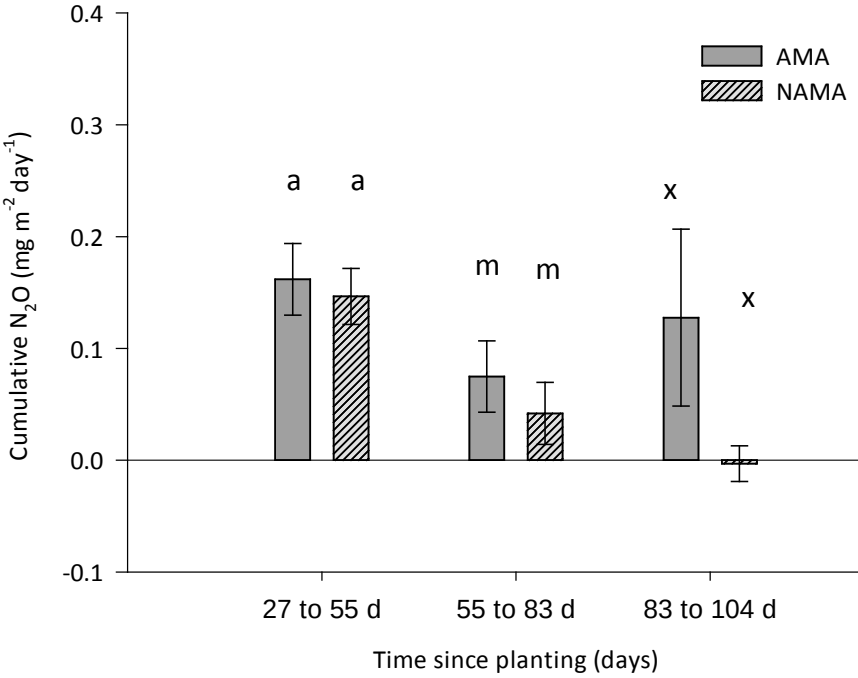


Figure 2.13. Mean cumulative N_2O production for AMF access (AMA; solid bars) and no AMF access (NAMA; hatched bars) compartments for the time preceding each harvest. Error bars represent ± standard error of the mean (n = 6). There were no significant differences between AMA and NAMA treatments within each harvest as indicated by letters (P > 0.05 in each case) as determined using one-sample t-tests to compare the AMA – NAMA values to zero.

The cumulative CH_4 oxidation did not differ over time in the AMA (Q_2 = 1.24, P = 0.539) or NAMA treatments (Q_2 = 5.81, P = 0.055). The difference between AMA and NAMA cumulative CH_4 oxidation was not significantly different from zero at any time point (Figure 2.14; P > 0.05 in each case), but the AMA and NAMA CH_4 oxidation rates were all significantly different from zero at each time point (P < 0.05 in each case). There was a significant difference among the AMA-NAMA values over the three time points (Q_2 = 7.68, P = 0.021), however, the source of this difference was not clear when a Bonferroni correction was applied as P > 0.016 in all cases.

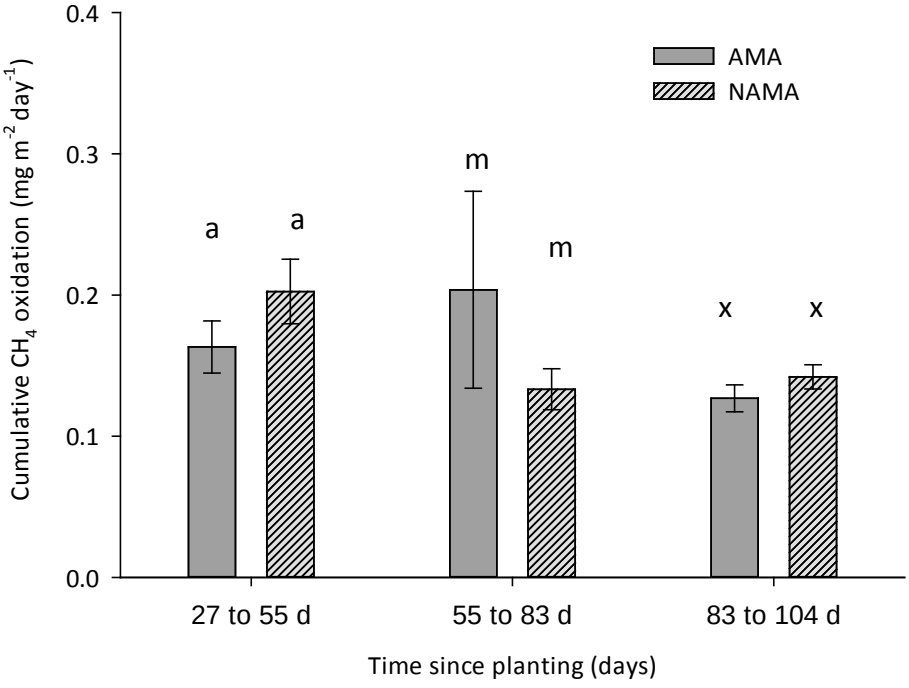


Figure 2.14. Mean cumulative CH_4 production for AMF access (AMA; solid bars) and no AMF access (NAMA) compartments for the time preceding each harvest. Error bars represent ± standard error of the mean (n = 6). There were no significant differences between AMA and NAMA treatments within each harvest as shown by letters (P > 0.05 in each case); determined using one-sample t-tests to compare the AMA – NAMA values to zero.

The pre-harvest CO_2 fluxes from the AMA compartments positively correlated against those from the NAMA compartments when controlling for harvest time (Figure 2.15; n = 18; r_s = 0.7620, P = 0.0004, r^2 = 0.6), but this was not significant within each harvest (P > 0.05 in each case). The pre-harvest CH_4 fluxes for AMA also positively correlated with those from the NAMA compartments overall when harvest time was controlled for (n = 18; r_s = 0.7734, P = 0.0003, r^2 = 0.6), but when each harvest was taken separately, the CH_4 fluxes from AMA and NAMA treatments were only positively correlated at the 105 d harvest (n = 6; r_s = 0.8286, P = 0.042, r^2 = 0.69). However, the pre-harvest AMA and NAMA N_2O fluxes did not correlate (r = 0.1964, P = 0.450) and the pre-harvest N_2O and CO_2 fluxes also did not correlate for either of the AMA or NAMA treatments (AMA: r = -0.0909, P = 0.729; NAMA: r_s = -0.2633, P = 0.307).

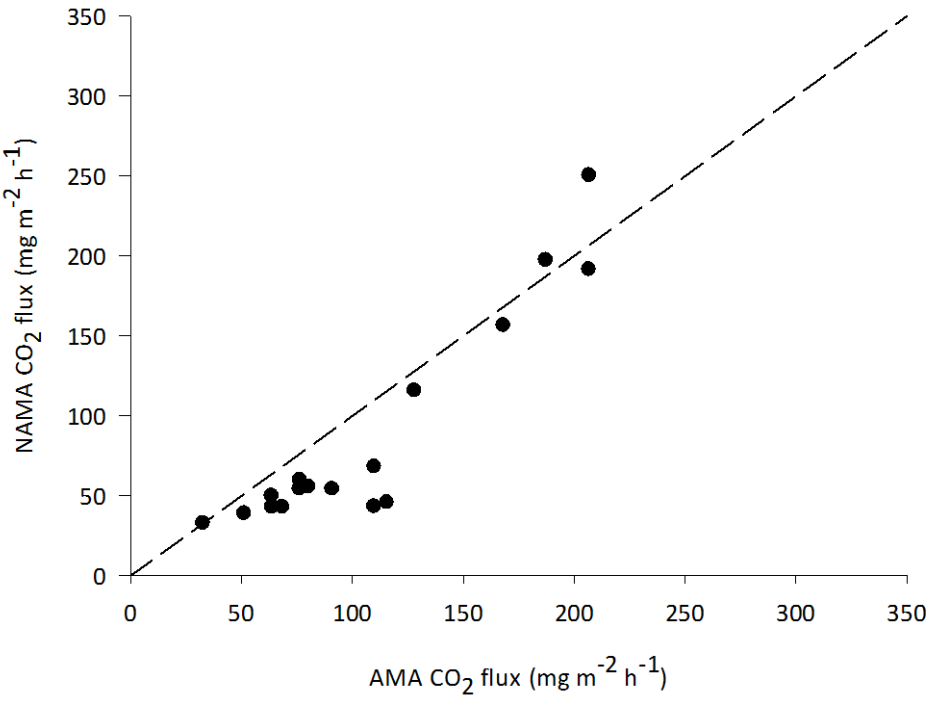


Figure 2.15. Carbon dioxide (CO_2) flux from the AMF access (AMA) compartment plotted against the CO_2 flux from the no AMF access (NAMA) compartment preceding each destructive harvest. Dashed line is a 1:1 line. A Spearman partial rank order correlation controlling for time was used to test the relationship between AMA and NAMA treatment CO_2 fluxes (n = 18, r^2 = 0.6).

The total ERM length of the outer compartment was estimated by multiplying the soil ERM length densities by the total mass of soil (estimated as 2000 g) and adding this to the total patch ERM length (calculated by multiplying the patch ERM length density by 15, as the patches were 15 g dw). The total ERM length estimates did not correlate with pre-harvest N_2O fluxes at any harvest, or overall ($P > 0.05$ in each case). The AMA total ERM lengths also did not correlate with the AMA CO_2 flux over all harvests when controlling for time ($n = 18$; $r_s = 0.4063$, $P = 0.106$), or preceding the 56 d or 84 d harvests (56 d: $r_s = 0.4286$, $P = 0.397$; 84 d: $r = 0.4661$, $P = 0.351$), but it did positively correlate with the CO_2 flux measured preceding the 105 d harvest (Figure 2.16; $r = 0.9806$, $P = 0.0006$, $r^2 = 0.9616$). There were also no significant correlations of the NAMA total ERM lengths against CO_2 flux, overall or for any harvest ($P > 0.05$ in each case).

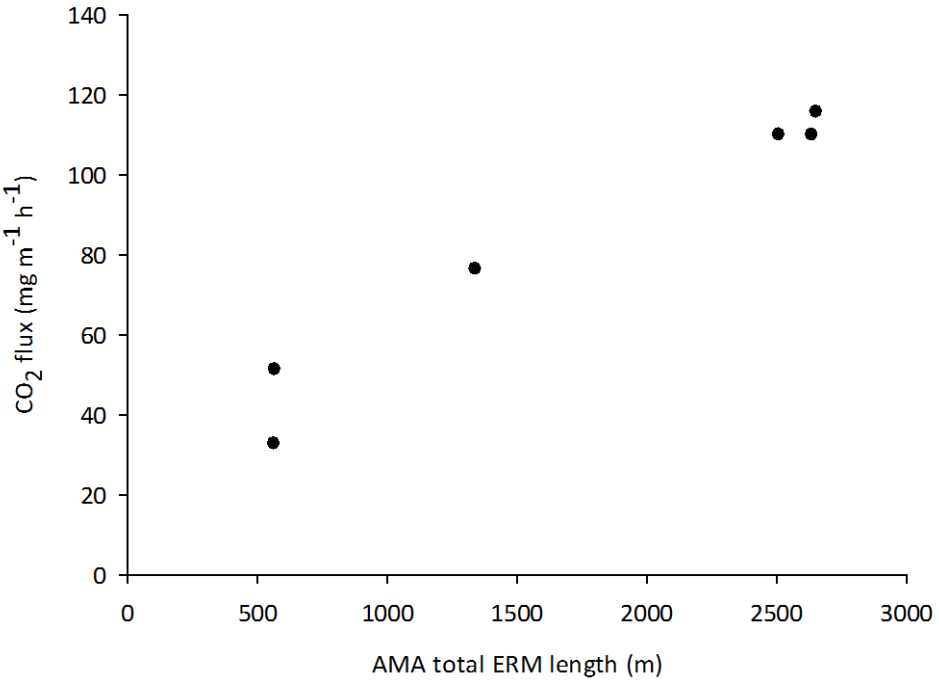


Figure 2.16. Total extraradical mycelium (ERM) length estimate for AMF access (AMA) compartments for the 105 d harvest plotted against the AMA compartment CO_2 flux measured 24 h preceding the harvest The significant relationship was assessed using a Pearson correlation ($n = 6$, $r^2 = 0.9616$).

2.3.3.3 Post-harvest patch greenhouse gas fluxes

The patch N_2O fluxes did not differ among harvests for AMA patches (Figure 2.17; Q_2 = 2.67, P = 0.264) but decreased significantly after the 56 d harvest for NAMA patches (Q_2 = 11.81, P = 0.0027). The differences between AMA and NAMA N_2O fluxes from the patches remained similar among harvests (Figure 2.17; Q_2 = 0.38, P = 0.827) and did not differ from zero at the 56 d or 84 d harvest (56 d: S_5 = 1.5, P = 0.844; 84 d: S_5 = 5.5, P = 0.313), but were significantly higher than zero at the 105 d harvest (S_5 = 10.5, P = 0.031). Thus, the AMA patches were producing more N_2O than NAMA patches by this stage. All N_2O fluxes were significantly greater than zero at every harvest (P < 0.05 in each case). There were no significant relationships between post-harvest patch N_2O fluxes and pre-harvest CO_2 fluxes in either the AMA or NAMA treatment when harvest time was controlled for using a partial correlation (AMA: r_s = -0.2079, P = 0.423; NAMA: r_s = 0.4464, P = 0.073).

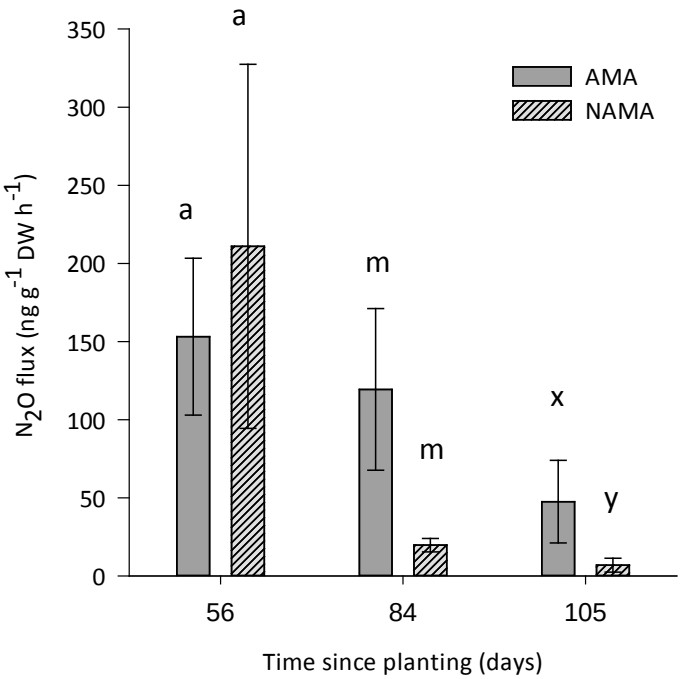


Figure 2.17. Mean AMF access (AMA; solid bars) and no AMF access (NAMA; hatched bars) organic patch N_2O fluxes at each harvest, (56 d, 84 d and 105 d post-planting). Error bars represent ± standard error of the mean (n = 6). Different letters show significant differences between AMA and NAMA patches within each harvest (P < 0.05) after Wilcoxon Signed Ranks tests comparing AMA – NAMA values to zero.

There was a significant negative correlation between N_2O fluxes from the AMA patches and corresponding ERM lengths when time was controlled for using a partial correlation (Figure 2.18; r_s = -0.653, P = 0.005), but only the 84 d harvest maintained the significant correlation when analyses were performed for each harvest (r = -0.8783, P = 0.021). There was no similar relationship in the NAMA patch treatment (P > 0.05). The patch N_2O fluxes for AMA and NAMA treatments were positively correlated for the 105 d harvest (Figure 2.19; r_s = 0.9429, P = 0.0048), but not at any other harvest or overall (P > 0.05 in each case). With the exception of one occasion (NAMA, 56 d harvest; S_5 = -10.5, P = 0.031) the patch CH_4 oxidation rate was not significantly different to zero. There were also no significant CH_4 fluxes measured from the post-harvest soils; therefore these data are not shown.

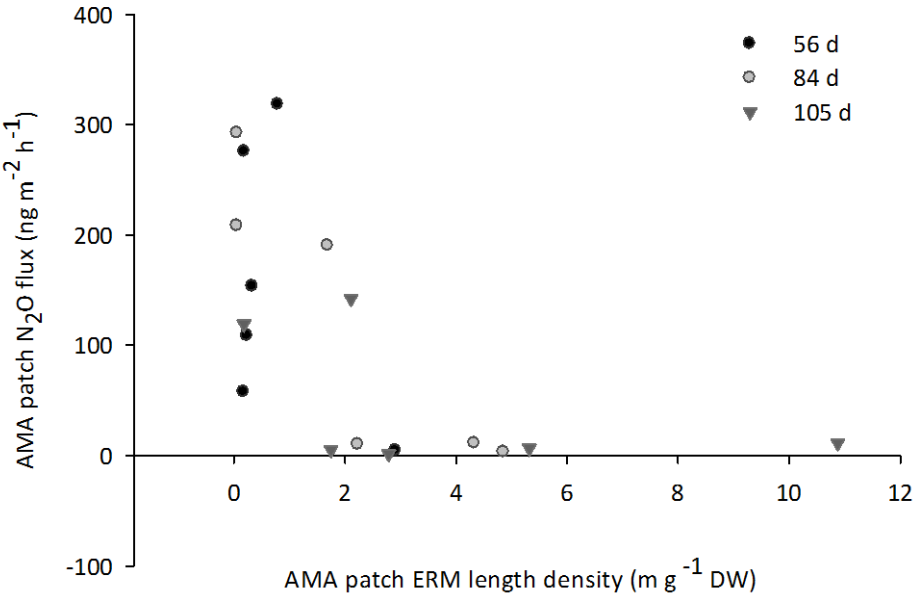


Figure 2.18. Nitrous oxide (N_2O) flux for AMF access (AMA) patches measured at each harvest (56 d, 84 d, 105 d post-planting) plotted against corresponding extraradical mycelium (ERM) length densities (m g^{-1} DW; n = 18, r^2 = 0.42). Different symbols represent the three harvests (56 d (black circle), 84 d (grey circle), and 105 d (black triangle) post-planting). A Spearman partial rank sum correlation controlling for harvest time was used to determine the relationship between the two variables.

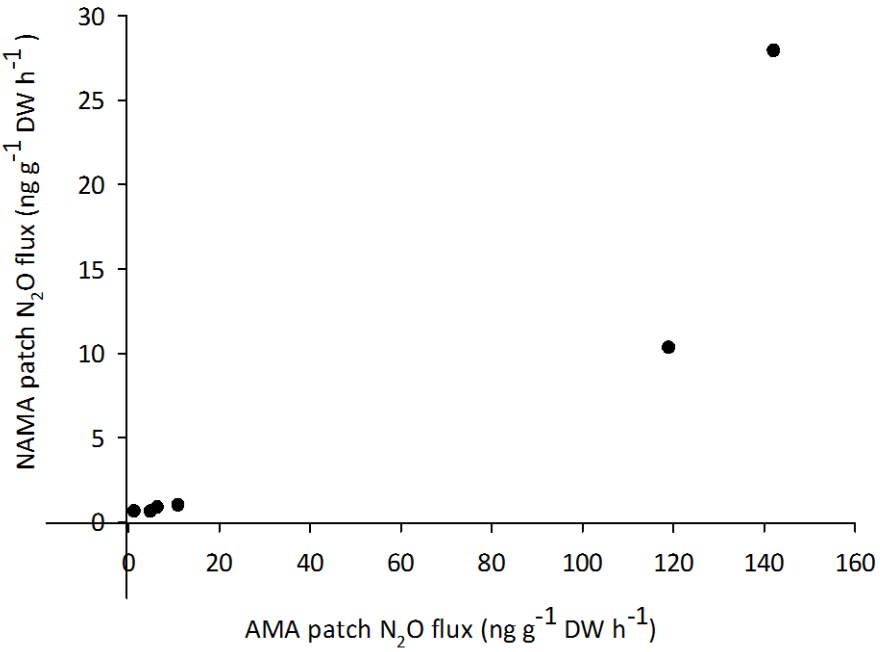


Figure 2.19. Nitrous oxide (N_2O) fluxes post-harvest for AMF access (AMA) and no AMF access (NAMA) patches following the harvest at 105 d post-planting. A Spearman rank order correlation was used to determine the relationship between the AMA and NAMA patch N_2O fluxes (n = 6, r^2 = 0.89).

2.3.3.4 Post-harvest soil greenhouse gas fluxes

The soil N_2O flux from both AMA and NAMA soils did not significantly differ among harvests (Figure 2.20; $P > 0.05$ in each case). There was no significant difference in N_2O flux between AMA and NAMA soils ($P > 0.05$ in each case, although note the 56 d harvest: $S_5 = 9.5$, $P = 0.063$) and consequently, no difference among harvests (Figure 2.20; $Q_2 = 5.81$, $P = 0.055$). The N_2O fluxes did not differ from zero at any harvest, although it was close in the AMA treatment at 56 d post-planting ($S_5 = 9.5$, $P = 0.063$) and in the NAMA treatment at 105 d post-planting ($S_5 = 9.5$, $P = 0.063$).

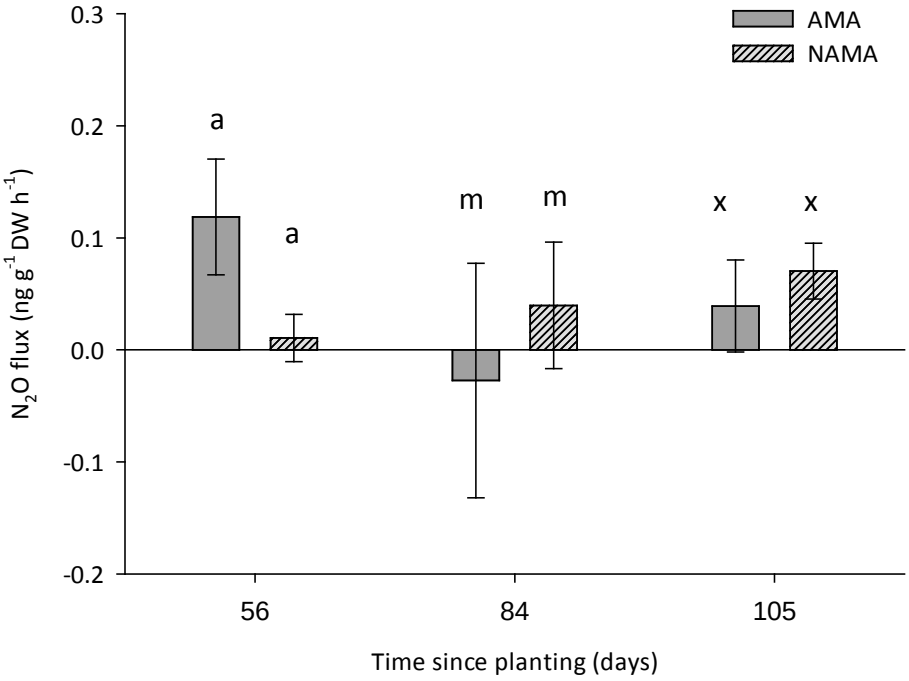


Figure 2.20. Mean soil N_2O fluxes from AMF access (AMA; solid bars) and no AMF access (NAMA; hatched bars) treatments following each harvest (at 56, 84 and 105 d since planting). Error bars represent ± standard error of the mean ($n = 6$). There were no significant differences in N_2O flux between AMA and NAMA treatments within each harvest (Wilcoxon signed ranks tests were used to compare AMA-NAMA values to zero; $P > 0.05$ in each case).

The mean post-harvest soil fluxes for the AMA and NAMA treatments (0.04 and 0.04 ng g^{-1} DW h^{-1} respectively) were 2450 and 1966 times smaller than the post-harvest patch fluxes for the same AMA and NAMA treatments (106.7 and 79.2 ng g^{-1} DW h^{-1} respectively).

Prior to the addition of KNO_3 to the soils sampled at the final (105 d post-planting) harvest, there was no difference in the N_2O fluxes from AMA and NAMA soils (Figure 2.21; t_5 = -1.24, P = 0.269). This was still not significant at 24 h (t_5 = 0.596, P = 0.596) or 48 h (t_5 =0.71, P = 0.512 after KNO_3 addition, but the N_2O fluxes from the AMA soils were 98.1% higher than those from the NAMA soils at 96 h after KNO_3 addition (t_5 = 3.01, P = 0.030).

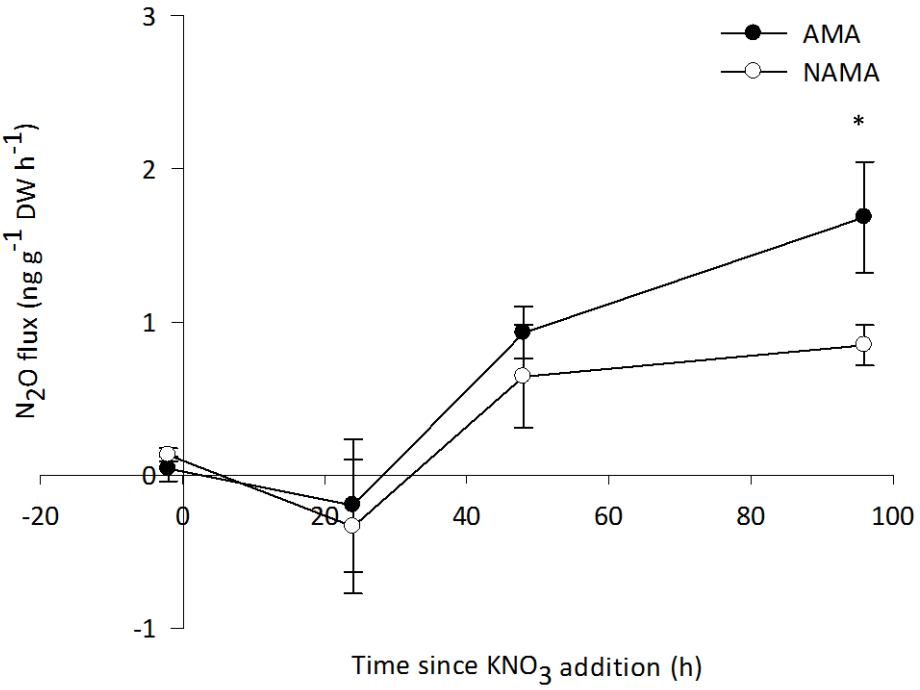


Figure 2.21. Mean N_2O flux from AMF access (AMA; solid circle) and no AMF access (NAMA; open circle) soils following the final (105 d post-planting) harvest, pre-KNO_3, and 24, 48 and 96 h post KNO_3 addition. Error bars represent ± standard error of the mean (n = 6). Asterisk indicates significant differences at the *P < 0.05 level for individual time points, determined using a paired t-test.

Glucose addition to post-harvest soils did not result in any difference in N_2O flux from AMA and NAMA treatments (Figure 2.22; pre-glucose: t_5 =0.29, P = 0.784; 24 h: t_5 = -1.61, P = 0.167; 48 h: t_5 = -0.66, P = 0.537; 96 h: t_5 = -0.29, P = 0.784), although there was an increase in N_2O flux over time in those soils following the addition of glucose (Time: $F_{3,18}$ = 77.3, P < 0.0001). However, there were no significant correlations between soil ERM length densities and N_2O fluxes following addition of KNO_3 or glucose, for any time point (P > 0.05 in each case).

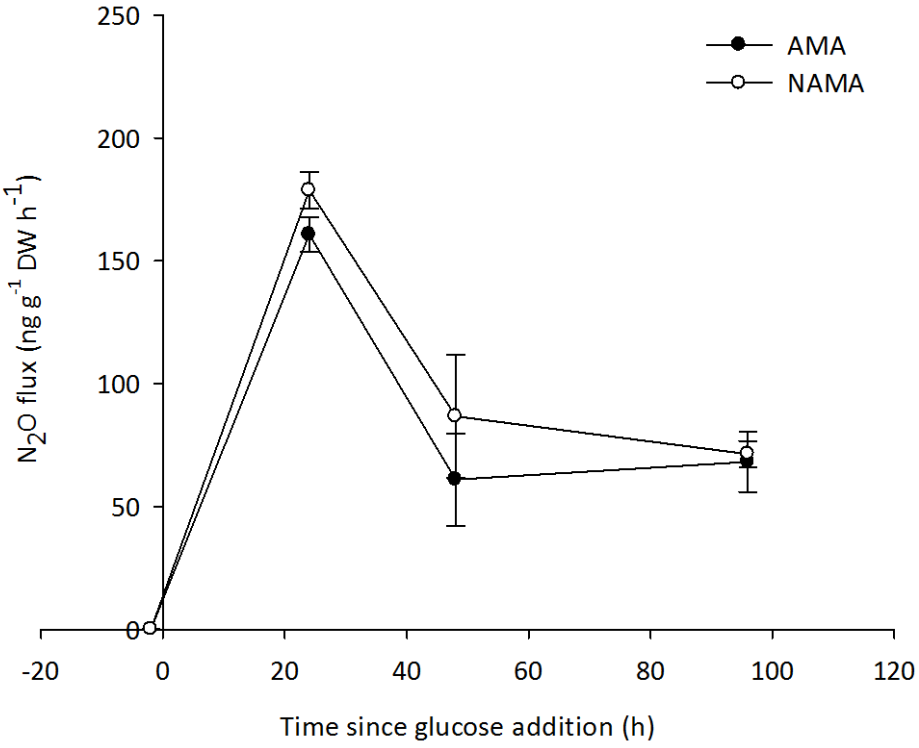


Figure 2.22. Mean N_2O flux from AMF access (AMA; solid circle) and no AMF access (NAMA; open circle) soils pre-glucose addition (following the third harvest at 105 d post-planting) and 24, 48 and 96 h post glucose addition (n = 6). Paired t-tests were used to determine that the AMA and NAMA treatment N_2O fluxes did not differ at any time point (P > 0.05 in each case).

2.3.3.5 Post-harvest patch and soil analysis

Post-harvest C:N ratios for the patches did not differ between the AMA and NAMA treatments at any harvest (Table 2.5; 56 d: S_5 = 4.5, P = 0.438; 84 d: S_5 = 0.5, P = 1.0; 105 d: S_5 = 1.5, P = 0.844); there was also no significant difference in the C:N ratio of patches among harvests (AMA: Q_2 = 1.81, P = 0.405; NAMA: Q_2 = 1.14, P = 0.565).

The patch C content did not differ between the AMA and NAMA treatments at any harvest (Table 2.5; 56 d: t_5 = 0.59, P = 0.583; 84 d: t_5 = -1.68, P = 0.154; 105 d: t_5 = 0.32, P = 0.762) and the C content of AMA and NAMA patches also did not differ among harvests (AMA: $F_{2,13}$ = 2.05, P = 0.169; NAMA: $F_{2,13}$ = 1.01, P = 0.391). There were also no differences among harvests or treatments for the patch C or N concentrations (Table 2.5; $P > 0.05$).

The N content of AMA and NAMA patches did not significantly differ at the 56 d or 105 d harvests (56 d: t_5 = 0. 0.13, P = 0.904; 105 d: t_5 = 0.73, P = 0.498), but the N content of AMA patches was slightly higher than that of the NAMA patches following the 84 d harvest (84 d: t_5 = -2.61, P = 0.048). The patch N content in both the AMA and NAMA treatments did not differ among harvests (AMA: $F_{2,13}$ = 0.56, P = 0.583; NAMA: $F_{2,13}$ =3.74, P = 0.052).

Table 2.5. Mean patch C and N contents, patch C:N ratio, patch C and N concentrations, and patch and soil gravimetric moisture contents (%) following each destructive harvest at 56, 84, and 105 d post-planting (30 d, 58 d and 79 d post-patch addition) and overall patch and soil gravimetric moisture content (%) for AMF access (AMA) and no AMF access (NAMA) treatments ± standard error of the mean. Different letters within each row and harvest indicate significant differences at $P < 0.05$, determined using either one-sample t-tests or Wilcoxon signed rank tests to compare the AMA-NAMA values to zero (further details in Section 2.3.3.5). The significant differences are highlighted in bold.

	56 d		84 d		105 d	
	AMA	NAMA	AMA	NAMA	AMA	NAMA
C content (mg)	658.5 ± 27.6^{a}	623.5 ± 47.9^{a}	537.1 ± 39.4^{m}	680.8 ± 64.8^{m}	603 ± 52.8^{x}	576.7 ± 63.3^{x}
N content (mg)	63.5 ± 4.0^{a}	62.7 ± 3.4^{a}	**57.3 ± 3.1^{m}**	**71.6 ± 5.5^{n}**	62.1 ± 5.3^{x}	57.8 ± 3.9^{x}
C concentration (mg g^{-1} DW)	46.3 ± 1.8^{a}	44.3 ± 3.6^{a}	39.1 ± 3.4^{m}	48.8 ± 4.5^{m}	43.0 ± 4.0^{x}	41.4 ± 4.6^{x}
N concentration (mg g^{-1} DW)	4.5 ± 0.3^{a}	4.5 ± 0.3^{a}	4.2 ± 0.3^{m}	5.1 ± 0.4^{m}	4.4 ± 0.4^{x}	4.2 ± 0.3^{x}
C:N ratio	10.5 ± 0.4^{a}	9.9 ± 0.4^{a}	9.4 ± 0.6^{m}	9.5 ± 0.3^{m}	9.7 ± 0.1^{x}	9.9 ± 0.5^{x}
Soil moisture content (%)	12.2 ± 0.1^{a}	11.8 ± 0.1^{a}	12.1 ± 0.5^{a}	11.6 ± 0.3^{a}	15.3 ± 2.1^{a}	11.9 ± 0.3^{a}
Patch moisture content (%)	37.7 ± 2.5^{x}	34.4 ± 0.6^{x}	38.0 ± 2.6^{x}	34.6 ± 1.2^{x}	**42.0 ± 2.7^{x}**	**32.8 ± 1.4^{y}**
Overall moisture content (%)	AMA			NAMA		
Soil	**13.2 ± 0.8^{a}**			**11.8 ± 0.1^{b}**		
Patch	**39.2 ± 1.5^{x}**			**33.9 ± 0.6^{y}**		

Overall the percentage (%) water content (g g^{-1} DW soil) of the patches was significantly higher for the AMA patches than for the NAMA patches (Table 2.5; n = 18; S_{17} = 59.5, P = 0.0077), but the % water content was not significantly different between AMA and NAMA treatments following the 56 d and 84 d (post-planting) harvests (Table 2.5; 56 d: S_5 = 6.5, P = 0.219; 84 d: S_5 = 3.5, P = 0.563). There was also no change in the % water content of the AMA and NAMA patches among harvests (AMA: $Q_{2,13}$ =1.81 P = 0.405; NAMA: Q_2 = 1.23, P = 0.539). However, by the 105 d post-planting harvest, the AMA patches were significantly wetter than the NAMA patches (S_5 = 10.5, P = 0.031). Even though the water content of the outer compartments was equalised weekly, following the destructive harvests, the soil % water content was higher overall when AMF hyphae had access (Table 2.5; n = 18; S_{17} = 51.5, P = 0.024), although this was not significant within each harvest (P > 0.05 in each case). The patch water content was not correlated with the patch ERM length densities at any point (P > 0.05) and the soil water content was also not correlated with the soil ERM length densities or N_2O flux in either AMA or NAMA treatments (P > 0.05 in each case).

The patch C:N ratios did not correlate with the pre- or post-harvest N_2O fluxes (P > 0.05 in each case). However, the N and C contents and N concentration of AMA patches positively correlated with the pre-harvest N_2O fluxes, but the same relationships were not found for the NAMA treatment (statistics in Table 2.6). There were also no significant relationships between post-harvest N_2O fluxes for AMA or NAMA treatments with patch C or N contents (Table 2.6). Although the C and N contents of the patches were positively correlated in both the AMA and NAMA treatments (Table 2.6). The patch % moisture for the AMA treatments was negatively correlated with the pre-harvest N_2O fluxes but the same relationship was not found for NAMA compartments, or for AMA or NAMA post-harvest patch N_2O fluxes (Table 2.6). Patch ERM length densities were also not related to the C:N, C content, N content, C or N concentration of the patches (P > 0.05 in each case).

Table 2.6. Correlation coefficients and r^2 values for correlations between pre- or post-harvest N_2O fluxes against other patch variables (C and N content, C and N concentrations and patch gravimetric moisture content). Correlation coefficients are from either Pearson's or Spearman's rank order partial correlations controlling for harvest time indicated by r or r_s respectively. Significant correlations are shown in bold and indicated by asterisks (*P < 0.05, **P < 0.01 ***P < 0.0001).

			AMA			NAMA		
	Patch variable		coefficient	P	r^2	coefficient	P	r^2
Pre-harvest N_2O flux	C content	r	0.4974	**0.042***	0.25	-0.0187	0.943	0.0003
	C concentration	r	0.4532	0.068	0.21	0.0127	0.962	0.0002
	N content	r	0.6792	**0.0027****	0.46	-0.0695	0.791	0.005
	N concentration	r	0.6470	**0.005****	0.42	-0.0538	0.838	0.003
	Moisture content	r	-0.5801	**0.015***	0.34	0.1095	0.676	0.01
Patch C content	Patch N content	r	0.8184	**<0.0001*****	0.67	0.8940	**<0.0001*****	0.80
Post-harvest patch N_2O flux	C content	r_s	-0.0158	0.952	0.0002	-0.2275	0.380	0.05
	C concentration	r_s	-0.0575	0.827	0.003	-0.2316	0.371	0.05
	N content	r_s	0.1799	0.490	0.032	-0.0458	0.861	0.002
	N concentration	r_s	-0.0724	0.783	0.005	-0.0889	0.734	0.008
	Moisture content	r_s	0.0785	0.765	0.006	0.3657	0.149	0.13

2.4 Discussion

2.4.1 Preliminary experiments

Plantago lanceolata is commonly used in AMF experiments with organic patches (Hodge *et al.*, 2001; Hodge & Fitter, 2010; Barrett *et al.*, 2011) and when in symbiosis with *G. intraradices,* the hyphae have previously proliferated in organic matter patches (Leigh *et al.*, 2009; Leigh *et al.*, 2011). In this case, the symbiosis with *Z. mays* produced the highest ERM length densities in the patches (Figure 2.9), and the highest colonisation of host plant roots (Figure 2.8). Therefore as *Z. mays* is an important agricultural crop world-wide (FAO, 2012), it was selected as the host plant for all future experiments on AMF interactions with trace gas fluxes in this thesis. Following the second preliminary experiment (outlined in Section 2.2.2), the microcosm units with gas tight lids were found to be suitable for use in cover boxing as the meshes did not impede the flow of CO_2. As CO_2 has a molecular weight of 44, it was therefore unlikely that N_2O and CH_4 would also not be impeded by the meshes as they were either the same molecular weight (N_2O: 44) or smaller (CH_4: 16). The discussion below relates to the subsequent experiment assessing the interactions between AMF hyphae and trace gas fluxes outlined in Section 2.2.3.

2.4.2 Experimental design

The colonization of the host plants and outer compartments by *G. intraradices* was comparable to that of previous studies using similar systems (Leigh *et al.*, 2009; Hodge & Fitter, 2010). The *Z. mays* host plants were highly colonised by AMF (Table 2.2) and the extraradical mycelium (ERM) length densities produced in the patches at 84 d and 105 d of 2.2 ± 0.84 m g^{-1} DW and 3.84 ± 1.57 m g^{-1} DW respectively (Figure 2.11; 58 d and 79 d post-patch addition respectively) were not dissimilar to those reported by Leigh *et al*. (2009) where patch ERM length densities were typically 3.1 m g^{-1} at 70 d post-planting (42 d post-patch addition). There was also a greater than 1000x difference between patch and soil N_2O fluxes in both AMA and NAMA treatments. As outlined in Section 2.2.3.4, the patches were designed to create a location in which

AMF and N_2O producers could interact and the combination of high ERM length densities and patch N_2O production should therefore have provided suitable conditions.

2.4.3 Pre-harvest gas fluxes

2.4.3.1 Carbon dioxide (CO_2)

The presence of AMF hyphae is known to increase CO_2 fluxes (Heinemeyer *et al.*, 2006; Heinemeyer *et al.*, 2012b), which was also the case here (Figure 2.12). If AMF hyphal respiration was the main cause of the difference between AMA and NAMA treatments, the CO_2 flux might have been expected to correlate with the total ERM lengths measured. This did occur at the 105 d harvest, but not at the 84 d harvest. It is therefore probable that the presence of AMF hyphae influenced soil respiration in more than one way. The most likely causes are carbon (C) and nitrogen (N) deposits from hyphal exudation (Toljander *et al.*, 2007) and turnover (Staddon *et al.*, 2003; Godbold *et al.*, 2006) causing increased mineralisation rates and microbial respiration in the hyphosphere.

Since neither the patch nor soil water content correlated with the CO_2 fluxes for either AMF treatment, the differences in AMA and NAMA CO_2 flux were unlikely to be a result of the AMA patches being wetter. Moreover, soil water content is only likely to affect CO_2 fluxes when conditions are very dry or very wet (Bowden *et al.*, 1998), which was not the case in the patches here (Table 2.5; see Curtin *et al*. (2012)). Although the soils were relatively dry (Table 2.5), the moisture contents did not differ between the AMA and NAMA treatments and thus moisture content was not likely to be a driver for the difference in CO_2 flux between the AMA and NAMA treatments. Additionally, root respiration was probably contributing to both the AMA and NAMA compartment fluxes, as the AMA and NAMA CO_2 fluxes were positively correlated. Similar plant compartment effects are also likely to have caused the correlation in AMA and NAMA CH_4 fluxes. It is also possible that the flow of air over the soils in the closed loop system

(CDC system) may have resulted in some purging of air from the soil which could also contribute to the similar fluxes of CH_4 and CO_2 in the AMA and NAMA compartments and would have reduced the possibility of finding a significant difference between the AMA and NAMA treatments. However, since the AMA and NAMA treatments shared a plant compartment, it was still possible to determine the effect of AMF hyphae on CO_2. In the case of CO_2 fluxes, the regression line falls below that of the 1:1 line (Figure 2.15), thus while related to the AMA CO_2 fluxes, the NAMA CO_2 fluxes were not increasing at the same rate. The microcosm design paired the AMF treatments by unit, and thus allowed AMF influence on CO_2 to be detected regardless of root respiration rates. This not only demonstrates that the AMF hyphae were influencing hyphosphere activity, but also that in this experiment and under the specific conditions used here, CO_2 could be used as an indicator of hyphal presence without the need for destructive harvest when using these microcosms.

2.4.3.2 Nitrous oxide (N_2O)

While AMF hyphae can take up N from organic sources, albeit as inorganic forms (Leigh *et al.*, 2009; Hodge & Fitter, 2010), the hypothesis that this reduces the availability of NH_4 and NO_3 and subsequently the production of N_2O, was not supported by this study. To date, only one other study has measured the impact of AMF presence on N_2O fluxes directly. Cavagnaro *et al*. (2012) found no difference in the $^{15}N_2O$ production from field based soils surrounding AM colonised tomato plants compared to those of AM defective tomato mutants following ^{15}N addition as ^{15}N-KNO_3. However, they did find that ^{15}N uptake by AM plants was higher than that of the non-AM mutants. Additionally, the $^{15}N_2O$ production was lower in the presence of roots compared to soil alone; thus the difference in available ^{15}N in soils from AM plants compared to the non-AM mutants may not have been large enough for it to affect the overall $^{15}N_2O$ production.

The relationships between pre-harvest N_2O fluxes and patch C, N and moisture content in the AMA treatments here indicate that, despite the lack of overall difference in N_2O flux rates between the AMA and NAMA treatments, the AMF hyphae were interacting with the N_2O production pathways (Table 2.6). N_2O producers have differing N, C and moisture requirements (Philippot *et al.*, 2009), and the r^2 values for these relationships were all below 0.5, thus at most these relationships explain no more than 50% of the observed variation. This may suggest that there was more than one pathway contributing to the pre-harvest N_2O fluxes for the AMA treatments.

Residue N content can be positively related to N_2O fluxes (Millar *et al.*, 2004; Baggs *et al.*, 2006) and this was also found for the AMA patches and pre-harvest N_2O fluxes here. In this case, as suggested by Millar *et al*. (2004) the mineralisation rates may have increased in the patches with higher N content, which would stimulate the pre-harvest N_2O fluxes. Increased mineralisation rates would result in higher NH_4 and NO_3 availability, which could potentially reduce the competition between autotrophic nitrifiers and other heterotrophic organisms for NH_4 (Verhagen *et al.*, 1995). While the ERM length densities in this study were not related to the pre-harvest N_2O fluxes, or to the patch N content, it has been demonstrated that mineralisation rates can increase in the presence of AMF hyphae, although the mechanism is unclear (Hodge *et al.*, 2001; Atul-Nayyar *et al.*, 2009). Herman *et al*. (2012) found that when AMF hyphae were present in an organic patch of ^{13}C labelled plant roots, the atom% ^{13}C-PLFA was lower than when the AMF hyphae were absent. The authors suggested that loss of ^{12}C plant carbon from AMF hyphae may have diluted the ^{13}C enrichment of the hyphosphere organisms, and this could have resulted in increased decomposition as a result of C 'priming'. However, root mediated priming effects are not always positive (Cheng & Kuzyakov, 2005), and probably depend on the quantity of labile C added (de Graaff *et al.*, 2010).

In the AMA treatment patch, C content was also positively correlated with the pre-harvest N_2O fluxes. Previous studies have found denitrification rates to correlate with

total organic C (Stanford *et al.*, 1975; Baggs & Blum, 2004), and AMF can influence the communities of denitrifiers in the mycorrhizosphere (Veresoglou *et al.*, 2012a). The presence of AMF hyphae may therefore have increased the available N via increased mineralisation, acted as a C source either by exudation (Toljander *et al.*, 2007; Herman *et al.*, 2012) or hyphal turnover (Staddon *et al.*, 2003), or acted as an additional N source (Hodge & Fitter, 2010). Alternatively, uptake of NH_4 by AMF has been proposed to increased decomposition rates in soils (Cheng *et al.*, 2012). Cheng *et al*. (2012) suggested that saproptrophs could be released from metabolic repression as NH_4 is removed by AMF, while also having access to more labile C. If this resulted in N mineralisation in these patches the N available for N_2O production could have increased. However, in the present study, decomposition rates were probably not that different in the AMA and NAMA treatments as the C:N ratio of the patches did not differ at the final harvest. Furthermore, the patch N and C contents were positively correlated in both the AMA and NAMA treatments, therefore only one of these may have been related to the N_2O flux and the other was probably a consequence of that.

In the AMA treatments, there was also a negative relationship between patch % moisture and pre-harvest N_2O fluxes which was predominantly driven by the highest moisture contents (> 43%) that only occurred in the AMA patches. The N_2O flux would be expected to increase at higher moisture contents if denitrification was the dominant source of N_2O (e.g. Abbasi & Adams, 2000; Bateman & Baggs, 2005), thus in this case, at least some of the N_2O produced probably originated from aerobic pathways such as nitrification which would require a source of O_2 (Bollmann & Conrad, 1998). As the N_2O flux decreased at higher moisture contents, any beneficial effect of AMF presence on N_2O production via mineralisation may have been neutralised by the negative effect of increasing moisture content. Taken together, the relationships between pre-harvest N_2O and patch moisture, N and C contents indicate a complex relationship between AMF hyphal presence and N_2O production pathways, which requires further investigation.

In the present study, the overall production of N_2O detected in the outer compartments was almost certainly driven by that of the organic patches, which following the destructive harvests, were found to be producing over 1000 times more N_2O than the surrounding soils. The positive relationship between the patch N and C contents with the pre-harvest N_2O fluxes also indicates that the N_2O fluxes measured were predominantly driven by the patches. The cover-box method used to measure pre-harvest N_2O fluxes from the outer compartments included both the soil and patch with a headspace volume of 600 cm^3, which is much larger than the patch volume (ca. 25 cm^3) and the patches were located 8 cm below the soil surface. Therefore there will have been significant dilution of any N_2O produced by these patches in measurements taken using the cover-box method. If the concentration of N_2O measured falls to that of atmospheric (ca. 324 ppb in 2011; Blasing, 2013), the reliability of the measured flux values declines. Furthermore, the N_2O flux values were calculated from only four time points. While precautions were taken by discarding outlying values (see Section 2.2.3.10), this had the potential to increase the variability between samples and was not optimal for identifying an effect of AMF hyphae on N_2O fluxes from these units. Use of a soil gas probe inserted into the patch bag, similar to those used by Kammann *et al*. (2001) or Mastepanov & Christensen (2008), may be a more appropriate method of quantifying patch N_2O fluxes. The patches had both the highest ERM length densities and N_2O fluxes and are clearly the predominant site where interactions between the AMF hyphae and N_2O producing organisms were occurring.

2.4.3.3 Methane (CH_4)

While dilution may also have been an important factor limiting the accuracy of CH_4 fluxes measured, there was a detectable rate of CH_4 oxidation in both AMA and NAMA compartments (Figure 2.14). While quite low, these fluxes were within the range of CH_4 oxidation rates measured in previous studies on forest (McNamara *et al.*, 2008), and arable (Dobbie & Smith, 1996; Priemé *et al.*, 1997) soils. Although there are many potential interactions between AMF hyphae and CH_4 fluxes, there was no evidence for any effects of AMF hyphal presence in this study either before or after destructive

harvest. Even the difference in water content between the AMF treatments did not appear to reduce the rate of CH_4 oxidation, yet increasing water availability is often found to reduce CH_4 oxidation rates (Castro *et al.*, 1994; Hiltbrunner *et al.*, 2012; Yu *et al.*, 2013). The post-harvest CH_4 fluxes were also very low despite the addition of 20 ppm CH_4. This suggests that the CH_4 oxidisers were not limited by CH_4 in this system, and that another factor was impacting on the rate of CH_4 oxidation. Again, the lack of difference between AMA and NAMA treatments suggests that it was not related to the presence of AMF hyphae. It may be that AMF do not have a large enough impact on soil conditions to affect methanotrophs or methanogens in this system, particularly since only the net CH_4 flux was measured. There is evidence suggesting that some saprotrophs can produce CH_4 (Lenhart *et al.*, 2012), however, at the time of writing, there is no literature available showing AMF hyphae interacting with soil CH_4 fluxes, possibly because no interactions have yet been found, or because the potential interactions have not yet been studied in sufficient detail.

2.4.4 Post-harvest patch N_2O fluxes

The post-harvest N_2O fluxes from patches at the 105 d harvest were markedly higher in the presence of AMF hyphae (Figure 2.17). The denitrifying community can be modified in the mycorrhizosphere (Veresoglou *et al.*, 2012a) and the number of denitrifying bacteria were lower in the mycorrhizosphere in another study (Amora-Lazcano *et al.*, 1998), possibly because the C available to denitrifiers was reduced as it was instead passed onto the AMF (Veresoglou *et al.*, 2012a). In the hyphosphere, as studied here, the opposite may be true as there were no roots present. The harvesting method used by Amora-Lazcano *et al*. (1998) has also come under criticism since it may have increased the C available for denitrifiers upon root severing (Veresoglou *et al.*, 2012b). Conversely, this criticism may present an explanation for the higher N_2O flux from the patches when the AMF hyphae were present in this study. When the patches were removed from the units, the hyphae must have been severed on all surfaces of the patch bag. Unlike autotrophic nitrifiers which only require a source of NH_4, denitrifiers require a C source alongside NO_3 (Hino *et al.*, 2010; Parkin, 1987).

Severing the AMF hyphae may therefore have increased the available C and/or N in the patch, which may have acted as a proximal (short term, *sensu* Wallenstein *et al.*, 2006) control, increasing rates of nitrification, denitrification or both.

AMF hyphae can affect soil structure and water status, possibly via the production of glomalin (Rillig & Mummey, 2006; Purin & Rillig, 2007) and this can lead to an increase in the number of bacteria in subsequent stable aggregates (Andrade *et al.*, 1998). As the AMA patches were wetter than the NAMA patches, the frequency of anaerobic microsites may have increased the rates of denitrification and consequently N_2O production as denitrification is an anaerobic process (Zumft, 1997). Mineralisation rates can also increase at higher moisture contents (Curtin *et al.*, 2012), possibly as a result of an increase in the mobility of mineralisation substrates, which in turn may result in higher levels of available NH_4 and NO_3 that could also fuel N_2O production. However, the patch water content did not correlate with the N_2O flux. AMF can also reduce pH in the hyphosphere (Li *et al.*, 1991a), and reduction in pH can reduce total denitrification rates (Šimek & Cooper, 2002; Čuhel *et al.*, 2010), and nitrification rates (e.g. Nugroho *et al.*, 2007; Cheng *et al.*, 2013). Unfortunately, the patches were too small to sample for pH, however as the N_2O fluxes increased in the AMA treatments, it is unlikely to be related to AMF mediated pH changes. The higher N_2O fluxes from the AMA patches post-harvest are therefore likely to be a consequence of a combination of availability of N and/or C and increased patch water content.

The difference in N_2O fluxes between AMA and NAMA treatments only became significant at the final (105 d) harvest, although the trend appeared by the 84 d harvest. The high N_2O production at the 56 d harvest in both treatments may have masked any effect of AMF presence as the initial mineralisation rates of the low C:N patches would have been high (Huang *et al.*, 2004). Over time, as the available N and C decreased, the overall mineralisation rates and subsequent N_2O fluxes decreased as the ERM length densities increased, resulting in a negative correlation between the two, but a partial correlation controlling for time demonstrated that this relationship

was not simply a consequence of time. AMF have been demonstrated to take up N from organic patches (Leigh *et al*. 2009), which may reduce the N available for N_2O production pathways, and therefore could explain the negative relationship between patch N_2O flux and ERM length. This seems to contradict the finding that the AMA patches were producing more N_2O than the NAMA patches post-harvest, but the interactions between N and C may help to explain this. After harvesting, C loss from the AMF hyphae may have released denitrifiers from C limitation and subsequently increased overall N_2O production. However, once the denitrifiers were no longer limited by C, they may have become limited by the availability of N. If the AMF hyphae were taking up N, they could have reduced the N available in the patches, and therefore the N_2O production was negatively related to the ERM length densities present, even though it was still higher in the AMA treatment than in the NAMA treatment. Thus it was probably the balance between available N and C that was the main factor controlling the N_2O fluxes and the relationship of these with the ERM length densities.

The correlation between the AMA and NAMA patch N_2O fluxes at the 105 d harvest was probably caused by influences from the shared plant compartment. Root exudates and rhizodeposits are likely to have been present in the planted compartments, and can act as a C source for denitrifiers (Mahmood *et al.*, 1997; Jones *et al.*, 2004; Mounier *et al.*, 2004). All planted compartments received the same amount of nutrient solution and water, but these were only separated from the outer compartments by fine mesh membranes, thus it is possible that rhizodeposits, nutrients and/or water may have entered the outer compartments from the plant compartment. These influences would be equally likely for both outer compartments, and are likely to have increased over the growing period, hence the correlation only being significant at the 105 d harvest.

2.4.5 Post-harvest soil N_2O fluxes

In contrast to the patches, the soils were not producing N_2O (Figure 2.20), probably because they were much lower in N and C content. However, the AMA soil N_2O flux was almost significantly higher than that of the NAMA soil at the 56 d harvest. Both NH_4 and NO_3 are mobile in soils, particularly NO_3 (Tinker & Nye, 2000). Thus, NH_4 and/or NO_3 may have passed into the soils which were N limited via diffusion or mass flow from the patches, which were producing high amounts of N_2O and thus had high levels of NH_4 and/or NO_3 available. The presence of hyphae may have increased the C availability in the AMA soils via hyphal exudation (Toljander *et al.*, 2007), thus resulting in slightly higher denitrification rates (and therefore N_2O production) in the presence of AMF hyphae. As the mineralisation rates in the patches decreased, so would the available NH_4 and NO_3 passing into the soil and therefore the N_2O fluxes ceased. Although the outer compartments were equalised for water content weekly, the AMA soil water content was higher overall then in the NAMA treatment, although not within each harvest. Thus the effect of water availability cannot be ruled out, but, as soil water content did not correlate with either soil N_2O or ERM length, there is no evidence to suggest that it was related to the soil N_2O production in this case.

Denitrifiers are a very diverse group of soil organisms (Chèneby *et al.*, 2000), they are present in most soil bacterial communities, and there are estimates that denitrifiers make up to 5% of the soils microbial community (Philippot *et al.*, 2007). However, denitrifiers are often limited by availability of C, NO_3, and/or water or low pH (Bollmann & Conrad, 1998; Šimek & Cooper, 2002), and thus denitrification does not always occur. If a rate limiting factor such as C or NO_3 is relieved, the response of denitrifiers can be rapid, often resulting in increased rates of N_2O production within hours (e.g. Gillam *et al.*, 2008; Čuhel *et al.*, 2010; Miller *et al.*, 2012). This was also the case here, with increased N_2O production only 24 h following glucose addition and 48 h following KNO_3 addition (Figure 2.21 and 2.22). Thus, it is unlikely that the denitrifying population was reduced in the absence of AMF as there were no significant differences in N_2O flux from AMA and NAMA soils following glucose

addition (Figure 2.22). Moreover the denitrifiers were likely to have been C limited in the soils used, and the addition of glucose removed this limitation (Parkin, 1987; Miller *et al.*, 2012). Furthermore, the glucose concentration used here (200 mg kg^{-1}) was unlikely to be high enough to result in an increase in bacterial community abundance (Miller *et al.*, 2008; Miller *et al.*, 2012). Miller *et al.* (2012) only found significant increase in bacterial community abundance if the C addition treatment exceeded 1000 mg kg^{-1}. However, it should be noted that there are cases of heterotrophic nitrification which may result in N_2O production (Laughlin *et al.*, 2008), and this pathway cannot be ruled out.

Additionally, 96 h following KNO_3 addition, the N_2O flux was higher in the presence of AMF (Figure 2.21). When the denitrifiers in the NAMA soils were no longer limited by availability of water or NO_3, they were probably limited by C availability (Gillam *et al.*, 2008). The AMF hyphae were therefore presumably acting as a C source, either as a result of severing, hyphal exudation (Toljander *et al.*, 2007), hyphal turnover (Staddon *et al.*, 2003) or most likely, all three. Since the KNO_3 additions did not have an equivalent salt control, and neither the glucose nor KNO_3 additions had an equivalent water control, the effect of K^+ and water addition on N_2O production, although unlikely, cannot be ruled out. For example, the glucose and KNO_3 additions were made in the same volume of water, yet the observed N_2O fluxes were very different between these treatments. While salt effects cannot be ruled out, other studies have used KNO_3 in a similar way (Bergstermann *et al.*, 2011; Cavagnaro *et al.*, 2012), therefore it is most likely that it was the NO_3^- and glucose additions, and not the added water and K^+ that caused the observed effects.

2.4.6 Conclusions

The presence of AMF hyphae resulted in higher cumulative CO_2 fluxes, and thus CO_2 fluxes could be used as an indicator of AMF hyphal presence in this microcosm study. However, CH_4 fluxes did not differ between AMF treatments, although as only the net

CH_4 flux was measured, interactions between AMF and CH_4 flux pathways could not be ruled out. There appeared to be no effects of AMF presence on N_2O production in intact microcosms, but it is unclear as to whether this was a result of imprecise gas flux measurement methods or a lack of influence of non-severed AMF hyphae on N_2O fluxes; the relationships with patch N, C and moisture content suggest the former. Thus, it is still unclear as to whether AMF can affect N_2O fluxes without being damaged. On the contrary, N_2O fluxes were increased in the presence of AMF hyphae following a destructive harvest, and this was probably a result of the increase in C available to heterotrophic N_2O producers (Johnson *et al.*, 2002b; Toljander *et al.*, 2007). This was supported by evidence of increased N_2O fluxes from soils taken from both AMA and NAMA treatments following glucose addition, and only N_2O increases in AMA soils following KNO_3 addition.

As AMF hyphae appeared to have a significant impact on N_2O production, the following two Chapters use novel methods of N_2O measurement to examine this interaction in more detail by attempting to:

i. determine whether AMF hyphae can affect N_2O fluxes when they have not been severed from their host plants and if this is a result of interactions with N availability
ii. determine if severing AMF hyphae from their host plant has a different effect on N_2O production when compared to leaving the AMF hyphae intact

Chapter 3. The interactions between the hyphae of arbuscular mycorrhizal fungi (AMF) and localised organic patch N_2O concentrations

3.1 Introduction

Arbuscular mycorrhizal fungi (AMF) can form an obligate symbiosis with over two-thirds of land plants, providing P and/or N in exchange for C (Kiers *et al.*, 2011; Fellbaum *et al.*, 2012). Olsson *et al*. (1999) found that the extraradical mycelium (ERM) of AMF in a *Linum usitatissimum* L. field made up the largest fraction of the soil microbial biomass, and in biomass terms, the ERM was 10 times larger than the intraradical mycelium (IRM). In Chapter 2 it was found that the presence of AMF hyphae increased nitrous oxide (N_2O) fluxes from patches of organic matter, but only once the hyphae had been severed from their host plant. However, it remains unclear as to whether an intact AMF mycelium can influence soil N_2O fluxes, and whether this would lead to an increase or decrease in N_2O flux, as the pre-harvest N_2O fluxes reported in the previous Chapter (Chapter 2, Section 2.3.3.2) were highly variable.

Cheng *et al*. (2012) proposed that the uptake of NH_4 and simultaneous release of labile C by AMF could release saprotrophic microorganisms from metabolic repression, and therefore increase decomposition rates. If there was subsequent net mineralisation of N this could, in turn, increase N_2O production from soils when intact AMF hyphae are present (Vinther *et al.*, 2004; Manzoni *et al.*, 2008). Denitrification has been found to be increased in the rhizosphere (Mahmood *et al.*, 1997), and evidence suggests that this is driven by increased C availability from root exudates (Mounier *et al.*, 2004; Henry *et al.*, 2008). Since AMF hyphae both exude C (Toljander *et al.*, 2007) and can have rapid turnover (Staddon *et al.*, 2003) a similar effect may also occur in the hyphosphere (the volume of soil influenced by AMF hyphae). Additionally, AMF can improve water retention of soils (Augé *et al.*, 2001), which could increase N_2O production through denitrification (Bateman & Baggs, 2005), but simultaneously decrease N_2O from nitrification (Bollmann & Conrad, 1998). In Chapter 2, the presence

of AMF hyphae may have modified the organic matter patch water content, and subsequently affected the N_2O flux, as the patch water content was negatively correlated with the pre-harvest N_2O flux. Nitrification can also be limited by the availability of CO_2 (the source of C for nitrifiers; Azam *et al.*, 2004), which is usually higher with AMF hyphae (Heinemeyer *et al.*, 2006).

In direct contrast, it is possible that N_2O production may decrease in the presence of AMF hyphae. Arbuscular mycorrhizal fungi can provide their host plant with significant quantities of N from organic matter patches to which only the AMF hyphae have access (Leigh *et al.*, 2009), but they also require large amounts of N for their own nutrition (Hodge & Fitter, 2010). Additionally, AMF hyphae can decrease the concentration of available inorganic N in soils (Tu *et al.*, 2006), and the presence of arbuscular mycorrhizal (AM) colonised tomato plants reduced NO_3 loss via leaching when compared to transgenic non-AM tomato plants (Asghari & Cavagnaro, 2012). Therefore, AMF may increase competition for N in the hyphosphere, and this competition may, in turn, reduce N_2O fluxes as the N_2O producers become N limited. Potential nitrification rates have been found to decrease in the presence of AM colonised plants (Veresoglou *et al.*, 2011a), which lends support to this hypothesis. In contrast, the number of ammonium oxidisers (AO) can both increase (Amora-Lazcano *et al.*, 1998), or remain unchanged (Cavagnaro *et al.*, 2007) in the presence of AMF. Veresoglou *et al*. (2011a) also suggested that AMF mediated allelopathy could limit nitrification rates, although to date, there is no evidence for this. However, AMF are probably best known for their ability to acquire and transfer P to their host plants (Smith & Read, 2008), and as nitrifiers can become P limited (Purchase, 1974), this could also reduce the N_2O produced via nitrification in the hyphosphere.

As copper (Cu) is immobile in soils (Mengel & Kirkby, 2001), AMF hyphae can also improve their host plants Cu nutrition by extending the accessible volume of soil (Li *et al.*, 1991c; Liu *et al.*, 2000a). A reduction in the availability of Cu can increase N_2O production as the activity of the nitrous oxide reductase (NOR) enzyme, which has a

Cu-based centre, is reduced (Zumft, 1997). In contrast, the nitrite reductase (NIR) enzyme in some organisms can contain Cu (Suzuki *et al.*, 2000; Stein, 2011). Thus, if the AMF are taking up Cu, they may reduce the production of N_2O by reducing the activity of the Cu-based NIR enzyme. The availability of iron (Fe) can also be important in determining the rate of N_2O production, for example the production of N_2O via the oxidation of hydroxylamine (during nitrification) is increased when the availability of Fe(III) is higher (Bengtsson *et al.*, 2002). A recent study found correlative evidence showing that the availability of Fe in soils can be positively linked to N_2O production (Zhu *et al.*, 2013b). Since AMF hyphae can also take up Fe and transfer it to their host plants (Caris *et al.*, 1998; Farzaneh *et al.*, 2011), they therefore have the potential to modify soil N_2O production via Fe uptake.

The pH of growth media in the presence of AMF hyphae has been found to decrease in a Petri plate system (aka. hairy root culture; Villegas & Fortin, 2001) and a microcosm based soil system (Li *et al.*, 1991a). Soil pH can affect rates of nitrification, denitrification, and dissimilatory nitrate reduction to ammonium, all of which produce N_2O (Šimek & Cooper, 2002; Cheng *et al.*, 2013; Giles *et al.*, 2013). Nitrification rates often decrease at lower pH (e.g. Nugroho *et al.*, 2007; Cheng *et al.*, 2013), although nitrifiers can actually cope with lower pHs than previously thought (De Boer & Kowalchuk, 2001). Additionally, the length of time following pH change can be important in determining the effect of pH on nitrification (Baggs *et al.*, 2010). In contrast, the ratio of N_2O:N_2 production via denitrification can increase at lower pH (Šimek & Cooper, 2002), although the overall rate of denitrification tends to be lower. As there are various opposing mechanisms by which AMF may interact with N_2O production, no change in net N_2O production may occur, yet the communities and pathways producing the N_2O may be significantly different between the AMA and NAMA treatments. The interactions between AMF hyphae and N_2O production are therefore varied, potentially complex, and counter-acting; they are summarized in Figure 3.1, which also shows the implications for N_2O production rates.

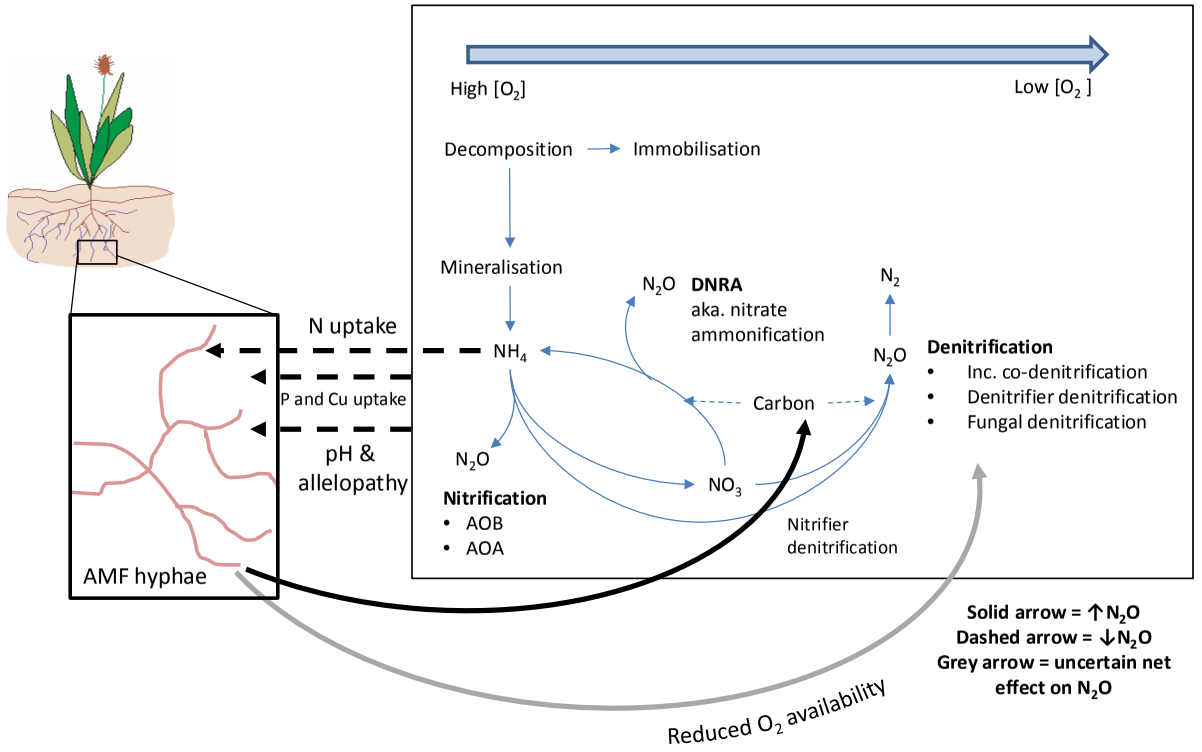


Figure 3.1. Summarized potential interactions between AMF hyphae and soil N_2O producing processes. The solid and dashed lines represent AMF effects that could result in an increase or decrease in N_2O production respectively. AMF can affect the availability of nitrogen (N), phosphorus (P), copper (Cu) and iron (Fe) in soils as well as having the potential to change soil pH and have allelopathic effects on soil microorganisms. Nitrifier nitrification is generally carried out by ammonia oxidising bacteria (AOB) and archaea (AOA). Dissimilatory reduction of nitrate to ammonium (DNRA) may produce N_2O as a side product. There are various pathways and organisms capable of carrying out these roles, but for simplicity, they are grouped by factors affecting the rate of N_2O production (i.e. availability of O_2, or carbon (C)).

In Chapter 2, it was demonstrated that the predominant location for interactions between AMF hyphae and N_2O production was in the organic matter patches. Therefore, it was decided to develop and use gas probes to sample the N_2O and CO_2 concentrations from directly inside the patches. Gas probes generally consist of a hollow metal tube sealed at the base with a gas sample port at the top and a small hole near the base covered with a gas permeable membrane such as silicone (e.g. Panikov *et al.*, 2007; Mastepanov & Christensen, 2008), expanded Teflon (DeSutter *et al.*, 2006), or polyvinylidene fluoride (PVDF; Albanito *et al.*, 2009). As both N_2O and CO_2 are soluble in water (Weiss, 1974; Weiss & Price, 1980), these membranes are used to prevent water access but allow equilibration of the internal and external concentrations of gases including CO_2 and N_2O over relatively short timescales (minutes or hours depending on the membrane used; Jacinthe & Dick, 1996; DeSutter *et al.*, 2006; Panikov *et al.*, 2007). Heinemeyer *et al*. (2012a) used a PVDF membrane as the gas permeable material in the 'Gas-Snake'; a gas sampling tube that is used to measure soil surface gas fluxes. Polyvinylidene fluoride is a semi-crystalline thermoplastic polymer that exhibits both chemical and mechanical resistance with a pore size of 0.2 µm, and a very fast diffusion rate of $3.95 \pm 0.31 \times 10^{-8}$ m^2 s^{-1} for CO_2 (Albanito *et al.*, 2009; Heinemeyer *et al.*, 2012a; Bio-Rad, Laboratories Inc. Hertfordshire, UK). The suitability of a PVDF membrane in this study was tested using an incubation chamber similar to that used by Jacinthe & Dick (1996) and PVDF was subsequently selected for use in the gas probe design used here.

As previously discussed, there are various mechanisms by which AMF hyphae could interact with N_2O production (see Figure 3.1), but the greatest effect of the presence of AMF hyphae is hypothesised to be the modification of NH_4, NO_3, C or O_2 availability as these are the most common limiting factors for N_2O producers (Gillam *et al.*, 2008). The addition of NH_4NO_3 usually results in increased N_2O production from soils as it simultaneously removes NH_4 and NO_3 limitations (Baggs *et al.*, 2003a; Bateman & Baggs, 2005), and was therefore used to test for N limitation in the organic matter patches. The hypothesis was that N_2O concentration would be lower in the AMA patches as N_2O producers would be N limited, even if C availability was higher.

Furthermore, following NH_4NO_3 addition, the N_2O production in the AMA patches was hypothesised to increase to equal that of the NAMA patches as the N_2O producers in the AMA patches would no longer be N limited.

Nuccio *et al*. (2013) demonstrated that when only AMF hyphae had access to an organic matter patch, they provided 7% of their host plants N content from the patch material. Total plant N content can also increase when the AMF hyphae are permitted access to organic matter patches (Hodge & Fitter, 2010; Barrett *et al.*, 2011; Nuccio *et al.*, 2013), although this has not always been found (see Leigh *et al.*, 2009). As *Z. mays* adopts a strategy where the leaf N concentration is directly lowered under N limitation (Vos *et al.*, 2005), the hypothesis tested was that the leaf N content would be higher when AMF hyphae were permitted access to an organic patch compared to when they were not. This was tested using a two-compartment microcosm design. It was also predicted that the lower N_2O concentration in the AMA patches, would be related to the leaf N content, as a result of the AMF transferring N from the patch to the plant.

3.2 Materials and Methods

3.2.1 Gas probe development

Following the discussion arising from Chapter 2 regarding dilution of the N_2O fluxes in large experimental units, measurement of the N_2O concentration directly in the organic matter patch was considered more informative, as this was where the highest ERM length densities were present, together with the highest N_2O fluxes post-harvest. Thus, initially, gas probes were developed to allow gas sampling in the patch only and these are outlined below.

3.2.1.1 Gas probe design

A stainless steel tube (9 cm long, outer diameter 1 cm, wall thickness 1 mm; Coopers Needle Works Ltd., UK) was welded at the base to form an airtight seal (see Figure 3.2). Two diametrically opposed holes, 6 mm, were drilled through each tube 13 mm from the base, making two holes through which air could enter the tube. These holes were then covered in a PVDF membrane (pore size 0.2 µm, Sequi-blot PVDF membrane, Bio-Rad Laboratories Inc.) that was air permeable but impermeable to water. The PVDF was stuck onto the stainless steel tube using aquarium sealant (Aquamate, Everbuild Building Products Ltd. Leeds, UK). This fine PVDF membrane was then housed in a supporting silicone tube (wall thickness 0.8 mm, outer diameter 8 mm; Silex Ltd., UK) with access holes exposing the membrane covering the holes in the stainless steel tube (Figure 3.2a, solid arrow); this silicone tube had a further layer of sealant sealing the top and bottom joins to ensure that the gas probe was water tight. Consequently, the only exposed area of PVDF membrane was that covering the hole in the stainless steel tube. The stainless steel tube was then sealed at the other (top) end with a white rubber Suba-Seal® (No. 13, Sigma-Aldrich, UK) to form a gas sampling port. The total internal volume of the gas probe was approx. 4.5 cm^3 (Figure 3.2).

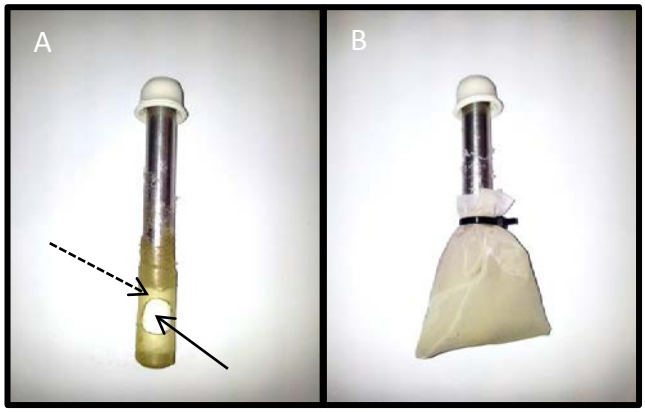


Figure 3.2. Gas probe with white rubber Suba-Seal® in the top. (A) At the base of each gas probe there was an outer housing of silicone tubing forming a water tight seal (dashed arrow) over the PVDF membrane exposed only at the drilled hole (solid arrow) and, (B) gas probe enclosed in the 20.0 µm bag that contained the organic matter patch material.

3.2.1.2 Gas probe testing

Gas samples removed from the gas probes needed to be in equilibrium with gas concentrations in the surrounding organic matter patch at the time of sampling. Before being used in the organic matter patches, the minimum length of time required for the gas probes to reach equilibrium with the surrounding air needed to be quantified. To do this, a gas probe (Figure 3.2a) was placed inside a Wheaton bottle with a modified crimp cap lid surrounding the gas probe and a rubber stopper to create both an air-tight seal around the gas probe, but also allowing the removal of gas samples from the Wheaton bottle (see Figure 3.3). The Suba-Seal® in the top of the gas probe allowed removal of gas samples from the probe using a needle and syringe. The Suba-Seal® ensured that the internal volume of the gas probe was not exposed to outside air, therefore only gas from inside the Wheaton bottle could diffuse through the PVDF membrane and into the gas probe volume. Three gas probe and Wheaton bottle units of this type (Figure 3.3) were used to provide adequate replication.

Known concentrations of CO_2 (523 ppm) and N_2O (9.4 ppm) were added to the Wheaton by flushing with a certified standard for at least 5 min. A 10 cm^3 syringe filled with the same standard was then attached to the Wheaton bottle via a needle port. The gas probe was then flushed with N_2 for 30 s and a second 10 cm^3 syringe filled with N_2 was attached to the gas probe in the same way. Gas samples (2 cm^3) were then taken at intervals (10 min up to 24 h) from both the Wheaton bottle and gas probe. To prevent pressure differences affecting the rate of diffusion the attached 'reservoir' syringes were depressed by 2 cm^3 each time a sample was removed from the gas probe or Wheaton. The gas probe units were stored at 20^oC throughout.

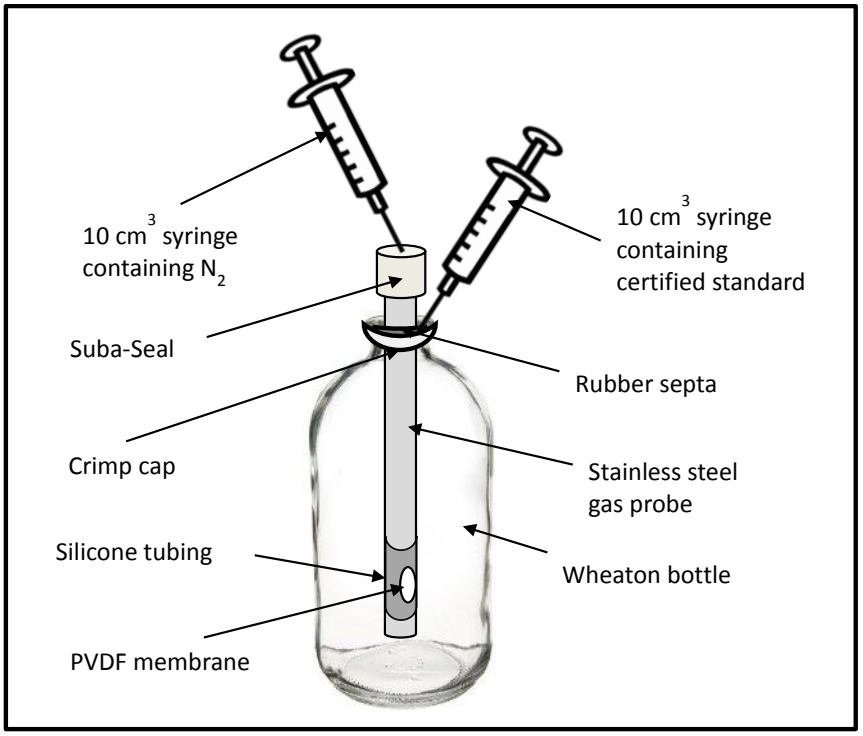


Figure 3.3. Unit design (not to scale) used to test the time taken for the concentration of N_2O inside the gas probe (described in Section 3.2.1.1) to reach equilibrium with that of a certified standard in the surrounding Wheaton bottle. The gas probe was secured inside a Wheaton bottle using a rubber septa and crimp cap creating an air tight seal (described in Section 3.2.1.2).

The samples were injected into Exetainers that had been pre-filled with 5 cm^3 N_2 before being analysed using a gas chromatograph (GC, Perkin Elmer ARNEL Autosystem XL Gas Chromatograph, Massachusetts, USA). The areas were then converted to concentrations by comparing to certified standards that had also been diluted in parallel in the same ratio (see Chapter 2, Section 2.2.3.9). The ppm concentration was plotted over time for both the Wheaton and gas probes, and the time at which they did not differ was identified as the length of time necessary for the probes to reach equilibrium. The gas probes were also tested for water retention. Three of each gas probes were left in wet soil for 3 d, dried externally with a paper towel, weighed, dried internally and then weighed again to assess for leaks into the tube.

The methods outlined by DeSutter *et al*. (2006) and Jacinthe & Dick (1996) were used to fit the data to a first order diffusion model (Equation 3.1),

$$\ln(C_h - C_i) = -kt_{eq} + \ln(q_0) \quad (3.1)$$

where C_h was the concentration of N_2O in the Wheaton bottle, and C_i was the concentration of N_2O in the gas probe at time t, k was the rate constant (min^{-1}) calculated from a plot of $\ln(C_h - C_i)$ against time (min) and q_0 was the difference between the N_2O concentration in the gas probe (C_i) and Wheaton bottle (C_h), as $(C_h - C_i)$ at time zero (t_0). Following Jacinthe & Dick (1996), Equation 3.1 was solved for time (t) using the first three time points by substituting $0.05q_0$ for (C_h - C_i) to calculate the time taken for the N_2O concentration inside the gas probe and Wheaton bottle to reach 95% equilibrium (t_{eq}). The diffusion coefficient (D) was calculated using Equation 3.2, following methods used by Jacinthe & Dick (1996) and DeSutter *et al*. (2006);

$$D = \frac{kVL}{A} \quad (3.2)$$

where k is the rate constant (min^{-1}) as above, V is the internal volume of the gas probe, L is the thickness of the PVDF (cm) membrane, and A is the surface area of the exposed PVDF membrane over which the N_2O can diffuse (cm^2).

3.2.2 Experimental design

Twenty-four, two-compartment microcosm units (see Section 3.2.3 below) were planted on 25th June 2012. One compartment contained the AMF host plant (*Z. mays*), and was separated from the second compartment (hyphal compartment) by either a 20.0 µm or 0.45 µm mesh that allowed (AMF access; AMA) or prevented (no AMF access; NAMA) hyphal access respectively, creating two AMF access treatments (n = 12 for each treatment). The organic matter patches were placed in the hyphal compartments after 29 d of plant growth and each patch contained a gas probe. At 44 d after patch addition, half of the patches within each of the AMF access treatments received NH_4NO_3 and half received deionised water, now creating four treatments (n =

6) as follows: AMA + NH_4NO_3, AMA + water, NAMA + NH_4NO_3, NAMA + water. Patch gas concentrations were measured using gas probes (Section 3.2.1 above) before (pre-NH_4NO_3) and after NH_4NO_3 addition (post-NH_4NO_3) and the units were destructively harvested on 10th September 2012 at 77 d after planting (48 d post-patch addition).

3.2.3 Microcosm design

The same microcosms were used as in Chapter 2, Section 2.2.1.1. Two 1 L plastic boxes (145 x 145 x 70 mm) were screwed together at 4 equidistant points with a 6 mm thick plastic plate separating them (Figure 3.4). This created two compartments, one compartment was planted with *Z. mays* (details in Section 3.2.4 below) and the other compartment was the 'hyphal' compartment, to which roots were prevented access. The plastic plate that separated the plant and hyphal compartments had a 70 x 70 mm hole cut out which was covered on both sides of the plastic plate with either a 20.0 µm (John Stanier & Co., Whitefield, Manchester, UK) or 0.45 µm (Osmonics Inc., Minnetonka, USA) mesh membrane. These mesh membranes either allowed (AMA) or did not allow (NAMA) AMF hyphal access from the planted to the AMF compartment respectively. In both cases, the mesh membrane was glued into place using industrial strength superglue (Everbuild Building Products Ltd. Leeds, UK). Aquarium sealant (Aquamate, Everbuild Building Products Ltd. Leeds, UK) was used between the surfaces of the boxes and the plastic plate to create a seal between the two compartments preventing AMF hyphal breakthrough. The unit lids were taped into place on either side of the microcosm after both sides had been filled with the growth medium (see Section 3.2.4 below). There were three 6 mm drainage holes in the base of each compartment and, once planted, the units were wrapped in three layers of aluminium foil to prevent any algal growth inside the units.

Figure 3.4 Microcosm unit at 73 d post-planting (44 d post-organic patch addition). The planted compartment (left) was separated from the organic patch compartment by a plastic plate that had a double layer of either a 0.45 µm or 20.0 µm mesh membrane covering a 70 x 70 mm window between the two compartments. The white rubber Suba-Seal® indicated by the white circle and arrow is in the right hand 'hyphal' compartment, denoting the top of the gas probe and associated patch which was located 7 cm below the surface, and at a distance of 2 cm from the membrane. The units were wrapped in aluminium foil to prevent algal growth inside the units.

3.2.4 Growth Media

Both the plant and hyphal compartments contained a 50/50 mix of sand/Agsorb® (v/v) (Agsorb® is a calcined attapulgite clay soil conditioner; previously called TerraGreen but now rebranded as Agsorb®; Oil-Dri, USA) that had been rinsed three times in deionised water to remove any excess soluble N and/or P. The planted compartments also had 50 g of a fresh *Glomus intraradices* inoculum (Schenck & Smith; Plantworks Ltd., Kent, UK) and 0.25 g L^{-1} bonemeal (a complex N and P source; 3.5% N, 8.7% P; Vitax, Leicestershire, UK) added. Three pre-germinated *Z. mays* seeds were added to each plant compartment on 25th June 2012 and thinned to one per pot after two weeks (11 d), as in Chapter 2, Section 2.2.3.3. At 8 d post-planting, the second (hyphal) compartments were filled with a 50/50 mix of sand/Agsorb® (v/v). Addition of the growth media to the hyphal compartments was delayed to allow the AMF time to

colonise the host plant roots in the plant compartment. Similar to Chapter 2 Section 2.2.3.4, a sterile 15 cm^3 centrifuge tube was added to the hyphal compartments to enable creation of a hole into which the organic matter patches could be added at a later date (see Section 3.2.6 below).

3.2.5 Growth conditions

Twenty-four microcosm units were housed in a temperature controlled glasshouse in a randomised block design. The photosynthetically active radiation (PAR) was measured weekly between 10 am and 2 pm and at plant level in the centre of each block using a hand-held PAR meter (Skye Instruments, UK); the mean PAR over all blocks was 141 ± 15 µmol m^{-2} s^{-1}. Overhead lights were used in the morning and evening to extend the photoperiod to 16 h per day and the mean daily temperature over the experimental period was 21.9 ± 0.02°C.

The planted and hyphal compartments for all microcosm units were watered daily as required. The water content of the outer compartments could not be equalised to the same extent as in Chapter 2 as the units were too small to allow insertion of a moisture probe. After two weeks of plant growth, the plant compartments received 50 cm^3 of a modified nutrient solution originally from Thornton and Bausenwein (2000) (1/10^{th} N and P; see Appendix 1 for details) once a week.

3.2.6 Organic matter patches

After four weeks (29 d post-planting), the centrifuge tubes were removed and an organic matter patch was inserted in the hyphal compartment of each microcosm (2 cm from the mesh membrane) that was composed of dried milled *Z. mays* shoots (F1 Earligold, Moles Seeds; 2 g dw) mixed with soil (equivalent to 13 g dw), as in Chapter 2 Section 2.2.3.4. The patch was contained in a 20.0 µm bag that also housed a gas probe (described in Section 3.2.1). The bags were made as in Chapter 2 (Section

2.2.1.4) but were 1 cm wider at the top to allow for the insertion of a gas probe (see Section 3.2.1 for details on gas probe design), and the top was sealed around the gas probe using a 100 mm x 2.5 mm plastic cable tie (Centurion Europe Ltd., UK). The patches were all inserted at a depth of 7 cm and at a distance of 2 cm away from the mesh window. The moisture content of each patch was adjusted to 20% moisture (w/w) content using deionised water. The remaining hole was then filled with 50/50 mix of sand/Agsorb® at approximately 20% moisture content. The C and N content of the mixed organic patches and milled *Z. mays* leaves used in the organic patches are outlined in Table 3.1.

Table 3.1. Carbon (C) and nitrogen (N) content and C:N ratio of the mixed organic patch material (13 g DW equivalent soil mixed with 2 g DW milled *Z. mays* leaves) and *Z. mays* leaves that were used in the organic patches before addition to the microcosms. Mean values ± standard error of the mean (*n* = 3).Total values are for 15 g (DW equivalent) mixed organic patches and 2 g DW of milled *Z. mays* leaves.

	Total C (mg)	% C	Total N (mg)	% N	C:N Ratio
Mixed patch	1435.4 ± 181.8	9.6 ± 1.2	116.4 ± 14.7	0.78 ± 0.1	12:1
Z. mays leaves	874.4 ± 3.5	43.7 ± 0.2	55.7 ± 1.8	2.79 ± 0.1	16:1

3.2.7 Inorganic nitrogen addition

Half of the AMA and NAMA patches were injected with 7 cm^3 of 30 mM NH_4NO_3 and the other half with 7 cm^3 of deionised water (*n* = 6 in each case) at 44 d after patch addition. This solution was administered in two batches of 3.5 cm^3 with an hour gap in between additions to reduce the risk of the solution diffusing away from the patches. Consequently, the treatments were: AMA + NH_4NO_3, AMA + water, NAMA + NH_4NO_3 and NAMA + water.

3.2.8 Gas sampling and analysis

The air in the gas probes was sampled before N addition at 73 d post planting (44 d post-patch addition). The NH_4NO_3 and water addition treatments were then added and the gas probes were sampled again at 24, 48 and 96 h post-NH_4NO_3 addition. Since the gas probes had a limited internal gas volume (approx. 4.5 cm^3), the samples taken during the experiment were reduced to 1 cm^3. Before sample removal, 1 cm^3 of N_2 was added to the probe via the Suba-Seal®, taking care not to disturb the surrounding media. This was left for 10 s before a 1 cm^3 sample was slowly removed from the gas probe, waiting for a further 5 s to allow the sample to mix inside the syringes. Each gas sample was then stored in a pre-filled 3 cm^3 Exetainer (with 6 cm^3 N_2), over-pressuring the sample to 7 cm^3 in total. All gas samples were analysed using a gas chromatograph (GC) as in Chapter 2 Section 2.2.3.9 which analysed the samples for CO_2 and N_2O. The ppm values for each sample were calculated by comparing to standards that were diluted in parallel in a 1 cm^3 standard: 6 cm^3 N_2 ratio and correcting for this dilution. The concentration values were also corrected for dilution from addition of N_2 to the gas probe just before gas sample removal (Section 3.2.8).

3.2.9 Post-harvest plant and AMF analyses

The plant compartments were harvested as described in Chapter 2 Section 2.2.3.7, except extraradical mycelium (ERM) lengths from the planted compartment were not quantified. After the plant shoots were removed, but before removal of the growth medium, a sharp metal blade was passed down the membrane at the planted compartment side to sever the hyphae from the hyphal compartment. The plant compartment was then emptied and the roots collected and stained, as in Chapter 2 Section 2.2.3.7. The hyphal compartments were then destructively harvested; the patches were removed and well mixed sub-samples of the patches were taken for ERM length quantification (again, as in Section 2.2.3.7). Following the destructive harvest, the dried plant leaves (green leaves only, defined as > 50% green) were milled and analysed for C and N content using an elemental combustion system (Costech Analytical Technologies Inc., California, USA).

3.2.10 Data analysis

To determine whether the data were suitable for analysis using parametric statistical tests, the data were first tested for normality and equality of variance assumptions using Kolmogorov-Smirnov and Levene's equality of variance tests respectively. All statistical analyses were carried out in SAS (v9.3 SAS institute Inc., North Carolina, USA).

Gas probe data were analysed for changes in concentration over time inside the gas probe and Wheaton bottle using a repeated measures ANOVA. At each time point, the difference between the concentration of N_2O or CO_2 in the gas probe and Wheaton bottle was compared to zero using a one-sample t-test.

Where N_2O concentration and ERM length data did not fulfil normality or equality of variance assumptions they were $\log_{10}$ transformed; all percentage colonisation data were arcsine transformed before analysis. All gas, AMF and plant data were analysed using a two-way ANOVA including block, with Duncans *post hoc* tests used to identify differences between treatments. However, transformations on percentage colonisation by vesicles, patch moisture and changes in N_2O concentration following N addition failed to normalise the data, and non-parametric equivalent Friedman's two-way ANOVAs, including block, with Wilcoxon *post hoc* tests and Bonferroni corrections were used. Where N_2O concentrations were measured over time, repeated measures ANOVA including treatment and block was used on $\log_{10}$ transformed data. Pearson's product moment correlations were used to determine the relationship between variables. Where variables were not normally distributed, Spearmen's rank order correlations were used. Untransformed data are presented in all figures.

3.3 Results

3.3.1 Gas probe testing

The time taken for the gas probe N_2O concentration to equal that of the Wheaton bottle was < 8 h when the PVDF membrane was used (time: $F_{4,16}$ = 18.76, P < 0.0001; time*treatment: $F_{4,16}$ = 16.88, P < 0.0001; Figure 3.5). Using a first order diffusion model, the diffusion coefficient (D) and time taken to reach 95% equilibrium (t_{eq}) for N_2O were 7.02 ± 0.46 x 10^{-3} cm^2 s^{-1} and 1.64 ± 0.1 h respectively. These values could not be calculated for CO_2 as this gas equilibrated too quickly; the CO_2 concentrations in the gas probe and Wheaton bottle were not significantly different following the first sample (t_0, up to 1 min after N_2 was no longer flushed through the gas probe; time: $F_{4,16}$ = 1.74, P = 0.191; time*treatment: $F_{4,16}$ = 0.22, P = 0.924; t_0: t_2 = 2.13, P = 0.167). After three days in wet soil, the gas probes contained a small amount of water (0.018 cm^3 ± 0.002), this equated to an input daily average of 0.006 ± 0.0008 cm^3.

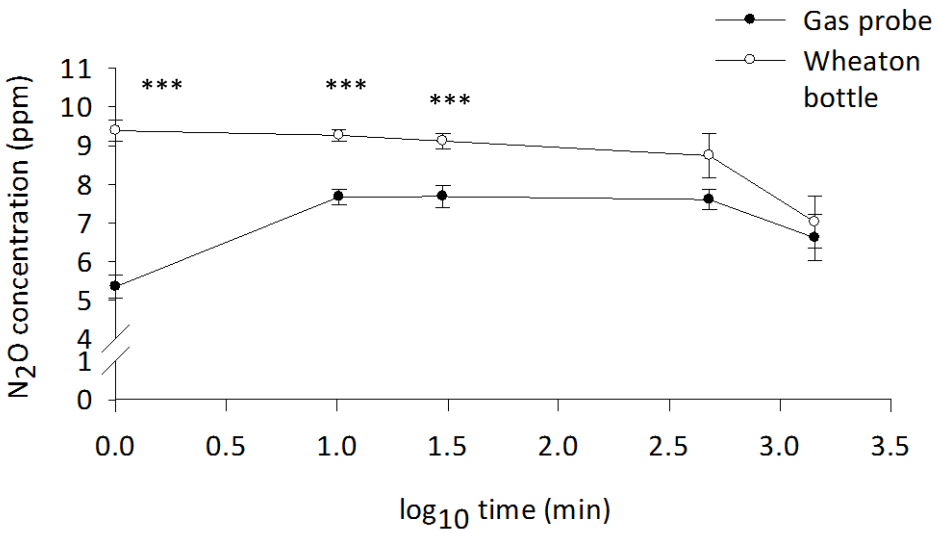


Figure 3.5. N_2O concentrations measured in gas probes (solid circle) and Wheaton bottles (open circle) at 0, 0.17, 0.5, 8 and 24 h after a known concentration of N_2O (9.4 ppm) was added to the Wheaton bottle and allowed to diffuse into the gas probe that had been flushed with N_2 for at least 30 s. Error bars represent ± standard error of the mean (n = 3). Asterisks indicate significant differences between treatments within each time point (*P < 0.05, **P < 0.01, ***P < 0.001), determined using a one-sample t-test to compare the difference between the treatments to zero.

3.3.2 AMF colonisation of *Z. mays* and production of hyphae

All of the *Z. mays* plants were well colonised by AMF hyphae, arbuscules and vesicles (Figure 3.6). The plants that grew in AMA microcosms had a higher percentage of their root length colonised by AMF than those in NAMA microcosms ($F_{1,12}$ = 10.62, P = 0.0068). There was no difference in the frequency of arbuscules or vesicles between the AMA and NAMA treatments (arbuscules: $F_{1,12}$ = 3.58, P = 0.083; vesicles: Q_1 = 0.4, P = 0.527), and no differences in the % RLC, arbuscule or vesicle colonisation as a result of N addition treatment (i.e. NH_4NO_3 or water addition; P > 0.05 in each case, data not shown). The ERM length densities measured in the AMA organic matter patches were greater than those in the NAMA patches (Figure 3.7; $F_{1,12}$ = 30.77, P = 0.0001).

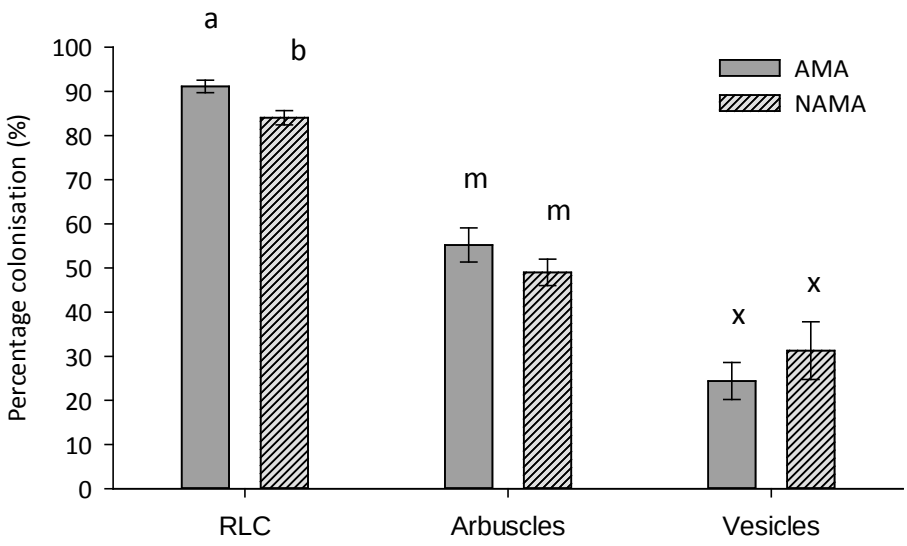


Figure 3.6. Mean percentage length of *Z. mays* roots colonised by AMF hyphae (RLC), arbuscules and vesicles for AMF hyphal access (AMA; solid bars) and no AMF hyphal access (NAMA; hatched bars) treatments. Error bars represent ± standard error of the mean (n = 12). Different letters within each group (RLC, arbuscules or vesicles) represent significant differences between AMA and NAMA treatments at P < 0.05 as determined using a Duncans *post hoc* test on the RLC data.

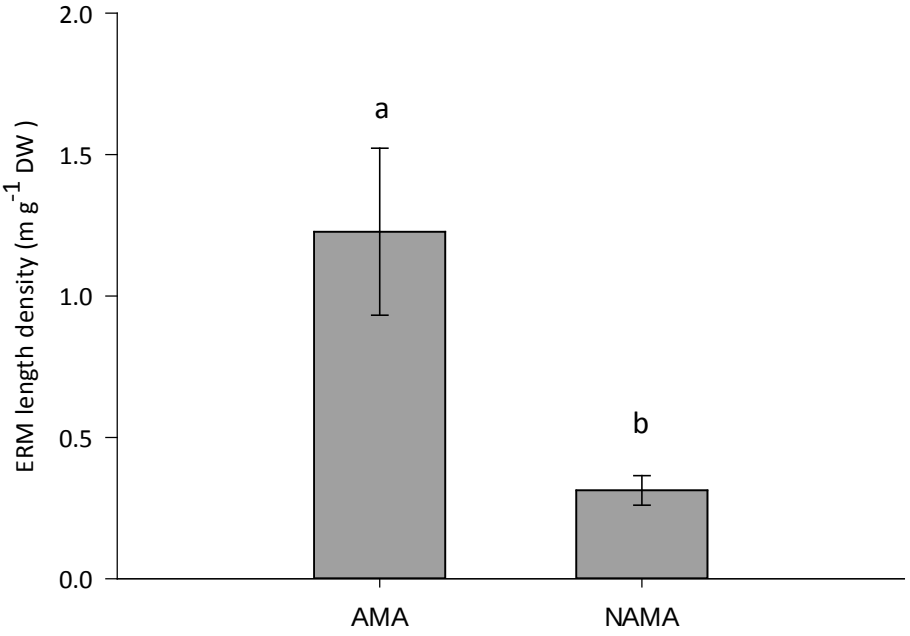


Figure 3.7. Mean extraradical mycelium (ERM) length density (m g^{-1} DW) in AMF access (AMA) and no AMF access (NAMA) patches. Error bars represent ± standard error of the mean (n = 12). Different letters represent significant differences at $P < 0.05$ as determined using a two-way ANOVA including block.

3.3.3 Patch CO_2 and N_2O concentrations

At 43 d post-organic patch addition, there was no significant difference in the CO_2 concentration measured from the AMA compared to the NAMA patches (Figure 3.8a; $F_{1,12}$ = 0.23, P = 0.637). There was also no relationship between the measured ERM length densities and CO_2 concentration in AMA patches (n = 12, r_s = 0.0559, P = 0.863, r^2 = 0.003). However, the N_2O concentration inside the NAMA patches was significantly higher than that of the AMA patches at 43 d post-organic patch addition (Figure 3.8b; $F_{1,12}$ = 6.46, P = 0.026). There was also a negative relationship between the concentration of N_2O and CO_2 in the organic matter patches preceding NH_4NO_3 addition (r_s = -0.6434, P = 0.024, r^2 = 0.41) but this was not significant in the NAMA treatment (r_s = -0.5315, P = 0.075).

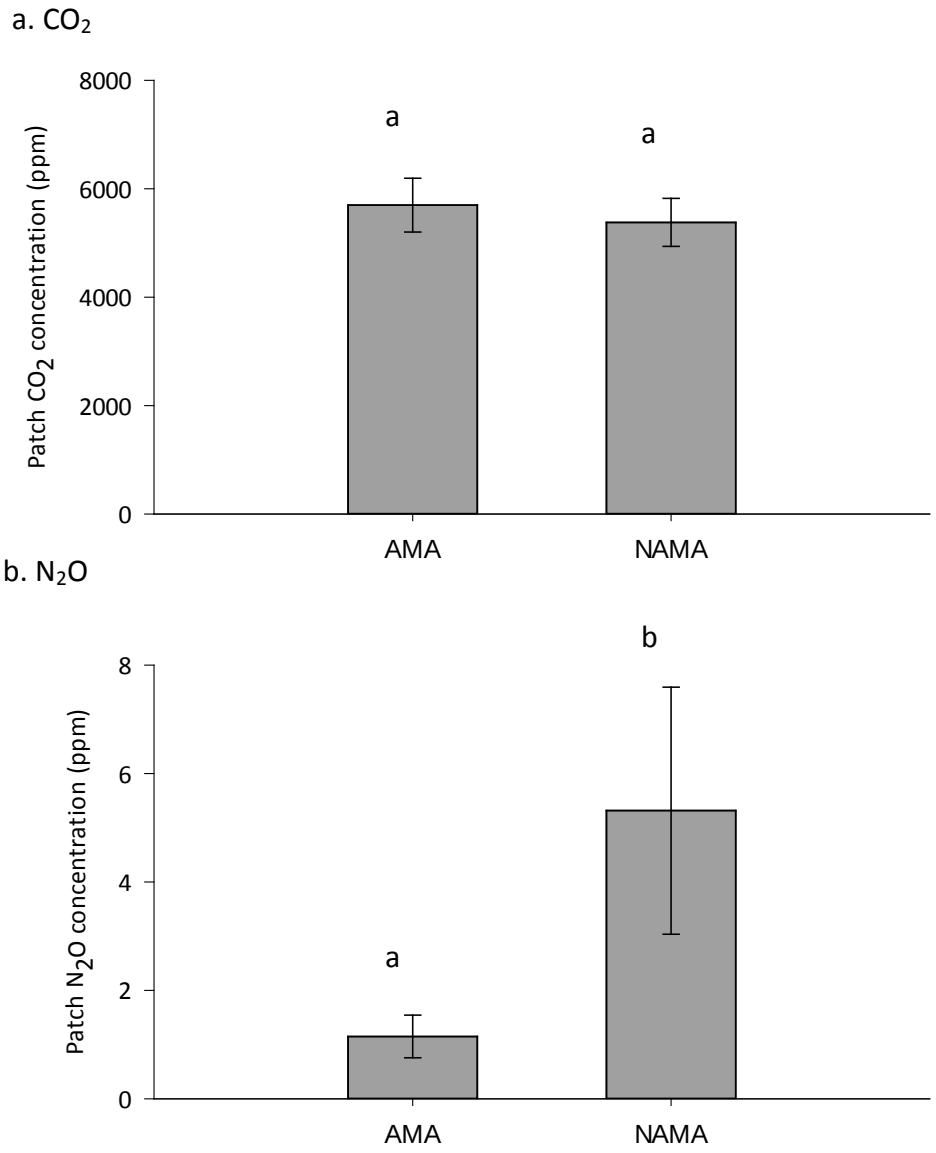


Figure 3.8. Mean (a) CO_2 and (b) N_2O concentration (ppm) in AMF access (AMA) and no AMF access (NAMA) organic matter patches at 43 d post-organic patch addition. Error bars represent ± standard error of the mean (n = 12). Different letters represent significant differences at $P < 0.05$ as determined using two-way ANOVAs.

The highest N_2O concentrations were observed at the first gas sample which was taken 24 h after the addition of NH_4NO_3 and water (Figure 3.9a). Subsequently, the N_2O concentrations in the patches decreased over time except in the AMA + water treatment which did not change (Figure 3.9a; time: $F_{2,30}$ = 4.37, P = 0.023). There was also a significant effect of the AMF access + N treatment (NH_4NO_3 or water) on patch N_2O concentration (Figure 3.9a,b; $F_{3,15}$ = 5.67, P = 0.0084) and the interaction between time and AMF access + N treatment was also significant (Figure 3.9a,b; $F_{6,30}$ = 3.23, P = 0.015). Two-way ANOVAs at each time point showed that the N_2O concentration of the AMA + water treatment was lower than all other treatments at 24 h post-NH_4NO_3 addition (Figure 3.9a,b; $F_{3,15}$ = 4.44, P = 0.020). This effect decreased by the 48 h sample, although the NAMA + water treatment still had a higher N_2O concentration that that of the AMA + water treatment ($F_{3,15}$ = 3.05, P = 0.030). At 96 h post-NH_4NO_3 addition, the AMA patch N_2O concentrations were no longer different from each other but were significantly lower than those of the NAMA patches ($F_{3,15}$ = 7.25, P = 0.0031). There was no relationship between the ERM length densities in AMA patches and N_2O concentration at any point, before or after NH_4NO_3 and water addition (P > 0.05 in each case).

At 24 h post-NH_4NO_3 addition, the N_2O concentration increased in both the AMA + NH_4NO_3 and NAMA + NH_4NO_3 treatments, but this increase did not occur in the AMA + water treatment (Figure 3.10; Q_3 = 8.2 P = 0.042). However, the NAMA + water treatment was not significantly different from the AMA + NH_4NO_3 treatment (Z = 1.92, P = 0.055) or NAMA + NH_4NO_3 treatment (Z = -1.76, P = 0.078) when a Bonferroni correction was applied (where P < 0.0083). There were also no significant changes in N_2O concentration at 48 h or 96 h post-NH_4NO_3 addition when compared to pre-N addition values (48 h: Q_3 = 7.4 P = 0.060; 96 h: Q_3 = 7.2 P = 0.066).

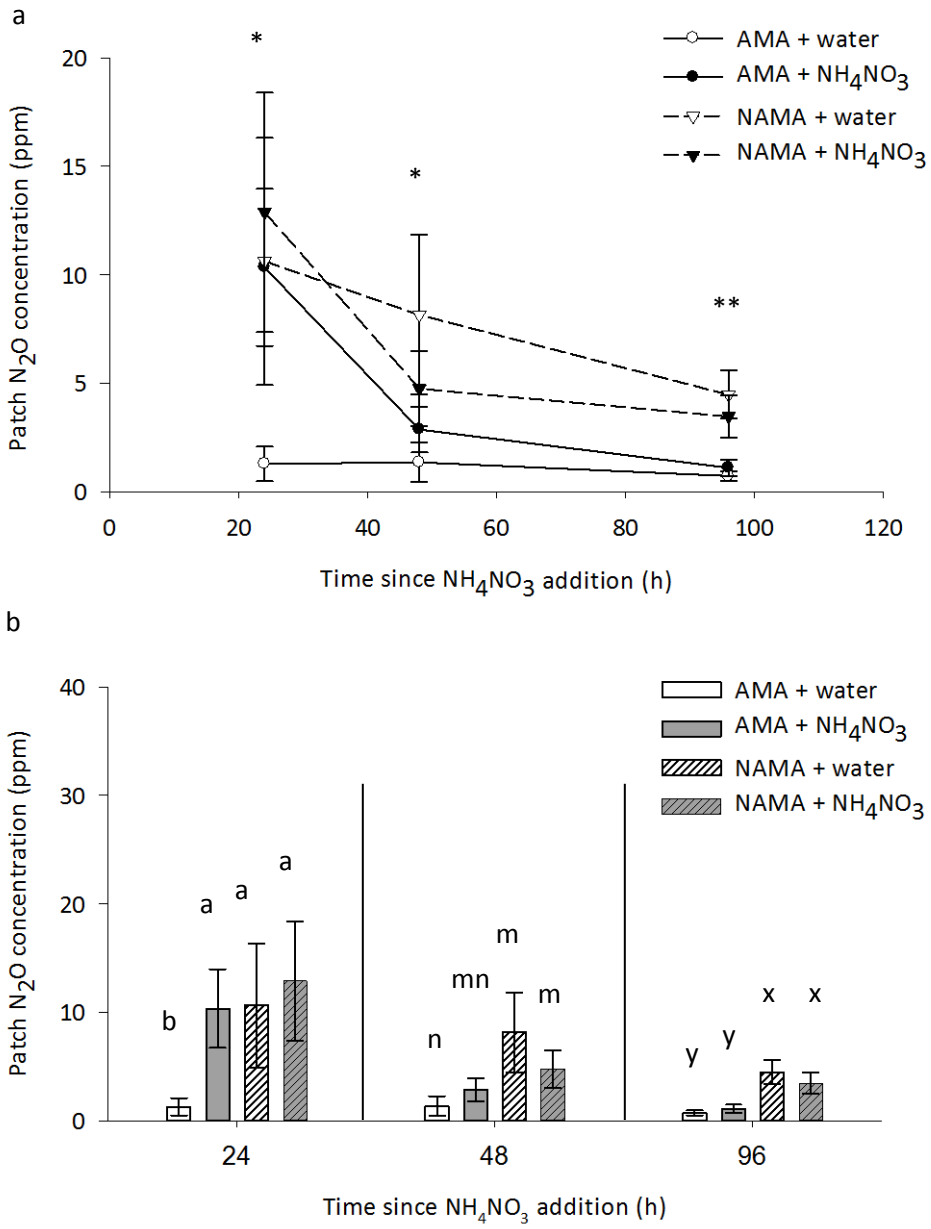


Figure 3.9. Mean patch N_2O concentration at 24, 48 and 96 h following NH_4NO_3 addition for AMF access (AMA; (a) solid lines or (b) solid bars) and no AMF access patches (NAMA; (a) dashed lines or (b) hatched bars) shown (a) over time and (b) separated by treatment for each sample (NH_4NO_3: (a) filled symbols or (b) grey bars; water: (a) open symbols or (b) white bars). Error bars represent ± standard error of the mean (n = 6). In Figure 3.9a, asterisks represent a significant difference between treatments within each sample period (*P < 0.05, **P < 0.01) as determined using a Friedman's two-way ANOVA. Different letters within each sample time in Figure 3.9b represent significant differences between treatments (P < 0.05).

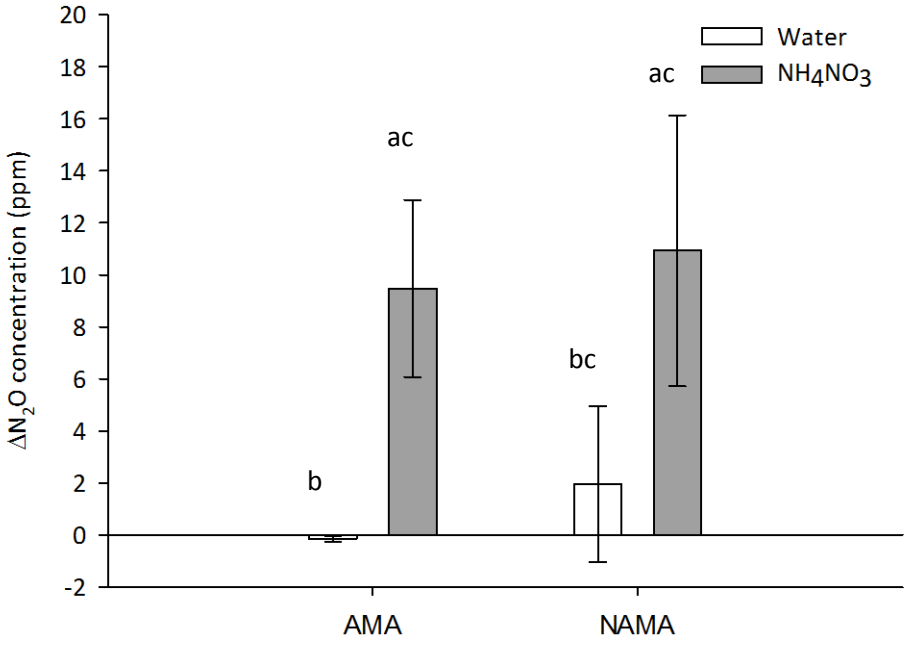


Figure 3.10. Mean change in organic patch N_2O concentration (ΔN_2O concentration) from pre-NH_4NO_3 addition to 24 h post-NH_4NO_3 addition (NH_4NO_3: grey bars, water: white bars) for the AMF access (AMA) and no AMF access (NAMA) patches. Error bars represent ± standard error of the mean (n = 6). Bars with different letters are significantly different ($P < 0.05$) as determined using Wilcoxon *post hoc* tests.

3.3.4 Patch moisture content

The moisture contents of the organic matter patches did not significantly differ between AMA and NAMA treatments (Table 3.2; n = 12, Q_1 = 0.05, P = 0.827) or among all treatments, including NH_4NO_3 and water addition (Table 3.2; n = 6, Q_3 = 0.05, P = 0.827). There was no relationship between the moisture content and pre-NH_4NO_3 addition N_2O concentrations for the AMA organic matter patches (n = 12, r_s = -0.3566, P = 0.255, r_2 = 0.13), but there was a significant negative relationship between the moisture content and N_2O concentrations before the NH_4NO_3 addition in the NAMA organic patches (n = 12, r_s = -0.6783, P = 0.015, r^2 = 0.46). There was a significant

negative correlation between organic patch moisture and N_2O concentration following NH_4NO_3 and water addition at the 24, 24 and 96 h samples for the AMA + water treatment and at the 24 and 48 h samples for the NAMA + water treatment (Table 3.3). However, this was not the case for the NH_4NO_3 treatments (Table 3.3).

Table 3.2. Mean gravimetric moisture content (%) for AMF access (AMA) and no AMF access (NAMA) patches following the harvest at 48 d post-organic patch addition. The means are separated by both NH_4NO_3/water addition and AMF access treatment (n = 6) or by AMF access treatment only (overall, n = 12) ± standard error of the mean. Different letters in the central section indicate significant differences between the four AMF access and NH_4NO_3/water addition treatments at $P < 0.05$ and different letters in the bottom row (overall) indicate a significant difference between AMA and NAMA treatments only ($P < 0.05$) as determined using a Friedman's two-way ANOVA.

	Mean gravimetric moisture content (%)	
Treatment	AMA	NAMA
Water	43.8 ± 3.2 [a]	53.2 ± 9.1 [a]
NH_4NO_3	50.5 ± 2.8 [a]	49.0 ± 1.8 [a]
Overall	47.1 ± 2.2 [x]	51.0 ± 4.5 [x]

Table 3.3. Spearman's rank order correlation coefficients and r^2 values for the relationship between organic patch N_2O concentration at 24, 48 and 96 h post-NH_4NO_3 addition and organic patch gravimetric moisture content (%) following the harvest at 48 d after organic patch addition for each of the four treatments, AMF hyphal access (AMA) + NH_4NO_3, AMA + water, no AMF hyphal access (NAMA) + NH_4NO_3 and NAMA + water. Significant relationships are highlighted in bold and are indicated by asterisks on the *P* values (* $P < 0.05$, ** $P < 0.01$).

AMF access treatment	NH_4NO_3 or water addition treatment	Gas sample (h post-NH_4NO_3 addition)	r_s	*P*	r^2
AMA	NH_4NO_3	24	0.1429	0.787	0.02
		48	0.3143	0.544	0.10
		96	0.5429	0.266	0.29
	Water	24	-0.8857	**0.019***	0.78
		48	-0.8286	**0.041***	0.69
		96	-0.9429	**0.005****	0.89
NAMA	NH_4NO_3	24	-0.0286	0.957	0.0008
		48	-0.3714	0.469	0.14
		96	-0.2000	0.704	0.04
	Water	24	-0.8857	**0.019***	0.78
		48	-0.8857	**0.019***	0.78
		96	-0.7714	0.072	0.60

3.3.5 Host plant response to AMF hyphal access to organic matter patches

Neither the addition of NH_4NO_3 or water had any effect on the leaf C and N content or concentrations or on the C:N ratios ($P > 0.05$ in each case), therefore, these data were combined for comparison of AMA and NAMA treatments. Leaf carbon (C) content was not different between AMA and NAMA host plants (Table 3.4; $F_{1,12} = 0.30$, $P = 0.595$),

although the C concentration of the leaves was lower in the AMA plants compared to the NAMA plants (Table 3.4; $F_{1,12}$ = 5.37, P =0.039). Both the N content (Table 3.4, $F_{1,12}$ = 14.81, P = 0.0023) and concentration (Table 3.4; $F_{1,12}$ = 20.06, P =0.0008) of the leaves were higher in AMA plants compared to the NAMA plants, and consequently the C:N ratio of the leaves was lower in the AMA plants when compared to the NAMA plants (Table 3.4; $F_{1,12}$ = 18.51, P = 0.001).

Table 3.4. Mean leaf N and C total content (mg), concentration (mg g^{-1} DW) and C:N ratio of AM host plants for AMF access (AMA) and no AMF access (NAMA) treatments ± standard error of the mean (n = 12). Different letters within rows represent significant differences at P = 0.05 (highlighted in bold) as determined using two-way ANOVAs.

		AMF access treatment	
		AMA	NAMA
Leaf N	Total content (mg)	**13.8 ± 0.8^{a}**	**10.2 ± 0.9^{b}**
	concentration (mg g^{-1} DW)	**11.3 ± 0.6^{f}**	**8.8 ± 0.5^{g}**
Leaf C	Total content (mg)	503.2 ± 19.9^{j}	488.1 ± 27.2^{j}
	concentration (mg g^{-1} DW)	**413.4 ± 2.8^{m}**	**422.9 ± 3.7^{n}**
	Leaf C:N ratio	**37.6 ± 2.0^{x}**	**50.0 ± 3.0^{y}**

The organic patch N_2O concentration was not related to the leaf C or N content or concentration, nor was the patch N_2O concentration related to the leaf C:N ratio, either before or after N addition for both AMA and NAMA treatments (P > 0.05 in each case). There were no significant differences between AMA and NAMA plant DW either overall or when compared by tissue type (leaf, total shoot, stalk, total root, root weight ratio, tassel), therefore these data are not shown (P > 0.05 in each case). There was also no relationship between ERM length densities and host plant leaf C and N status for the AMA treatment (P > 0.05 in each case).

3.4 Discussion

3.4.1 Gas probe design

The time taken for the concentration of N_2O inside the gas probe to reach equilibrium (t_{eq}) with the N_2O concentration in the Wheaton bottle was 1.64 ± 0.1 hours and the diffusion coefficient for N_2O was 7.02 ± 0.46 x 10^{-7} m^2 s^{-1}. This diffusion coefficient is in a similar range to the value for CO_2 calculated by Albanito *et al*. (2009), yet Albanito *et al*. (2009) measured equilibration times closer to 14 min for CO_2. The diffusion coefficients for CO_2 in this study could not be calculated as the gas equilibrated too quickly. This may have been a result of various factors; a CO_2 concentration of 523 ppm was used which could increase the rate of diffusion into the gas probe, variation in sampling CO_2 at a concentration of 523 ppm may have masked the diffusion rate as the gas concentration would not have been diluted by much when diffusing into the gas probe (i.e. it is difficult to quantify a small change in a large number), or contamination of the gas in the gas probe with CO_2 from lab air or human error may have contributed.

Additionally, to prevent pressure issues, the gas samples in this thesis were not mixed (by pumping the syringe) upon removal from the gas probe or Wheaton bottle. While this may not have affected the CO_2 (at a concentration of 523 ppm), it is likely to have affected the N_2O measurements as this was only present at ca. 1.4 ppm. Consequently, each time a gas sample was taken, the N_2O concentration measured inside the gas probe was probably diluted, as N_2 had been pushed into the gas probe from the reservoir syringe, and therefore the t_{eq} was overestimated. Ideally, these diffusion rates could be calculated using a more sophisticated automated gas monitoring system. However, as gas sampling was not planned to be more frequent than once every 24 h, the measured diffusion coefficient and t_{eq} values were deemed acceptable for N_2O which was the focus of this study. The water permeability rate of 0.006 ± 0.0008 cm^3 d^{-1} was also minimal, and following the destructive harvest of the microcosm experiment, the probes did not show any signs of leaking, thus water permeability was not an issue.

The gas sample removed from the gas probe was large compared to the volume (1 cm^3 from a 4.5 cm^3 volume), which could not be avoided to obtain a large enough sample for GC analyses. Thus, it is likely that gas beyond the patch was sampled. However, since all units were identical and the gas probes were inserted into the centre of each patch in every case, it should not have affected the comparisons between the AMA and NAMA patch gas concentrations. Additionally, the addition of N_2 to the probe before gas sampling may have escaped through diffusion, this was intended to reduce the gas sampling from beyond the patch but may have increased the variation and thus made it less likely for differences between AMA and NAMA treatments to be observed. Nonetheless, the AMA and NAMA comparisons are valid, but the absolute concentration values should be considered with caution.

3.4.2 Density of AMF hyphae and CO_2 concentrations in organic matter patches

The ERM length densities from the AMA patches in this experiment (1.23 ± 0.25 m g^{-1} DW; 48 d post-patch addition) were similar to those measured in Chapter 2 at the 56 d (0.77 ± 0.44 m g^{-1} DW; 30 d post patch addition) and 84 d harvest (2.20 ± 0.84 m g^{-1} DW; 58 d post-patch addition). These values were also comparable to other studies using organic matter patches in a sand/Terra-Green® medium. Nuccio *et al*. (2013) measured hyphal lengths of ca. 1 m hyphae g^{-1} DW soil at 42 d post-patch addition, or between 1.5 to 2 m hyphae g^{-1} DW soil at 70 d post-organic patch addition, and Hodge *et al*. (2001) measured ca. 1 m hyphae g^{-1} DW soil at 42 d post-organic patch addition. Therefore, AMF hyphal lengths reported in the present study were comparable to other previous studies and demonstrated that AMF did proliferate in the organic patch zone when permitted access. However, there were measured values of ERM length densities in the NAMA treatments. It is not always possible to distinguish AMF hyphae from non-AMF hyphae (Leake *et al*., 2004), and since there was no evidence of AMF hyphal breakthrough on the 0.45 µm mesh membranes, these ERM length density values in the NAMA treatment are probably representative of the non-AMF

component included in the ERM length density measurements. This is discussed in further detail in Chapter 4, Section 4.4.1).

The CO_2 concentration inside the AMA patches however did not increase with AMF hyphal presence (Figure 3.8a). Soil CO_2 concentrations are normally very high (Pumpanen *et al.*, 2003; Kusa *et al.*, 2010), and this study was no exception with concentrations in the range of 3021 to 9462 ppm. One study with forest soils measured soil CO_2 concentrations ranging from 580 to 780 ppm in the humus layer, up to 13620 to 14470 ppm in the C-horizon (Pumpanen *et al.*, 2003), whereas CO_2 concentrations in two agricultural soils from Japan ranged from 2100 to 22000 ppm, increasing with depth (Kusa *et al.*, 2010). The CO_2 flux (and concentration) would be expected to increase in the presence of AMF hyphae as found in Chapter 2. However, as discussed in Chapter 2, the CO_2 fluxes may have been influenced by the presence of host plant roots in neighbouring compartments, which in this unpaired design would not be shared by the AMA and NAMA treatments. Therefore, as root respiration exceeds hyphal respiration, (Karasawa *et al.*, 2012), AMF hyphal influences may have been masked, particularly as in Chapter 2, hyphal effects on CO_2 were only measured leading up to the 105 d harvest (79 d post-organic patch addition), by which point the ERM length densities (3.84 ± 1.57 m g^{-1} DW) exceeded the values measured here (1.23 ± 0.25 m g^{-1} DW; 48 d post-organic patch addition). The level of PAR was quite low in this study and the others discussed in this thesis. It could be argued that this could impact upon the activity of the AMF partner. However in a microcosm based study Hodge and Fitter (2010) showed that shading a host plant had no impact upon AMF uptake of N or ERM length densities, and therefore the slightly lower PAR levels were unlikely to have a major impact on the studies outlined in this thesis.

3.4.3 Organic patch N_2O concentrations in the presence of AMF hyphae

When AMF hyphae were permitted access to the organic patches, the patch N_2O concentration was significantly reduced (Figure 3.8b). Thus, at least one, if not all, N_2O

producing pathways in the AMA patches must have been negatively affected by the presence of AMF hyphae, which has significant implications for N cycling, particularly our understanding of controls on N_2O production in soil systems. This fits with the findings of Veresoglou *et al*. (2011a) who measured lower potential nitrification rates in soils surrounding AM plants compared to soils from low-AM or non-AM plants. A field study on the effect of ectomycorrhizal (ECM) mycelia on soil N_2O fluxes in an afforested organic soil similarly found that the exclusion of roots and ECM mycelia significantly increased the soil N_2O fluxes, whereas the exclusion of roots alone did not (Ernfors *et al*., 2011). Ernfors *et al*. (2011) suggested that the ECM mycelia were either reducing the water availability to anaerobic denitrifiers, or reducing N availability for N_2O producers but this was not determined. Since N is essential for N_2O production (Prosser, 2007; Thomson *et al.*, 2012), N limitation of N_2O producers may have been a result of competition for N by AMF hyphae in the present study. Veresoglou *et al*. (2011a) also proposed that the presence of AMF may increase competition for N in the mycorrhizosphere, reducing the PNR rates as the slow growing ammonium oxidisers (AO; Woldendorp & Laanbroek, 1989) were suppressed by N limitation (Veresoglou *et al.*, 2011a). Similarly, NO_3 is the essential substrate for denitrification (Groffman *et al.*, 2006). While in fertilised systems, NO_3 availability may not affect N_2O production unless both O_2 and C availability are optimised (Gillam *et al.*, 2008), Firestone & Davidson (1989) proposed that in non-fertilised systems, NO_3 availability is only second to O_2 availability in controlling denitrification rates, and particularly the N_2O:N_2 ratio; thus if NO_3 is limiting, the rate of N_2O production is likely to decrease. In fact, in some cases, cropped systems can produce less N_2O than fallow soils as a result of competition for N (Duxbury *et al.*, 1982). Nitrogen limitation of N_2O producers was therefore further investigated in the current work by the addition of NH_4NO_3 to release any potential N limitations.

The majority of studies to date have found that the main factors limiting N_2O production is often the availability of N, C or O_2 (Parton *et al.*, 1996; Gillam *et al.*, 2008; Enwall *et al.*, 2010), most probably because N and C are substrates required for the production of N_2O (Wrage *et al.*, 2001; Giles *et al.*, 2013), and O_2 availability

determines if aerobic or anaerobic organisms dominate (Khalil *et al.*, 2004). Unfortunately, it was impossible to determine if the water content of the patches increased in the AMA treatment, as reported in Chapter 2 (section 2.3.3.5), due to the addition of NH_4NO_3 solution or water to the organic patches just before harvest in the present study. Consequently, the patch moisture contents did not differ between the AMA and NAMA treatments at the exact time of measurement. But, if the moisture contents had increased in the AMA treatments prior to NH_4NO_3 addition, it is possible that the rate of N_2O production from nitrification could have decreased with the declining availability of O_2 as the patches became more anaerobic (Bollmann & Conrad, 1998). The negative correlation between the N_2O and CO_2 concentrations in the organic matter patches preceding NH_4NO_3 addition in the AMA treatment may also be an indication of this occurring. Similarly, it was not possible to determine the pH of the organic patches, as they were too small to sub-sample, but AMF mediated decreases in pH (Li *et al.*, 1991a) may also be responsible for the observed decreases in N_2O concentration (reviewed by Šimek & Cooper, 2002; Nugroho *et al.*, 2007; Cheng *et al.*, 2013), although in comparison to changes in N availability, the relative impact of pH on N_2O production is not clear.

The availability of other nutrients that are also required by N_2O producers such as P (Purchase, 1974), Cu (Zumft, 1997; Enwall *et al.*, 2010) or Fe (Zhu *et al.*, 2013b) can also be reduced by AMF (Caris *et al.*, 1998; Li *et al.*, 1991b; Liu *et al.*, 2000a). However, these nutrients may be secondary in the control of N_2O production to the availability of N, C and O_2. For example, in a field study Enwall *et al*. (2010) found that, although the abundance of Cu containing NIR genes was correlated with the soil Cu content, the activity of denitrifiers was predominantly correlated with availability of N and C. However, AMF can dominate their host plants P supply (Smith *et al.*, 2003b), extending the P depletion zone beyond the plant roots (Li *et al.*, 1991b), and the plants in this study received reduced P in the added nutrient solution (Section 3.2.5). Nitrifiers can be limited by the availability of P (Purchase, 1974), therefore, the potential for N_2O producers to become P limited in the presence of AMF hyphae, and consequently produced less N_2O, cannot be ruled out, but is further investigated in Chapter 5.

3.4.4 The effect of NH_4NO_3 addition on patch N_2O concentrations in the presence of AMF hyphae

The addition of 7 cm^3 of 30 mM NH_4NO_3 was equivalent to 5.88 mg N per patch, which is equivalent to N additions in similar previous studies (e.g. Hodge *et al.*, 1999; Hodge, 2001). Furthermore, the concentration is not dissimilar to those used to encourage growth of denitrifiers and nitrifiers under optimal conditions (Vilcáez & Watanabe, 2009; Arnaout & Gunsch, 2012), and therefore should not have had any adverse effects on these groups of organisms; thus the NH_4NO_3 addition should have released N transforming organisms from any N limitation. It was expected that since the NH_4NO_3 was added as liquid media, some of this would drain out of the patch to the base of the microcosm. However, it was expected that this would be equal for the AMA and NAMA treatments, and therefore should not have impacted upon the findings presented here.

Within 24 h of NH_4NO_3 addition, the N_2O concentrations in the AMA and NAMA organic patches increased by up to 27 times the pre-NH_4NO_3 addition N_2O concentrations (Figure 3.10). Baggs *et al*. (2003a) also measured the highest N_2O production within one day following NH_4NO_3 addition to *Lolium perenne* L. swards. By ^{15}N labelling the NH_4NO_3, Baggs *et al*. (2003a) identified that 56% of the total denitrified ^{15}N-N_2O emission over a 7 d period occurred on the first day following the NH_4NO_3 addition. However, overall, nitrification was the dominant source of N_2O over the 7 d studied. Similarly, in a soil incubation study, Bateman & Baggs (2005) measured peak N_2O fluxes from both nitrification and denitrification 24 h after NH_4NO_3 addition. Thus, in the present study, it is likely that both nitrification and denitrification contributed to the N_2O concentrations measured over the 4 d period following NH_4NO_3 addition. The addition of NH_4NO_3 to the AMA patches demonstrated that the N_2O producers were limited by the supply of NH_4 and/or NO_3. In contrast, when water alone was added, the N_2O concentration was negatively related to the patch water content (Table 3.3). This suggests that in the absence of NH_4NO_3 addition, the water content of the organic patches may have been an important controller of N_2O

production. However, water content can be linked to the availability of O_2 (Bollmann & Conrad, 1998) and also to the diffusion of nutrients, including NH_4, NO_3 and P (Tinker & Nye, 2000), which can all influence N_2O production (Gödde & Conrad, 2000; Hino *et al.*, 2010).

The N_2O concentration in the NAMA organic patches also increased following the NH_4NO_3 addition, but while the N_2O concentration didn't increase following water addition to the NAMA patches, these values were not significantly different from each other (see Figure 3.9b and Figure 3.10). Preceding NH_4NO_3 and water addition, the N_2O concentrations in the NAMA patches were very variable (0.4 to 25.4 ppm; Figure 3.8b), and this variability may partly explain why the change in N_2O concentration following water addition was not significantly different from that following NH_4NO_3 addition (although a trend is apparent; Figure 3.10). N_2O production can be dominated by a single controlling factor such as water availability, which can mask the effect of other treatments unless it is tightly controlled (e.g. Gödde & Conrad, 2000). Furthermore, N_2O production in soils can be highly variable both spatially and temporally, with factors such as soil structure, and water content determining the availability of compounds (reviewed by Giles *et al.*, 2013). In this case, if AMF hyphae reduced the availability of N in the AMA treatment, the N_2O concentration (and associated variation) would decrease. In contrast, in the NAMA treatment, as there was less competition for N, the control of N_2O production may not have been N availability, but a more spatially variable factor such as O_2 availability, and subsequently the variability in N_2O production was higher in the NAMA treatment (Figure 3.8b and at 96 h after treatment addition in Figure 3.9a).

If O_2 concentrations are low, and C is readily available, then NO_3 availability is often the main controller of N_2O production, via denitrification (Gillam *et al.*, 2008). In this system, the patches were very wet following NH_4NO_3 and water addition (Table 3.2), and they initially had a high C content (Table 3.1). Thus, it is feasible that in the presence of AMF hyphae, a reduced availability of NO_3 resulted in lower N_2O

concentrations, and upon NO_3 addition, this effect was removed. Alternatively, autotrophic nitrifiers can struggle to compete with heterotrophic bacteria and plants for NH_4, particularly under conditions of increased C availability (Verhagen *et al.*, 1995). One possible explanation for this is the slow growth of nitrifying bacteria (e.g. ammonia oxidising bacteria (AOB)) means that they are therefore unlikely to obtain the numbers that would be able to exploit the soils maximum nutrient supply (Woldendorp & Laanbroek, 1989), particularly following a short term pulse of NH_4 as provided here. Conversely, ammonia oxidising archaea (AOA) can cope with very limiting NH_4 availabilities in marine systems and therefore successfully compete with heterotrophic bacterioplankton and phytoplankton (Martens-Habbena *et al.*, 2009). Although this has not been demonstrated for soil AOA, the wide range of soil conditions that AOA inhabit could indicate a similar adaptation to low NH_4 (Leininger *et al.*, 2006). However, an adaptation to low NH_4 conditions suggests that AOA may not be able to respond to a pulse of NH_4 any more successfully than AOB; therefore, the nitrifiers in the AMA patches may not have been able to respond to the NH_4 pulse. As the N was added in the form of NH_4NO_3, it was not possible to distinguish whether nitrification or denitrification was limiting N_2O production in both the AMA and NAMA patches. Nevertheless, as both AMF access treatments responded positively to NH_4NO_3 addition, at least one pathway of N_2O production in each AMF access treatment must have been N limited.

It is noteworthy that the patch N_2O concentrations for AMA treatments declined to levels below those of the NAMA patches 96 h after NH_4NO_3 addition. At this time, the patch moisture contents were equal (Table 3.2), therefore O_2 availability was unlikely to be the cause. This response suggests a long term inhibitory effect of AMF hyphae on nitrifier and/or denitrifier activity that continued to limit N_2O production even after N addition. One explanation may be that the heterotrophic denitrifiers responded rapidly in both the AMA and NAMA treatments to NH_4NO_3 addition. When NH_4NO_3 was added to *L. perenne* swards there was a rapid increase in denitrification within 24 hours of NH_4NO_3 addition (Baggs *et al.*, 2003a). In contrast, the nitrifiers may have responded more slowly and continued to produce N_2O in the NAMA treatments at the 96 h post-

NH_4NO_3 addition sample. In Bateman & Baggs (2005), nitrification was found to stimulate N_2O production for up to a week following NH_4NO_3 addition, thus nitrification can be stimulated for a longer period following N addition than denitrification. However, neither of these studies included an AMF treatment (Baggs *et al.*, 2003a; Bateman & Baggs, 2005).

Wallenstein *et al*. (2006) defined proximal and distal controls on denitrifiers and denitrification. Long term structural changes in the denitrifying community are controlled by 'distal' controls including environmental and biotic factors such as C and O_2 availability and pH, but not NO_3 availability (Wallenstein *et al.*, 2006). In contrast, short term changes in denitrifying rates are controlled by 'proximal' factors which will affect the activity of the existing denitrifying community such as a peak in C availability (Wallenstein *et al.*, 2006). These terms could also be applied to nitrifiers and nitrification rates, but whereas NO_3 may not be a distal control on denitrifier communities (Mergel *et al.*, 2001), NH_4 may be a distal control for nitrifier communities since AOA and AOB may be differentially adapted to NH_4 availability (Leininger *et al.*, 2006; Verhamme *et al.*, 2011).

The slow growing nitrifiers in the AMA patches in the present study may not have been capable of responding to the proximal control of NH_4 addition, possibly because the presence of AMF hyphae had reduced their numbers or activity in the longer term via a distal control such as competition for NH_4, reduced O_2 availability, or decreased pH (as discussed in Section 3.4.3). Therefore, once the denitrification peak declined, the N_2O concentration in the AMA treatment returned to the pre-NH_4NO_3 addition value, as was found here (Figure 3.9a & b). In the mycorrhizosphere, the number of ammonia oxidisers (AO) increased in one study (Amora-Lazcano *et al.*, 1998), but did not change in another (Cavagnaro *et al.*, 2007). In contrast, the number and community of denitrifiers can decrease (Amora-Lazcano *et al.*, 1998) and change (Veresoglou *et al.*, 2012a) respectively in the mycorrhizosphere. Thus, to date there is no consensus of the effect of AMF on the nitrifying or denitrifying populations, and these interactions

have only been studied in the mycorrhizosphere, which can be a different environment to the hyphosphere that was studied here (Johansson *et al.*, 2004). Thus, the idea that AMF hyphae have a distal effect on nitrifiers, while possible in theory, is highly speculative, and would require the use of either ^{15}N techniques (e.g. Bateman & Baggs, 2005) or separate additions of NH_4 and NO_3 (e.g. Bergstrom *et al.*, 2001; Li *et al.*, 2005) to identify the predominant source of N_2O in the different AMF access treatments; this will be further investigated in Chapter 4.

AMF hyphae can reduce the extractable inorganic N in the hyphal compartment of a microcosm system (Tu *et al.*, 2006), and there is evidence for AMF hyphal uptake of NH_4 and NO_3 (Tanaka & Yano, 2005), but AMF are thought to preferentially take up N as NH_4 (Govindarajulu *et al.*, 2005). In the current experimental system, there would have been very little N available to the AMF hyphae outside the patch as the washed sand/Agsorb® medium has a very low N and available P content (Oil-Dri, USA; Leigh *et al*., 2009). The percentage of the root length colonised by the AMF hyphae was also higher in the AMA treatments, which agrees with the findings of previous studies (e.g. Blanke *et al.*, 2005; Hodge & Fitter, 2010). This could be a result of host plant N limitation, as Blanke *et al*. (2005) found that plants with lower plant N concentrations (e.g. leaf concentrations of 1.76 and 2.03%) had higher rates of internal colonisation by AMF hyphae, even under high P conditions. It was proposed that the AMF were aiding in the plant N nutrition as N was limiting in that system (Blanke *et al.*, 2005). The very low N concentration of *Z. mays* leaves in the present study (only 1.13% ± 0.06 in the AMA treatment and 0.88% ± 0.05 in the NAMA treatment compared to 3.5% under high N supply (Vos *et al.*, 2005)) also supports this.

Zea mays adopts a strategy of reducing leaf N concentration when N is limiting, and at higher N supply leaf N concentration can reach values of up to 3.5% (Vos *et al.*, 2005). Thus, the increased N content and concentration in the leaves of the AMA treatment host plants, indicates that the AMF were contributing to the plant N nutrition as they had access to additional N in the organic patch. This is supported by the fact that all

planted compartments received the same amount of nutrient solution, and similar studies using ^{15}N labelled organic patches with non-AMF access treatments have shown that the risk of diffusion of inorganic N from the patch to the plant roots was very low (Leigh *et al.*, 2009; Hodge & Fitter, 2010). This AMF to host plant transfer of N in the AMA treatment, alongside the fact that the N rich AMF hyphae will also require a high N supply (Hodge & Fitter, 2010), may have increased competition for N in the AMA patches. It is therefore probable that competition for NH_4 would be high within the organic patch, which was likely to, at least, affect the AOB (Woldendorp & Laanbroek, 1989), if not the AOA.

3.4.5 Conclusions

The presence of AMF hyphae in an organic patch resulted in a decreased N_2O concentration compared to when AMF hyphae were absent. Addition of NH_4NO_3 removed the effect of AMF hyphal presence on N_2O concentration for up to 48 h by increasing the N_2O concentration in both AMA and NAMA organic patches, indicating that the N_2O producers in both treatments were limited by NH_4 and/or NO_3. The high variability in the N_2O concentration measured in NAMA patches, and rapid response to NH_4NO_3 addition in both AMA and NAMA treatments, suggested that there may have been more than one N_2O producing pathway stimulated by N addition. By 96 h after NH_4NO_3 addition, the N_2O concentration in AMA patches returned to a value below that of the NAMA patches. This suggests that the N_2O producing pathways in both AMA and NAMA patches were N limited, but that the AMA patches had a longer term limitation to N_2O production. The AMA host plants had a higher leaf N content and concentration, indicating that the AMF was supplying the host plant with additional N, presumably from the organic patch. Thus the lower N_2O concentration in AMA patches was probably a result of N limitation as the AMF hyphae removed N from the organic patch for both their own use, and that of their N limited host plants. Although unlikely, other causes including pH changes, long term water availability and availability of P and Cu could not be ruled out.

The results from this experiment are in contrast to those from Chapter 2 where the N_2O flux was higher from AMA patches compared to NAMA patches, once the organic patches had been removed from the microcosm units, and therefore the hyphae had been severed from the host plant. The following Chapter (Chapter 4) considers the effect of severing hyphae on patch N_2O fluxes by modifying the three compartment microcosm used in Chapter 2. The results from this chapter will be further explored by incorporation of NH_4 and NO_3 treatments to assess whether the main effect of AMF hyphal presence is linked to nitrification or denitrification pathways, and particular, whether or not this differs between severed and intact hyphal treatments.

Chapter 4. The effect of severing arbuscular mycorrhizal fungal (AMF) hyphae and the addition of NH_4 or NO_3 on organic patch N_2O fluxes

4.1 Introduction

Nitrous oxide (N_2O) is an important greenhouse gas which has a global warming potential that is 310 times greater than that of CO_2 over a 100 years (Forster *et al.*, 2007). The results presented in Chapter 2 demonstrated that the flux of N_2O from an organic matter patch was higher when AMF hyphae were present, although it was unclear if this was because the AMF hyphae were severed during the harvest process. Severing the AMF hyphae may increase the C and N input into the growth medium in two ways. Firstly, by increasing decomposition; Staddon *et al*. (2003) used ^{14}C analysis to determine the turnover rate of AMF hyphae in a semi-sterile system and found that fine AMF hyphae had a rapid turnover rate of only 5-6 days and suggested that in the field could be even more rapid. If the hyphae are severed from their host, the rate of decomposition may increase, and could therefore increase N_2O production. Secondly, severing AMF hyphae could release labile C. Between 1 and 20% of photosynthetically derived C can be transferred to the AMF (Paul & Kucey, 1981; Jakobsen & Rosendahl, 1990; Johnson *et al.*, 2002b), although values around 5-10% are more common (Bryla & Eissenstat, 2005), and up to 25% of this is transferred to the AMF hyphae (Hamel, 2004) where it is found in the form of chitin, lipids or hexoses (Pfeffer *et al.*, 1999; Bago *et al.*, 2000). Therefore severing AMF hyphae has the potential to result in leakage of a significant quantity of low molecular weight C into the soil which could fuel heterotrophic denitrification (Parkin, 1987; Hino *et al.*, 2010). In the current experiment, by severing the AMF hyphae, without destructively harvesting the organic matter patches, confounding factors including aeration of the patches and disturbance could be eliminated and the effect of severing AMF hyphae on patch N_2O production and how this compares to the effect of undisturbed AMF hyphal presence on patch N_2O production could be determined.

In contrast, the presence of intact AMF hyphae in an organic patch in Chapter 3 resulted in decreased N_2O concentrations within the organic patches. The addition of NH_4NO_3 removed this effect, suggesting that N limitation in the presence of AMF hyphae was the main cause of the reduction in N_2O concentration. However, as shown in Figure 4.1, the addition of NH_4NO_3 could have resulted in a stimulation of N_2O production from any of the known N_2O producing pathways in soil (Baggs, 2011; Zhu *et al.*, 2013a). Therefore, it is unclear as to whether the N_2O producers were limited by the availability of both or either of NH_4 or NO_3 in the presence of AMF, and which of the main N_2O producing pathways were involved. However, the main nitrification and denitrification pathways are under microbial control (except for chemodenitrification) and as indicated in Figure 4.1, they can be broadly separated by their requirement for either NH_4 or NO_3.

The main 'nitrification' pathways in soil potentially resulting in N_2O release are nitrifier nitrification, and nitrifier denitrification. Nitrifier nitrification is an aerobic process carried out by autotrophic nitrifiers that converts NH_4 or NH_3 to NO_3 via NO_2 (Wrage *et al.*, 2001). Ammonium oxidation is the first stage of nitrification (NH_4 to NO_2), carried out by ammonia oxidising bacteria (AOB), or archaea (Leininger *et al.*, 2006) and it is during this process that incomplete aerobic hydroxylamine oxidation can result in N_2O production as a side product (Hooper & Terry, 1979; Jiang & Bakken, 1999; Stein, 2011). Nitrite is then further oxidised to nitrate by nitrite oxidising bacteria (NOB). Autotrophic nitrification is an energetically expensive process, and therefore nitrifiers are generally slow growing (Wrage *et al.*, 2001). While nitrification is generally an autotrophic process, there are cases of heterotrophic nitrification carried out by both fungi (Laughlin *et al.*, 2008) and bacteria (Brierley & Wood, 2001). In contrast to the well-known aerobic nitrification processes, autotrophic nitrifiers have also been found to denitrify under conditions of low O_2 and this process is termed nitrifier denitrification (Wrage *et al.*, 2001; Bateman & Baggs, 2005; Zhu *et al.*, 2013a). Nitrifier denitrification follows an almost identical pathway to denitrifier denitrification (Baggs, 2011), but NO_3 is not produced as an intermediate, instead the NO_2 produced during nitrifier nitrification is converted to N_2 via N_2O (Wrage *et al.*, 2001).

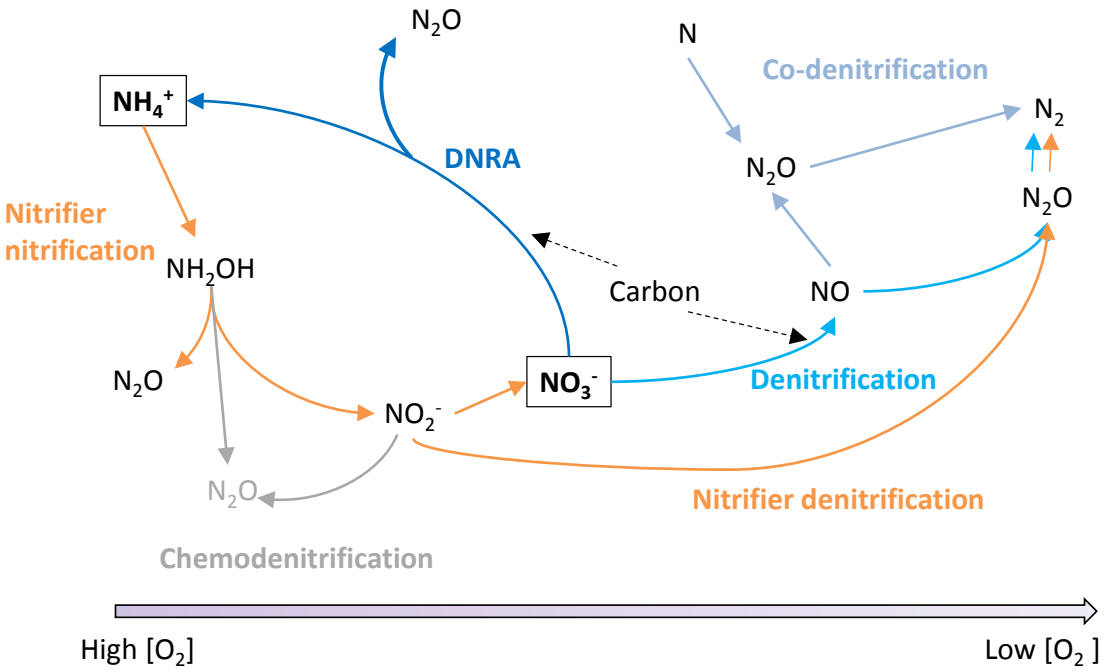


Figure 4.1. Pathways of N_2O production in soil. Pathways coloured orange are controlled by the provision of NH_4 and nitrifier activity (broadly termed nitrification pathways), whereas pathways coloured in blue are reliant upon the provision of NO_3 (broadly termed denitrification pathways). The grey pathways are non-microbial sources of N_2O (chemodenitrification: the chemical decomposition of nitrite (NO_2^-) or hydroxylamine (NH_2OH)) that are still potentially linked to provision of NH_4. The general control of oxygen (O_2) availability on these pathways is indicated by the gradient arrow at the bottom of the diagram, and pathways that require a source of carbon are indicated by the black dashed arrows. While this diagram includes what are considered to be the main N_2O producing pathways in soil at present, there are also cases of aerobic denitrification (reviewed by Chen & Strous, 2013), and heterotrophic nitrification (Laughlin *et al.*, 2008) which are not included here. NH_2OH = hydroxylamine, NO_2^- = nitrite, NO = nitric oxide, DNRA = dissimilatory nitrate reduction to ammonium. Adapted from pathways described in Baggs (2011) and Zhu *et al.* (2013a).

Denitrifier denitrification is a facultative anaerobic process carried out by a wide range of organisms including bacteria (Dandie *et al.*, 2008), fungi (Herold *et al.*, 2012), and archaea (Cabello *et al.*, 2004). This process is predominantly heterotrophic, although autotrophic denitrifiers have been identified (Claus & Kutzner, 1985; Trouve *et al.*, 1998), which use inorganic sources (e.g. sulphur compounds or Fe^{2+}) as electron donors (Straub *et al.*, 1996; Trouve *et al.*, 1998). There is also evidence for aerobic denitrification, although this appears to be under conditions of low O_2 and at rates generally much lower than anaerobic denitrification (reviewed by Chen & Strous, 2013). However, as both aerobic and anaerobic denitrification follow the same pathways they are both included in denitrification here. Denitrification is a process where NO_3 is reduced in stages to N_2 via NO_2, NO, and N_2O (Groffman *et al.*, 2006); a different group of facultative anaerobes control each stage and N_2O is only produced when the process does not complete to form N_2 (Ye *et al.*, 1994; Singh *et al.*, 2010). Denitrifiers can utilise the outputs of nitrification (NO_2 or NO_3) to carry out denitrification, termed 'nitrification-coupled denitrification' to acknowledge the difference between this and nitrifier denitrification. Dissimmilatory nitrate reduction to ammonium (DNRA; also known as nitrate ammonification) can also yield N_2O as a side product in a stepwise reduction process with NO_2 as the intermediate stage at which N_2O can be released (Kelso *et al.*, 1997; Stremińska *et al.*, 2012). This is a heterotrophic process carried out by both bacteria and fungi that occurs under anaerobic conditions. Although, in contrast to denitrification, DNRA can occur at low O_2 levels (Fazzolari *et al.*, 1998). Finally co-denitrification is currently considered an extension of denitrification, where NO joins with N from another substrate (e.g, amino acids, azide, ammonia etc.) to form N_2O (Su *et al.*, 2004). This process is poorly understood at present, but may contribute a significant quantity of N_2O to denitrification outputs (Baggs, 2011).

By measuring the change in N_2O production following the addition of NH_4 or NO_3 to organic matter patches (as used in Chapters 2 and 3) that contain AMF hyphae, it was possible to determine if the rate of N_2O production by nitrifiers, denitrifiers or both is limited in the presence of AMF hyphae. For example, increases in N_2O production

following NO_3 addition could be attributed to denitrification. Conversely, if NH_4 addition resulted in a large N_2O flux whereas NO_3 addition did not, it could be assumed that the main N_2O source was a nitrification pathway (Figure 4.1). If, upon severing, the turnover of AMF hyphae resulted in a release of C, by adding NO_3, it is expected that denitrifiers would be released from both C and N limitation (Figure 4.1), resulting in increased N_2O produced in the AMF hyphal access (AMA) treatment. Alternatively, the reduced N_2O production in the AMA treatments is predicted to be a result of reduced nitrification rates in the presence of AMF hyphae. Arbuscular mycorrhizal fungi (AMF) are thought to preferentially take up N in the form of NH_4 (Govindarajulu *et al.*, 2005; Hodge *et al.*, 2010) which could increase competition for NH_4 in the hyphosphere and limit the supply for nitrifiers, acting as a long term 'distal' control (*sensu* Wallenstein *et al.*, 2006) on their numbers and/or activity. Therefore, in the non-severed treatments, it was expected that the slow growing nitrifiers (Woldendorp & Laanbroek, 1989) would not be able to respond to the addition of a pulse of inorganic NH_4 and consequently less N_2O would be produced in the AMA patches compared to the NAMA patches following NH_4 addition.

Following the hyphal severing treatment, solutions containing NH_4 or NO_3 were injected directly into the patches as these were the main sites of N_2O production (as demonstrated in Chapter 2). The concentrations of $(NH_4)_2SO_4$ and KNO_3 (15 mM and 30 mM respectively) were selected to optimise growth conditions for nitrifiers (Stanier, 1987; Yue *et al.*, 2007; Arnaout & Gunsch, 2012) and denitrifiers (Stanier, 1987; Vilcáez & Watanabe, 2009; Arnaout & Gunsch, 2012). Nitrous oxide concentrations were measured in the organic patches using gas probes as in Chapter 3 both before, and after, hyphal severing and N addition treatments. A Los Gatos Isotopic N_2O analyser (Los Gatos Research, Inc., California, USA) was delivered days before gas sampling began, and could therefore be used to measure N_2O fluxes from the AMA and NAMA treatments following the closed dynamic chamber (CDC) approach outlined in Chapter 2 (Section 2.1). While the N_2O fluxes measured using the closed static chamber (CSC) chamber method in Chapter 2 were unreliable due to dilution effects (Section 2.4.3.2), the N_2O analyser used here had a much better accuracy at lower N_2O concentrations

with a measurement range of 0.3 – 100 ppm and a precision of 0.2 ppb (Los Gatos Research, Inc., California, USA), therefore eliminating any dilution issues, and making it technically possible to accurately measure the compartment N_2O fluxes and compare these to the patch N_2O concentrations. The AMA and NAMA compartment CO_2 fluxes were also quantified, and it was intended that the CO_2 fluxes would be used as an indicator of AMF hyphal presence as demonstrated in Chapter 2. In order to confirm the findings in Chapter 2 that AMF hyphae do not affect CH_4 production, the CH_4 fluxes from the outer compartments were also measured.

The hypotheses tested in this study were as follows;

1. The organic patch N_2O concentration and compartment N_2O flux would decrease in the presence of AMF hyphae compared to when they were absent.
2. Severing the AMF hyphae, thus increasing the availability of C and/or N to other soil organisms, would reverse this effect and the N_2O flux would increase in the presence of AMF hyphae.
3. Nitrate (NO_3) addition to the severed AMA treatment would result in a higher N_2O flux than that of the NAMA treatment, as the severing of AMF hyphae would release C into the patch, therefore increasing N_2O produced via denitrification in the AMA treatment.
4. Ammonium (NH_4) addition to the non-severed treatment would stimulate N_2O production from the NAMA organic patches, but not the AMA organic patches as the activity of the nitrifiers would be limited by a distal control in the presence of AMF hyphae.

4.2 Materials and Methods

4.2.1 Experimental design

Forty, three-compartment microcosm units (described in Section 4.2.2 below) were planted on 25th June 2012 in the central compartment with three pre-germinated a *Zea mays* L. seeds (Incredible F1, Mr Fothergills, Newmarket, UK) that were thinned to one per pot after two weeks (14 d) as in Chapter 2, Section 2.2.3.3. All microcosm compartments contained a 50/50 mix of sand/Agsorb® (v/v) (Oil-Dri, USA) and the planted compartment also contained 90 g of a live *Glomus intraradices* inoculum (Schenck & Smith; Plantworks Ltd., Kent, UK). The sand/Agsorb® and *G. intraradices* inoculum were prepared as in Chapter 3, Section 3.2.4. Each unit had two outer compartments, one on either side of the central compartment. In each unit, one outer compartment allowed AMF hyphal access (AMA) or prevented AMF hyphal access (NAMA) creating a paired design (described in Chapter 2, section 2.2.3.2). A sterile 50 cm^3 centrifuge tube was added to the outer compartments to create a hole into which an organic matter patch with a gas probe inserted (n = 40) was added at 28 d post-planting. These organic patches were made as in Chapter 3, Section 3.2.6 using 2 g of dried milled *Z. mays* shoots (Incredible F1, Mr Fothergills, Newmarket, UK), mixed with 13 g DW equivalent of a sieved (2 mm) agricultural soil (Chapter 2, Section 2.2.1.3; 53˚92'N, -1˚00'E). The organic patch C and N contents are outlined in Table 4.1.

Table 4.1. Mean carbon (C) and nitrogen (N) content and C:N ratio of the mixed organic patch material (13 g DW equivalent soil mixed with 2 g DW milled *Z. mays* leaves) and *Z. mays* leaves that were used in the mixed patches before addition to the microcosms. Mean values ± standard error of the mean (n = 3).Total values are for 15 g (DW equivalent) mixed organic patches and 2 g DW of milled *Z. mays* leaves.

	Total C (mg)	% C	Total N (mg)	% N	C:N Ratio
Mixed patch	1199.7 ± 78.5	8.0 ± 0.5	99.1 ± 15.2	0.7 ± 0.1	12:1
Leaf material	870.4 ± 2.1	43.5 ± 0.1	61.7 ± 3.7	3.1 ± 0.2	14:1

At 87 d after planting (59 d after patch addition), twenty of the units had a blade inserted alongside the mesh membrane separating the central and outer compartments to sever the hyphae from the host plant (severing treatment *n* = 20). Three days later, NH_4 (as $(NH_4)_2SO_4$), NO_3 (as KNO_3), K_2SO_4 or water were applied directly to the patches (N addition treatments, *n* = 5). The experimental design is outlined in Table 4.2. The plants were destructively harvested over three days at 100 d after planting (72 d after patch addition).

Table 4.2. Experimental design. There were two AMF access treatments (AMF hyphal access; AMA, no AMF hyphal access; NAMA; *n* = 40) which were paired within each microcosm unit. Half of the microcosms had blades added to sever the AMF hyphae in both the AMA and NAMA compartments creating the severed (S) or non-severed (NS) treatments (*n* = 20). Inorganic nitrogen (N) was then added to each of these treatments as $(NH_4)_2SO_4$ or KNO_3 to form the NH_4 and NO_3 addition treatments with K_2SO_4 and water controls (N addition treatments; *n* = 5).

Treatment					
AMF access	*n*	Severing	*n*	N addition	*n*
AMA	40	Severed (S)	20	$(NH_4)_2SO_4$	5
				KNO_3	5
				K_2SO_4	5
				Water	5
		Non-severed (NS)	20	$(NH_4)_2SO_4$	5
				KNO_3	5
				K_2SO_4	5
				Water	5
NAMA	40	Severed (S)	20	$(NH_4)_2SO_4$	5
				KNO_3	5
				K_2SO_4	5
				Water	5
		Non-severed (NS)	20	$(NH_4)_2SO_4$	5
				KNO_3	5
				K_2SO_4	5
				Water	5

4.2.2 Microcosm design

The three-compartment microcosms used in this experiment were slightly modified from those used in Chapter 2 (Section 2.2.3.2) through the incorporation of blade guides to facilitate the severing of the hyphae. The blade guides were two pieces of stainless steel mesh (100 x 130 mm; woven stainless wire cloth 60 mesh, 0.25 mm aperture; Hanscan Ltd. Stoke on Trent, UK) that were screwed into the outer compartment and sealed around three edges using aquarium sealant (Everbuild Building Products Ltd. Leeds, UK). This created a housing into which a stainless steel blade (110 x 65 mm; Figure 4.2a) could be tightly inserted (Figure 4.2b), thus severing any AMF hyphae that were growing through the fine mesh membrane. The blades were inserted into both the AMA and NAMA sides of the severing units to account for any possible disturbance resulting from blade insertion.

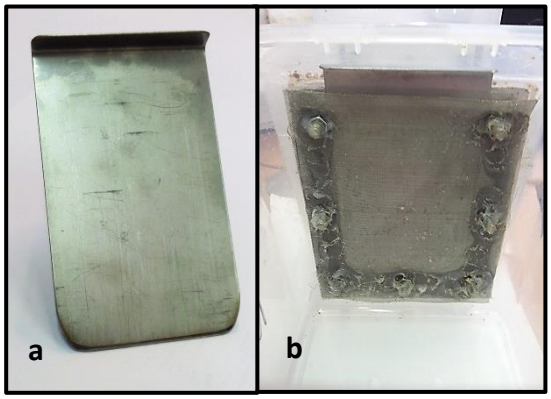


Figure 4.2. Blade (a) used in the microcosms to sever AMF hyphae and (b) the blade inserted into the stainless steel mesh housing inside the microcosm unit.

4.2.3 Growth conditions

Forty, three-compartment microcosms were placed in a temperature controlled glasshouse in a randomised block design. The mean daily temperature was 21.5 ± 0.3^{o}C and daily PAR was measured at plant height between 10 am and 2 pm once a week and averaged 251 ± 45 µmol m^{-2} s^{-1}. Lights (high pressure sodium 400 W; Philips SON-T AGRO) were used to extend the photo-period to 16 h per day. The moisture contents

of the outer compartments were equalised weekly as described in Chapter 2, Section 2.2.3.5. The plants were watered with equal volumes of deionised water daily as required. After 14 d of plant growth, the plant compartments received 50 cm^3 of a nutrient solution modified from Thornton & Bausenwein (2000) ($1/10^{th}$ N and P; Appendix 1) once a week. This was increased to twice weekly at 49 d after planting and to full N at 55 d after planting as the plants were showing symptoms of N deficiency. At 76 d the plants were showing P deficiency symptoms, so a $3/10^{th}$ P, full N solution was used once a week in addition to two $1/10^{th}$ N and P additions. The plants received no further nutrient solution after the blades were added on 20^{th} September 2012 to reduce unequal mass flow of nutrients to the patch compartments.

4.2.4 Severing and nitrogen addition treatments

At 59 d after organic patch addition, stainless steel blades were inserted into twenty (half) of the microcosm units in both the AMA and NAMA compartments. The blades were sharpened at the base and were inserted into the stainless steel housings that were incorporated into the microcosm design (described in Section 4.2.2). These blade guides protected the fine mesh membranes (0.45 µm or 20.0 µm for NAMA and AMA compartments respectively) from the blade and also acted as a guide to ensure that the blades were inserted into the same location in all units, ensuring equal disturbance and severing of all hyphae between treatments. Once the blades were inserted they were left in place. At 63 and 69 d after organic patch addition (19 and 97 d post-planting) the blades were lifted by 2 cm and then re-inserted to ensure that the hyphae had not reconnected.

At 62 d after organic patch addition (90 d after planting) each patch was injected with one of 7 cm^3 of 15 mM $(NH_4)_2SO_4$ (NH_4 treatment), 30 mM KNO_3 (NO_3 treatment), 15 mM K_2SO_4 or deionised water, where the N treatments were equivalent to 0.196 mg N g^{-1} DW patch. Two 3.5 cm^3 aliquots of solution were injected into each organic patch

with an hour gap between each addition to reduce the risk of flooding out into the surrounding sand/Agsorb®.

4.2.5 Gas sampling

Gas sampling was carried out using both gas probes (described in Chapter 3, Section 3.2.1) and continuous flow loop sampling (CDC sampling, outlined in Chapter 2, Section 2.2.3.6) with an attached Infra-Red Gas analyser (IRGA; LI-COR® Biosciences, Lincoln, USA), Los Gatos Rackmount CH_4 recorder (LGR CH_4; Los Gatos Research, Inc., California, USA) and Los Gatos Isotopic N_2O analyser (LGR N_2O; Los Gatos Research, Inc., California, USA) which together provided CO_2, CH_4 and N_2O concentrations once per second. The isotopic N_2O analyser also provided continuous measurements of the site specific isotopic ratios $\delta^{15}N^{\alpha}$ and $\delta^{15}N^{\beta}$ as well as $\delta^{18}O$ of N_2O, however these isotopic data proved to be very variable at the low concentrations of N_2O measured throughout this study, therefore these data are not included. The modified microcosm lid was attached to each of the 80 soil compartments in block sequence for a minimum of 5 minutes, with a minimum of 2 min flushing the system with lab air between each compartment measurement. The gas sampling timetable is outlined in Table 4.3.

Table 4.3. Experimental schedule including addition of hyphal severing (blade insertion) and inorganic nitrogen (N) addition treatments presented as days since planting and organic patch addition. The gas probes were sampled at 86 d post-planting and the N_2O fluxes were measured at 87 d post-planting. Gas samples were then taken at 2, 3, 5, 7 and 11 d (hours in brackets; h) following blade insertion to sever AMF hyphae and at 0, 48, 96 and 192 h after N addition (gas sample post-N addition i, ii, and iii respectively). n.a. = not applicable as treatment not yet applied. All microcosm units were harvested at 100-103 d post-planting (72-75 d post-patch addition).

	Time since planting	Time since organic patch addition	Time since severing	Time since N addition
	days	days	days (h)	days (h)
Gas sample pre-treatment	86-87	58-59	n.a.	n.a.
Blade insertion	87	59	0	n.a.
Gas sample post-severing	89	61	2 (48)	n.a.
N addition	90	62	3 (72)	0
Gas sample post-N addition i	92	64	5 (120)	2 (48)
Gas sample post-N addition ii	94	66	7 (168)	4 (96)
Gas sample post-N addition iii	98	70	11 (264)	8 (192)
Harvest	100-103	72-75	13 (312)	10 (240)

4.2.6 Gas flux and concentration calculation

Gas concentration measurements from the CDC system were calculated as in Chapter 2, Section 2.2.3.10 and N_2O concentrations in the gas probes were corrected for dilution as in Chapter 3, Section 3.2.8. All regressions were calculated using SAS (v9.3 SAS institute Inc., North Carolina, USA) to obtain mg s^{-1} values. The headspace in the microcosm unit (0.6 L), volume of connecting tubing (0.274 L) and internal volume for each instrument (IRGA, 0.019 L; LGR CH_4, 0.408 L; LGR N_2O, 0.850 L) along with the surface area of the soil sampled (0.024 m^{-2}) were used in the calculation of the flux

rates for each of the gases, in mg m^{-2} h^{-1}. These fluxes were calculated using values measured between 120 to 200 s after the lid was attached for both CO_2 and CH_4 and 200 to 280 s for N_2O; the N_2O analyser took slightly longer to settle as it was more sensitive to the unavoidable slight pressure change occurring upon lid closure; hence the later sample time. Since there were no significant effects of AMF hyphae on the CH_4 fluxes measured at any point, these data have not been included.

4.2.7 Post-harvest analysis

At each harvest the following data were collected; fresh and dry weights of shoots, roots, stalk, flowers, ear, soil, sand/Terra-Green® and patches, soil and patch % water content (g g^{-1} DW). The shoot material was cut off and separated into leaves, dying leaves (> 50% of the leaf dried up), stalk, ear and tassel and the roots were picked out of the growth medium using forceps for 5 min before washing to clear away any attached growth medium, patting dry and weighing. Two 5 g (FW) samples of each of the AMA and NAMA organic matter patches were taken to determine the extraradical mycelium (ERM) length densities of the AMF. The units were harvested as in Chapter 2, Section 2.2.3.7, omitting post-harvest gas sampling and quantification of root length colonisation as these would not provide any further information in this experiment.

4.2.8 Data analysis

All data were first tested for normality and equality of variance using a Kolmogorov-Smirnov and Levene's Homogeneity of Variance tests in SAS (v. 9.3, SAS institute Inc., North Carolina, USA) respectively and SAS was also used for all subsequent data analyses. There was hyphal breakthrough in one of the NAMA compartments (treatment: non-severed + K_2SO_4) as significant quantities of AMF hyphae were found in the ERM length density sample for this treatment therefore this microcosm was not included in any analyses.

The gas probe and gas flux data for CO_2 and N_2O preceding the severing of AMF hyphae or N treatment addition, were log_{10} transformed and analysed using a Spearman rank order correlation as the data failed normality assumptions. As some of the N_2O fluxes were slightly negative values, a positive integer constant was added to all N_2O flux (+3) values before log_{10} transformation as described in Field and Miles (2010). Differences between AMA and NAMA treatments were tested by comparing the AMA-NAMA values to zero using a one-sample t-test, or if the AMA-NAMA data were not normally distributed, a Wilcoxon Signed Ranks test was used; these tests were also used to compare absolute values (ERM length densities, N_2O fluxes etc.) to zero.

Differences among treatments where the number of treatments exceeded two (e.g. comparing all four treatments after hyphal severing) were analysed using a two-way ANOVA including block with Duncan's multiple range tests applied for *post hoc* analysis. Where the data failed normality and/or equality of variance assumptions, and transformations did not improve this, non-parametric tests were used. As there were more than two replicates per block of each treatment before the N addition treatments were added, a Friedman's test could not be used to control for block (as it requires one replicate per block/treatment combination), therefore the Cochran-Mantel-Haenszel (CMH) statistic was calculated for the ranked data; this is a generalization of Friedmans's test, controlling for block (SAS Institute Inc., 2008). Extraradical mycelium length density data failed normality assumptions and were therefore log_{10} transformed before analysis. The CO_2 concentration and CO_2 flux data for the post-severing gas sample (89 d post-planting) also failed normality assumptions and were therefore log_{10} transformed before analysis using a two-way ANOVA.

The pre-N addition fluxes or concentrations (48h post-hyphal severing, at 89 d post-planting) were subtracted from the post-N addition fluxes or concentrations respectively (at 48 h, 96 h or 192 h post-N addition, 92, 94 or 98 d post-planting) to obtain the change in N_2O flux or concentration following N addition (referred to as the

ΔN_2O flux or ΔN_2O concentration respectively). This reduced the variability within treatments and increased the precision in finding any N addition effects. These data were not normally distributed, even after transformation, and therefore a Friedman's non-parametric two-way ANOVA, controlling for block with Mann Whitney U (a.k.a. Wilcoxon rank sum) *post hoc* tests and Bonferroni corrections (where $P = 0.05$/number of tests performed), was used on the untransformed data. Where comparisons in ΔN_2O flux or ΔN_2O concentration data were made over time, a non-parametric Friedman's repeated measures analysis was used.

The paired AMA-NAMA values were compared to zero using Wilcoxon signed ranks tests. The N_2O concentration for one experimental unit in the AMA treatment (treatment: severed + $(NH_4)_2SO_4$) was out of range on the GC at the 48 h post N addition sample (92 d post-planting) and therefore the AMA and NAMA N_2O gas probe values were not included in the analysis for this sample. To compare flux values to zero, either a one-sample t-test or Wilcoxon signed rank test was used, depending on normality of data. The relationship between the change in N_2O flux and change in patch N_2O concentration for each gas sample following N addition (48 h, 96 h and 192 h post-N addition) was determined using a Spearman's rank order correlation for these non-normally distributed data.

4.3 Results

4.3.1 Growth of AMF hyphae in organic matter patches and patch moisture content

There were significantly higher ERM length densities measured in the AMA treatment compared to the NAMA treatment (Figure 4.3a; $t_{39} = 8.993$, $P < 0.0001$) and both the AMA and NAMA treatments were significantly greater than zero (AMA: $t_{39} = -3.22$, $P = 0.0026$; NAMA: $t_{39} = -15.49$, $P < 0.0001$). The ERM length densities from the AMA patches remained higher than those of the NAMA treatments when split by severing and all treatments compared (Figure 4.3b; $F_{3,58} = 24.61$, $P < 0.0001$).

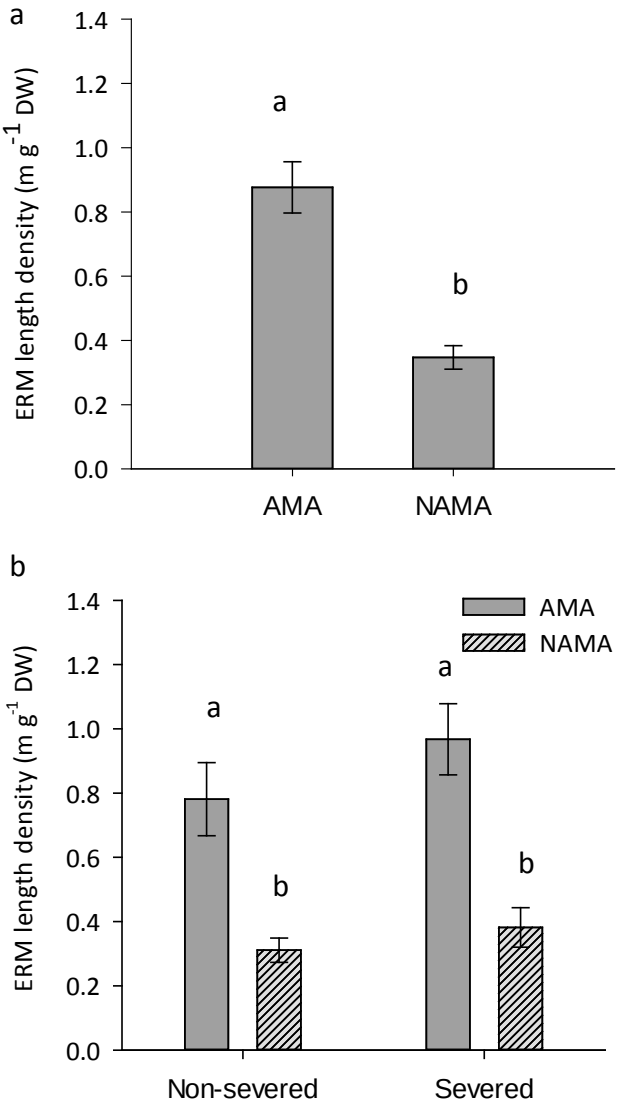


Figure 4.3. Mean extraradical mycelium (ERM) length densities (m g^{-1} DW) in (a) AMF access (AMA) and no AMF access (NAMA) patches (*n* = 40) and (b) split by hyphal severing treatment (*n* = 20; AMA: solid bars; NAMA: hatched bars) following the harvest at 100 d post-planting. Different letters represent significant differences at *P* = 0.05 (in a: comparing the AMA-NAMA value to zero using a one-sample t-test; in b: using a two-way ANOVA with Duncan's multiple range *post hoc* tests). Error bars represent ± standard error of the mean.

In the AMA treatment, the patch moisture content was positively correlated with the ERM length densities (Figure 4.4; r_s = 0.5937, P < 0.0001), but not in the NAMA treatment (r_s = 0.2032, P = 0.215).

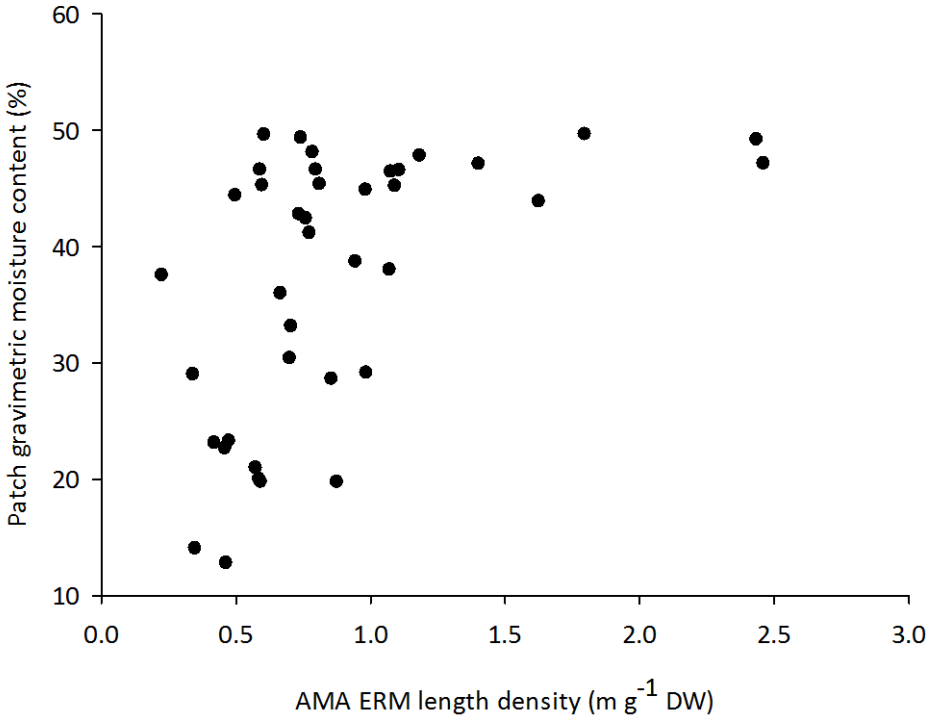


Figure 4.4. AMF access (AMA) organic matter patch extraradical mycelium (ERM) length density (m g^{-1} DW) plotted against the gravimetric moisture content (%) of the AMA organic patches. The ERM length densities in the AMA patches were positively correlated with the patch moisture content as determined using a Spearman's rank order correlation (n = 40; r^2 = 0.35).

There was no significant difference between the AMA and NAMA organic patch % moisture contents following the destructive harvest at 100 d post-planting (Table 4.4; t_{39} = -0.26, P = 0.799). There was also no significant difference in the % moisture content of the sand/Agsorb® medium when the AMA and NAMA compartments were compared (Table 4.4; S_{39} = -47, P = 0.519).

Table 4.4. Mean gravimetric patch and sand/Agsorb® moisture contents (%) for AMF access (AMA) and no AMF access (NAMA) compartments following the harvest at 100 d post-planting. There was no significant difference between the AMA and NAMA treatments for either organic patch or sand/Agsorb® % moisture content. This is indicated by the letters within each column and was determined using a one-sample t-test or Wilcoxon signed ranks test to compare the AMA-NAMA values to zero for patch and sand/Agsorb® data respectively (at $P = 0.05$).

	Organic patch % moisture content	Sand/Agsorb® % moisture content
AMA	37.1 ± 1.8^{a}	22.7 ± 0.5^{x}
NAMA	37.6 ± 1.8^{a}	23.2 ± 0.2^{x}

4.3.2 Pre-treatment gas concentrations and fluxes

Preceding the addition of blades and inorganic N treatments, there was no significant difference between the AMA and NAMA CO_2 fluxes (Figure 4.5a; $t_{38} = -0.196$, $P = 0.846$) or patch CO_2 concentrations (Figure 4.5b; $t_{38} = -1.58$, $P = 0.123$) but the CO_2 fluxes did positively correlate with the patch CO_2 concentrations (Figure 4.5c; $r_s = 0.4887$, $P < 0.0001$). There was also a positive relationship between the AMA and NAMA CO_2 fluxes ($r = 0.5286$, $P = 0.0005$), but not between the AMA and NAMA organic patch CO_2 concentrations ($r_s = 0.2773$, $P = 0.087$). The ERM length densities measured in the AMA compartments were not correlated with the CO_2 fluxes or concentrations before severing and N treatment addition ($P > 0.05$ in both cases). However, the organic patch moisture content was negatively correlated with the CO_2 flux in the NAMA treatment ($r_s = -0.3296$, $P = 0.041$, $r^2 = 0.11$), but not in the AMA treatment ($r_s = -0.2024$, $P = 0.217$).

The N_2O concentrations in the patches were significantly higher in the NAMA patches than the AMA patches before the severing or N addition treatments were added (Figure 4.6a; $S_{38} = -186$, $P = 0.0076$). The same trend was apparent in the N_2O fluxes,

although it was only significant at $P = 0.1$ (Figure 4.6b; $S_{38} = -128$, $P = 0.074$). The N_2O fluxes also positively correlated with the patch N_2O concentrations (Figure 4.6c; $r_s = 0.7495$, $P < 0.0001$). The N_2O fluxes and concentrations were all significantly greater than zero in both AMA and NAMA treatments ($P < 0.0001$ in each case) but the ERM length densities measured in the AMA compartments were not correlated with the N_2O fluxes or concentrations before severing and N treatment addition ($P > 0.05$ in both cases). The N_2O concentrations in the AMA and NAMA treatments were also not correlated ($r_s = 0.1352$, $P = 0.412$), but the N_2O fluxes were ($r_s = 0.3249$, $P = 0.044$).

In the NAMA treatment the CO_2 and N_2O concentrations were weakly positively correlated ($r_s = 0.3464$, $P = 0.031$, $r^2 = 0.12$), as were the CO_2 flux and N_2O concentration ($r_s = 0.3812$, $P = 0.017$, $r^2 = 0.15$). However, the N_2O flux and CO_2 flux were not related in the NAMA treatment ($r_s = 0.1253$, $P = 0.447$) and there were no relationships between the CO_2 and N_2O concentrations or fluxes in the AMA treatment ($P > 0.05$ in each case). Following the harvest, the patch moisture content was negatively correlated with the pre-treatment N_2O flux (before severing or N addition) in the AMA treatment ($r_s = -0.3577$, $P = 0.025$, $r^2 = 0.13$) but this relationship was not present in the NAMA treatment ($r_s = -0.1850$, $P = 0.260$), or between the patch moisture and the AMA or NAMA N_2O concentrations ($P > 0.05$).

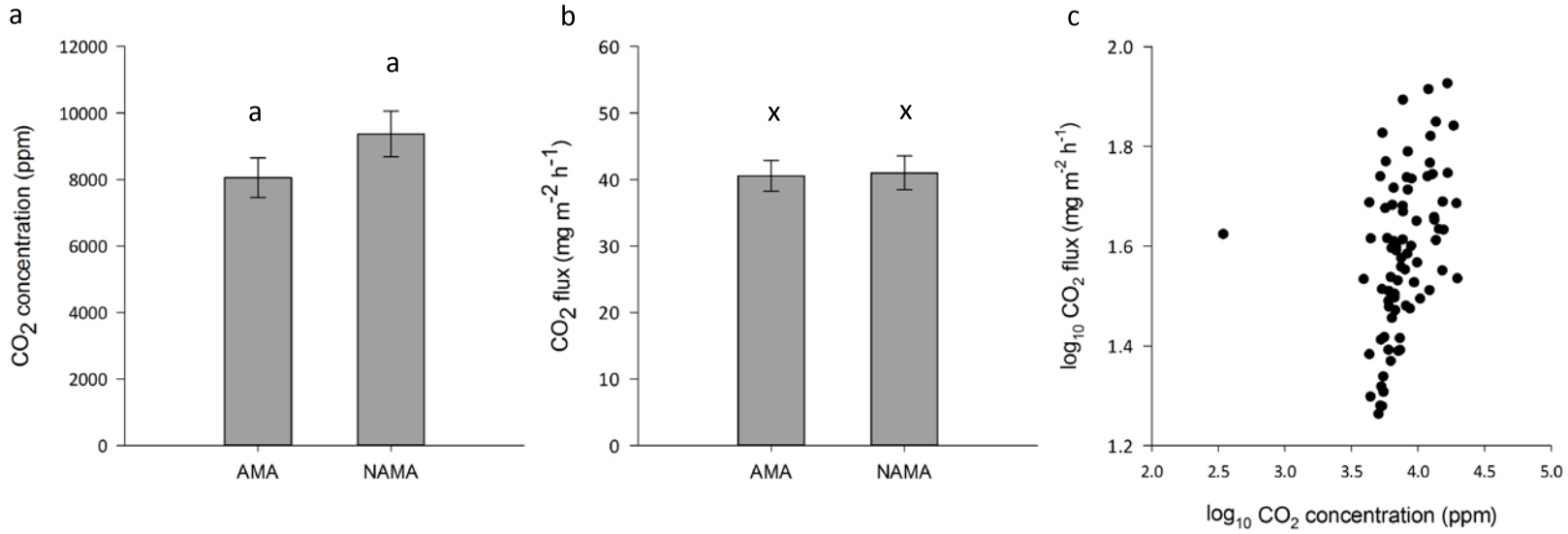


Figure 4.5. Mean (a) CO_2 concentration at 86 d post-planting and (b) CO_2 flux at 87 d post-planting measured in the AMF access (AMA) and no AMF access (NAMA) patches and compartments (before severing or N treatment addition). There were no significant differences between AMA and NAMA treatments in (a) or (b) indicated by the lettering. The AMA-NAMA values were compared to zero using a one-sample t-test (P = 0.05). The CO_2 concentrations and fluxes were positively correlated (c) as determined using a Spearman rank order correlation (n = 80; r^2 = 0.24). Error bars represent ± standard error of the mean (n = 40).

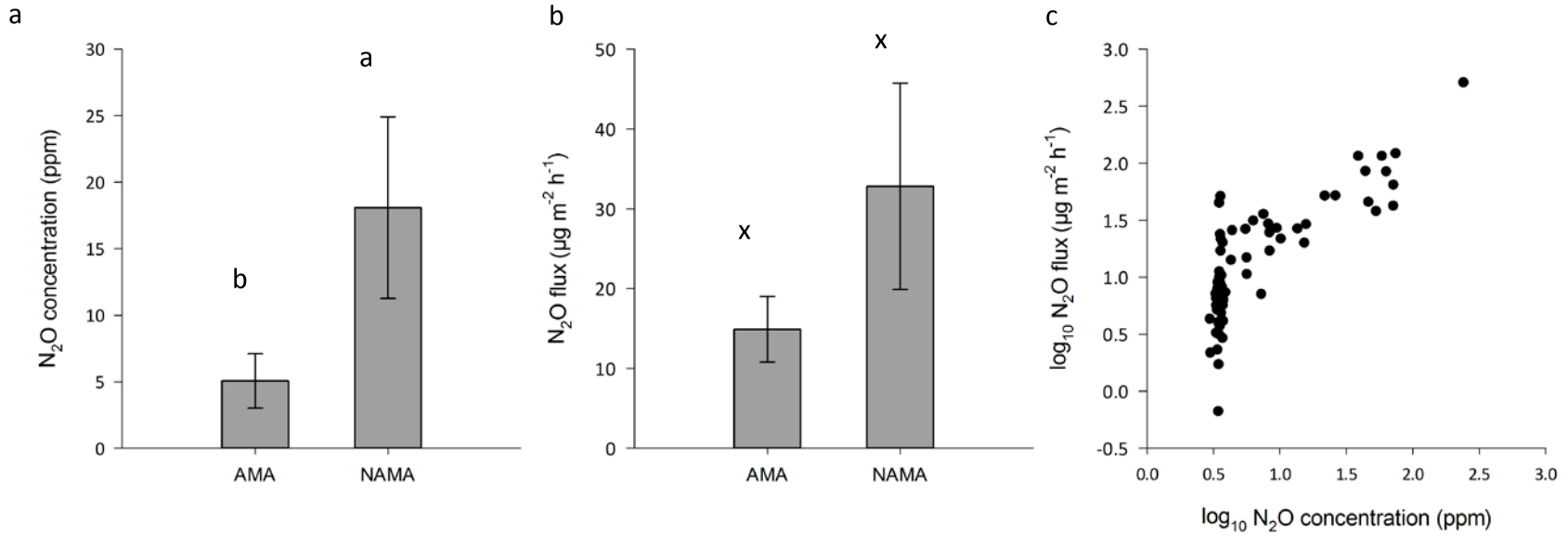


Figure 4.6. Mean (a) N_2O concentration at 86 d post-planting and (b) N_2O flux at 87 d post-planting measured in the AMF access (AMA) and no AMF access (NAMA) patches and compartments. Significant differences between AMA and NAMA treatments in (a) and (b) are indicated by different letters and were determined by comparing the AMA-NAMA value to zero (Wilcoxon Signed Rank test; $P = 0.05$). The N_2O concentrations and fluxes were positively correlated (c) as determined using a Spearman rank order correlation ($n = 80$; $r^2 = 0.56$). Error bars represent ± standard error of the mean ($n = 40$).

4.3.3 Post-severing gas concentrations and fluxes

Forty-eight hours after the severing treatment was applied, there were no significant differences between the AMA and NAMA (within the severing treatments) CO_2 concentrations (Figure 4.7a; severed, t_{19} = -0.58, P = 0.571; non-severed, S_{18} = -24, P = 0.353) or fluxes (Figure 4.7b; non-severed, t_{18} = 1.58, P = 0.131; severed, t_{19} = 0.27, P = 0.788). There were also no significant differences among the CO_2 concentrations over all treatments (Figure 4.7a; $F_{3,58}$ = 0.20, P = 0.899), but the CO_2 flux was higher from the AMA non-severed treatment than from both the AMA and NAMA severed treatments (Figure 4.7b; $F_{3,58}$ = 3.48, P = 0.022). There was also a positive relationship between the CO_2 fluxes from the AMA and NAMA compartments in both severing treatments (non-severed: r = 0.5819, P = 0.009; severed: r = 0.5189, P = 0.019).

There was no difference between the N_2O concentration or N_2O flux measured from the AMA and NAMA treatments in either the non-severed (Figure 4.8a,b; N_2O concentration: S_{18} = -41, P = 0.104; N_2O flux: S_{18} = -30, P = 0.241) or severed (N_2O concentration: S_{19} = -22, P = 0.430; N_2O flux: S_{19} = -8, P = 0.784) treatments and when compared overall, the N_2O concentration or fluxes of all four treatments did not differ (N_2O concentration: Q_3 = 2.45, P = 0.484; N_2O flux: Q_3 = 3.43, P = 0.330).

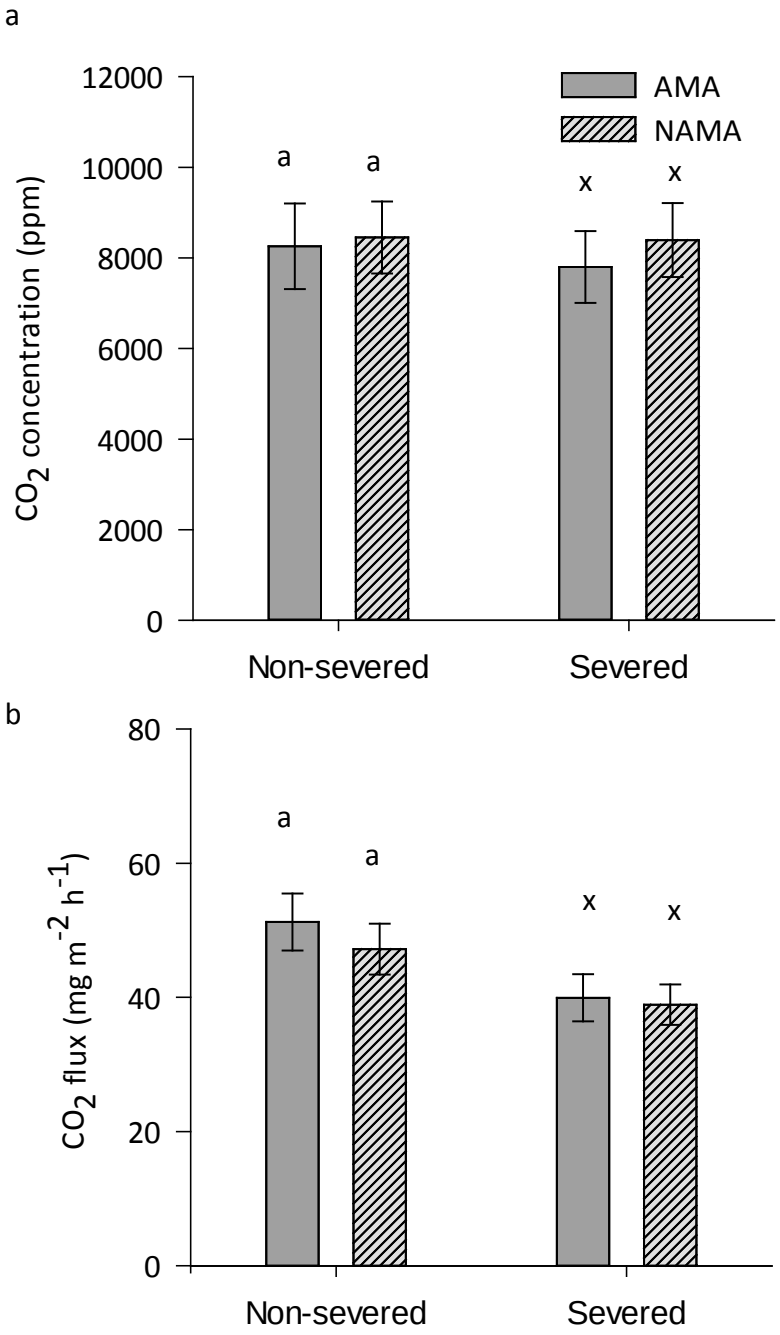


Figure 4.7. Mean CO_2 concentrations inside the organic matter patches (a) and CO_2 fluxes from outer compartments (b) for AMF access (AMA; solid bars) and no AMF access (NAMA; hatched bars) treatments split by non-severed and severed treatments at 89 d post-planting. Gas samples were taken 48 h after blade insertion. Different letters indicate significant differences ($P < 0.05$) between AMA and NAMA treatments within each severing treatment determined by comparing the AMA-NAMA value to zero using either a one-sample t-test or Wilcoxon signed ranks test depending on normality of the data. Error bars represent ± standard error of the mean ($n = 20$).

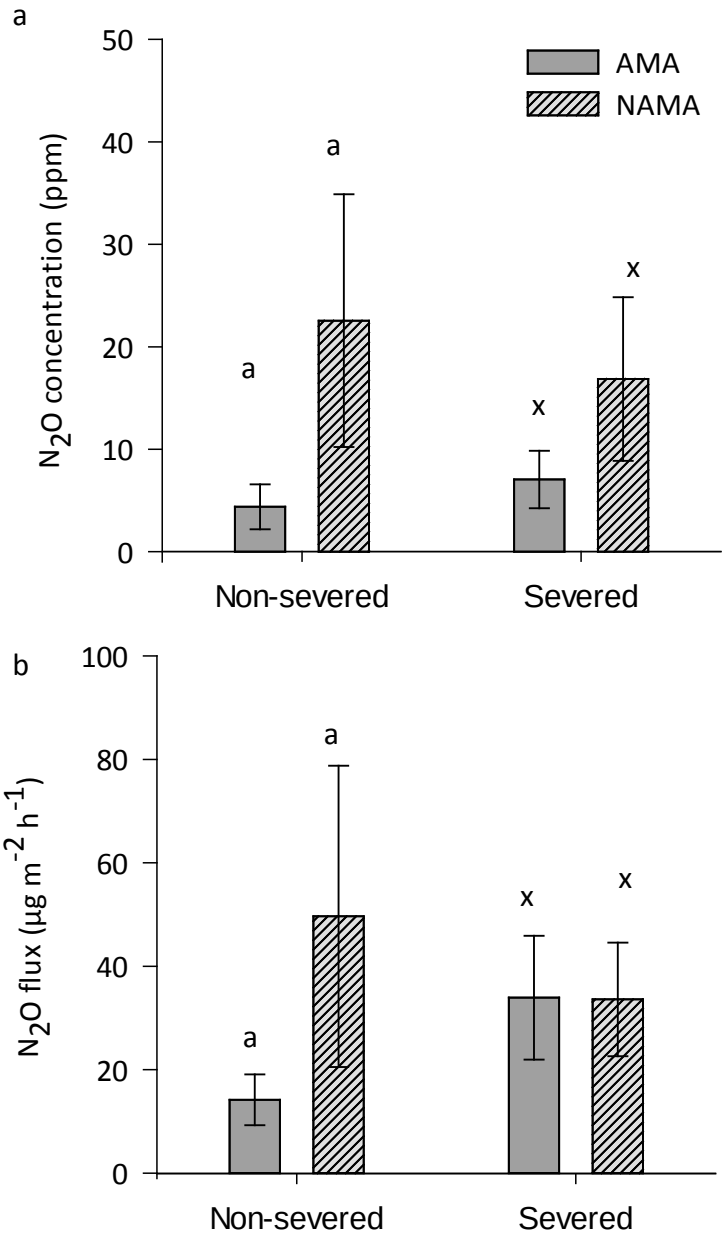


Figure 4.8. Patch N_2O concentration (a) and outer compartment N_2O flux (b) for AMF access (AMA; solid bars) and no AMF access (NAMA; hatched bars) treatments split by severed and non-severed treatments at 89 d post-planting. Gas samples were taken 48 h after blade insertion. There were no significant differences between AMA and NAMA treatments within each severing treatment as determined by comparing the AMA-NAMA value to zero using a Wilcoxon signed ranks test and indicated with the lettering. Error bars represent ± standard error of the mean (*n* = 20).

4.3.4 Changes in N_2O production following the addition of inorganic N

The ΔN_2O fluxes and concentrations changed over time following N addition, both peaked at the 48 h sample following N addition and then decreased back towards pre-N addition values at the 96 h and 172 h samples (ΔN_2O flux: $Q_6 = 79.30$, $P < 0.0001$; ΔN_2O concentration: $Q_6 = 81.86$, $P < 0.0001$). There was also a significant effect of treatment on ΔN_2O fluxes and concentrations when all gas samples were combined in a repeated measures analysis (ΔN_2O flux: $Q_{15} = 84.98$, $P < 0.0001$; ΔN_2O concentration: $Q_{15} = 55.62$, $P < 0.0001$).

The ΔN_2O flux values differed among all treatments at 48 h following N addition (Figure 4.9; Q_{15}, = 46.59, $P < 0.0001$), but due to the high number of *post hoc* tests, when a Bonferroni correction was applied (where $P = 0.00042$) the source of this significant result could not be identified. However, even when a Bonferroni correction was not applied, there were no significant differences between the non-severed and severed values for each AMF access + N addition combination ($P > 0.05$ in each case, see Table 4.5) at the 48 h and 96 h post-N addition gas samples. Therefore, as the severing treatment had no effect on the N_2O production, the data from the non-severed and severed treatments were combined to increase *n*.

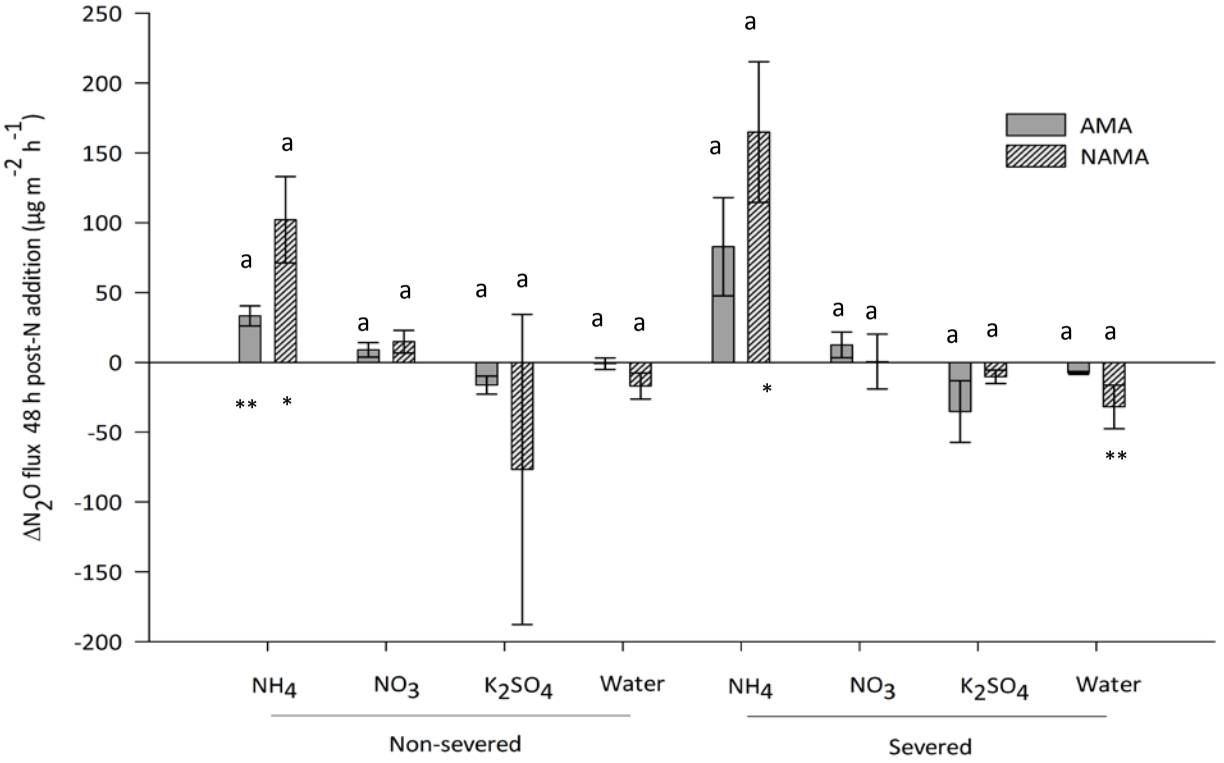


Figure 4.9. Mean difference between 48 h post-N addition (92 d post-planting) and pre-N addition (89 d post-planting) N_2O flux (ΔN_2O flux) for AMF access (AMA; solid bars) and no AMF access (NAMA; hatched bars) treatments split by severing treatment. Error bars represent ± standard error of the mean (n = 5). There were no significant differences between treatments as determined using Mann Whitney U tests and indicated by the lettering. Asterisks below the bars indicate significant differences to zero (*P < 0.05, **P < 0.01) determined using either a one-sample t-test or Wilcoxon signed rank test depending on normality of data.

Table 4.5. Wilcoxon rank sum (Mann Whitney U) statistics comparing the post-N addition N_2O flux response (at 48 h, 96 h and 192 h post-N addition/92 d, 94 d 98 d post-planting) of the non-severed treatment with those from the severed treatment for each AMF access (AMA: AMF hyphal access; NAMA: no AMF hyphal access) and N addition treatment. Significant differences between non-severed and severed treatments are indicated by bold text (*P < 0.05, **P < 0.01).

		48 h post-N addition		96 h post-N addition		192 h post-N addition	
AMF access	N addition	Test statistic (Z)	P	Test statistic (Z)	P	Test statistic (Z)	P
AMA	NH_4	-0.208	0.841	1.253	0.222	2.507	**0.0079****
	NO_3	<0.0001	1.0	-0.836	0.421	<0.0001	1.0
	K_2SO_4	<0.0001	1.0	0.612	0.556	-0.612	0.556
	Water	1.253	0.222	0.418	0.691	-0.418	0.691
NAMA	NH_4	-0.627	0.548	-0.418	0.691	-1.671	0.095
	NO_3	0.418	0.691	0.209	0.841	0.836	0.421
	K_2SO_4	<0.0001	1.0	-0.367	0.730	-0.367	0.730
	Water	0.836	0.421	0.627	0.548	1.880	0.056

The ΔN_2O flux and ΔN_2O concentration data were compared between treatments at each gas sample (Table 4.6). The same trends were apparent in the ΔN_2O concentration data as for the ΔN_2O flux data, but higher variation in the ΔN_2O concentration values resulted in reduced P values (Table 4.6), therefore, only the ΔN_2O flux data are presented graphically.

Table 4.6. Friedman's test statistics controlling for block comparing the post-N minus pre-N patch N_2O concentrations (ΔN_2O concentrations) or compartment N_2O fluxes (ΔN_2O fluxes) among N addition and severing treatment combinations (all treatments), among N treatments for non-severed units only (non-severed), among N treatments for severed treatments only (severed) or among N treatments when severed and non-severed treatments were combined (combined) for each of the gas sampling events. N_2O concentration and flux values that were analysed were post-N addition (48 h, 96 h, 102 h post-N addition or 92 d, 94 d, 98 d post-planting) minus pre-N addition values (89 d post-planting) for each of the gas sampling events at 48, 96 and 192 h post-N addition. *Q* = Friedman's test statistic, df = degrees of freedom. Significant results are indicated in bold at $P = 0.05$ (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

Time since N addition		48 h			96 h			192 h		
		Q	df	*P*	*Q*	df	*P*	*Q*	df	*P*
Patch N_2O concentration	All treatments	30.32	15	**0.011***	23.06	15	0.083	14.96	15	0.455
	Non-severed	12.65	7	0.081	11.80	7	0.107	5.49	7	0.601
	Severed	14.33	7	**0.046***	10.20	7	0.178	5.93	7	0.548
	Combined	28.89	7	**0.0002*****	14.35	7	**0.045***	3.79	7	0.804
Compartment N_2O flux	All treatments	46.59	15	**<0.0001*****	29.55	15	**0.014***	18.71	15	0.227
	Non-severed	20.08	7	**0.0054****	12.22	7	0.094	7.61	7	0.368
	Severed	23.40	7	**0.0015****	15.13	7	**0.034***	10.60	7	0.157
	Combined	44.85	7	**<0.0001*****	25.63	7	**0.0006*****	4.80	7	0.684

When the severing treatments were removed there was a significant difference in ΔN_2O fluxes among the N addition treatments at 48 h post-N addition (Figure 4.10; Q_7 = 44.85, $P < 0.0001$). In both the AMA and NAMA patches, the NH_4 addition treatments produced more N_2O than any other N addition treatment. While the AMA – NAMA values were not significantly different from zero in any treatment, it almost was in NH_4 treatment when a paired Wilcoxon signed ranks analysis was used (Figure 4.11; S_9 = -26.5, P = 0.0039), whereas the AMA – NAMA values for all other treatments had $P >$ 0.05.

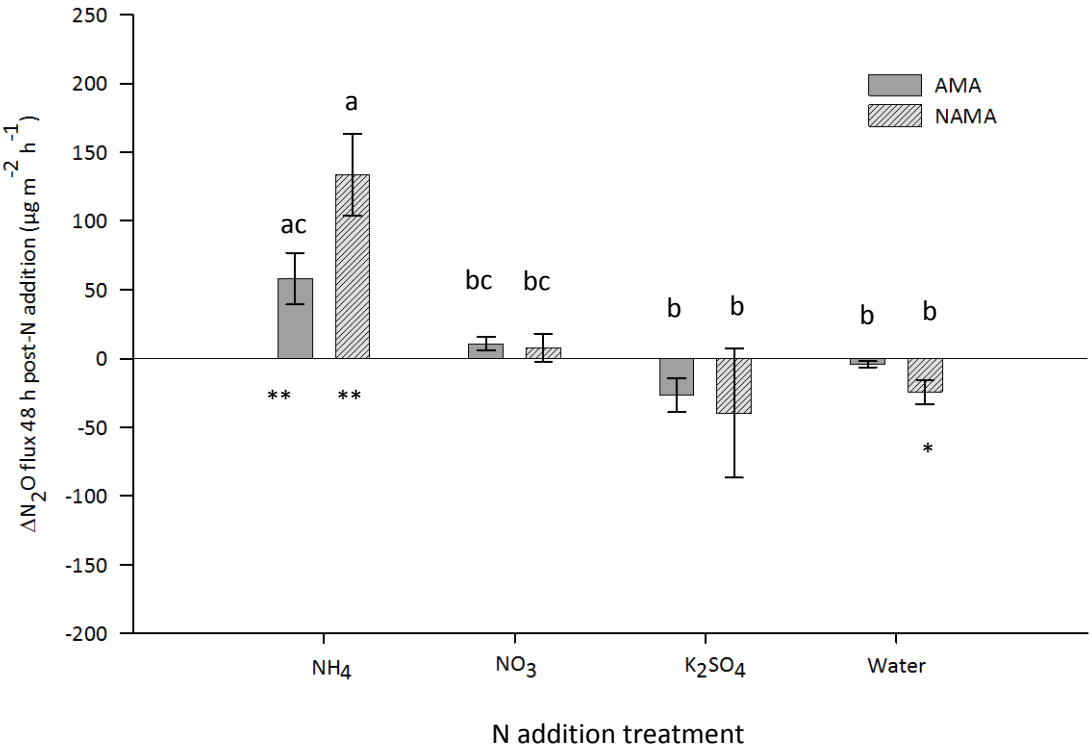


Figure 4.10. Mean difference between 48 h post-N addition (92 d post-planting) and pre-N addition (89 d post-planting) N_2O flux (ΔN_2O flux) for AMF access (AMA; solid bars) and no AMF access (NAMA; hatched bars) treatments, split by N addition treatment.The N addition treatments were: $(NH_4)_2SO_4$ (NH_4), KNO_3 (NO_3), K_2SO_4 or water. Bars with different letters are significant at P = 0.0018 as determined using Mann Whitney U *post hoc* tests and a Bonferroni correction. Asterisks below the bars indicate significant differences to zero (*$P < 0.05$, **$P < 0.01$). Error bars are ± standard error of the mean (n = 10).

When the AMA-NAMA values were compared between the N addition treatments, there was a significantly higher difference between the AMA and NAMA treatment post-N minus pre-N N_2O fluxes when NH_4 was added than when NO_3 or water was added, although not when K_2SO_4 was added (Figure 4.11; $Q_3 = 10.57$, $P = 0.014$).

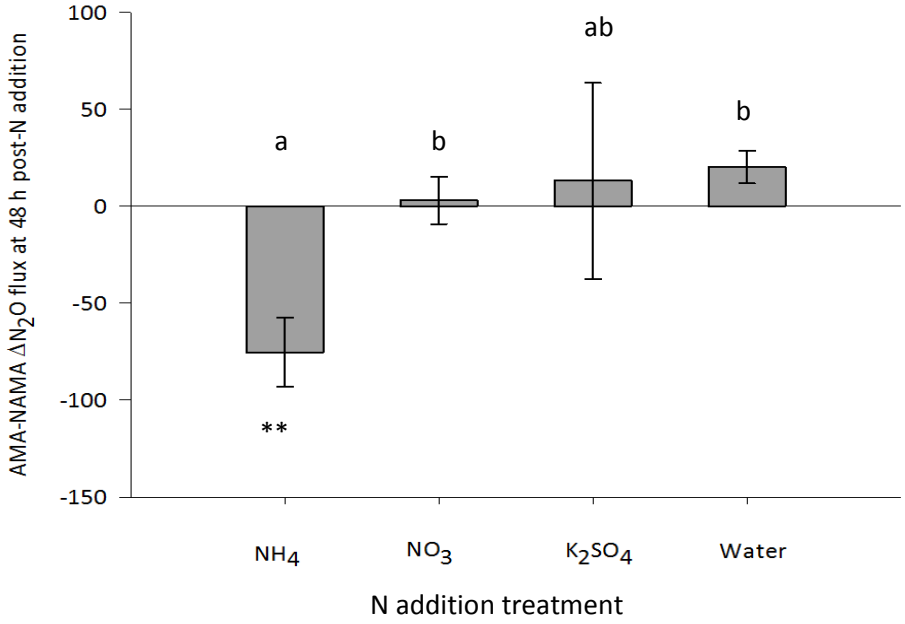


Figure 4.11. AMF access (AMA) – no AMF access (NAMA) change in N_2O flux (ΔN_2O flux) calculated from the 48 h post-N addition (92 d post-planting) minus pre-N addition values (89 d post-planting). The error bars represent ± standard error of the mean ($n = 10$). Bars with different letters are significantly different at $P = 0.0083$ (Mann Whitney U tests). Where a treatment was significantly different from zero (Wilcoxon signed rank test), this is indicated by an asterisk below the bar (*$P < 0.05$, **$P < 0.01$).

The ΔN_2O concentration data following N addition were positively correlated with the ΔN_2O flux data following N addition at all three gas samples (48 h: $r_s = 0.7993$, $P < 0.0001$, $r^2 = 0.64$; 96 h: $r_s = 0.6217$, $P < 0.0001$, $r^2 = 0.39$; 192 h: $r_s = 0.7561$, $P < 0.0001$, $r^2 = 0.57$). There was no relationship between the ΔN_2O fluxes or concentrations with patch moisture content when the data were combined by severing treatment for any of the eight AMF access + N treatments ($n = 10$, $P > 0.05$ in each case) at 48 h or 96 h post-N addition.

4.4 Discussion

4.4.1 Extraradical mycelium length densities and CO_2 fluxes

There were significantly higher ERM length densities in the AMA organic patches than in the NAMA organic patches, demonstrating successful growth into the organic patches by the AMF hyphae (Figure 4.3). However, the organic patch ERM length densities would generally be expected to exceed 1 m g^{-1} DW by 45 d post-patch addition, and be closer to 2 m g^{-1} DW by 70 d post-patch addition (Hodge *et al.*, 2001; Nuccio *et al.*, 2013). Yet, in the present study at 72 d post-patch addition, the mean ERM length density in the AMA patches was only 0.88 ± 0.08 m g^{-1} DW. In a glasshouse study using *Z.mays* as an AMF host plant, Liu *et al*. (2000b) found that peak ERM lengths occurred at intermediate N levels (70 mg kg^{-1}) rather than when high (140 mg N kg^{-1}) or no N fertiliser was applied, possibly because when N is limiting for both the plant and AMF, the plant may not invest C in AMF if they do not provide N (Fitter, 2006; Hodge *et al.*, 2010). While in the present study it was an intended effect to reduce the N and P available to the host plant, the plants received no further nutrient solution following insertion of the blade into the microcosm unit which may have increased their N and/or P limitation, possibly to levels at which plants no longer support AMF growth as in Liu *et al*. (2000b), which may have resulted in the reduced ERM length densities in the patches.

There are a number of issues with the method used here for quantification of ERM length densities (Cheng & Baumgartner, 2006). It can be difficult to identify the AMF hyphae when they are present in a complex medium such as the organic patches used here, particularly as the acid fuchsin stain is not AMF specific (Thies *et al.*, 2002; Vierheilig *et al.*, 2005), and AMF structures can be easily mistaken for other organisms (Leake *et al.*, 2004; Cheng & Baumgartner, 2006). However, this is the best method available for quantifying AMF ERM length densities in soil (Leake *et al.*, 2004), but results need to be considered with caution. The ERM length densities in the NAMA patches in this study were probably not AMF in origin, as there was no indication of hyphal breakthrough on the mesh membranes, but as these structures could not be

distinguished from AMF hyphae, they should therefore be considered as an indication of possible background non-AMF organisms included in the ERM measurements. At low ERM length densities, the CO_2 fluxes would not be expected to increase in the presence of AMF hyphae as found in Chapter 2 and this was also the case here (Figure 4.5a,b). Nonetheless, even when low ERM length densities were present in the AMA organic patches, they still had a significant effect on patch N_2O production.

4.4.2 Pre-treatment N_2O production in the presence of AMF hyphae

Even when low amounts of AMF hyphae were present in the organic patches, N_2O production was significantly reduced (Figure 4.6a). In the AMA treatment, patch moisture content was negatively correlated with both the N_2O flux and ERM length density. At higher moisture contents, O_2 availability is reduced, and the rate of aerobic nitrification can also decrease (Bollmann & Conrad, 1998; Baggs, 2011), which may have been occurring here. However, the r^2 for the correlation between the N_2O flux and patch moisture was very low (0.13) and the organic patch N_2O concentration was not correlated with the patch moisture content which would have been expected if the patch moisture was significantly affecting the N_2O production. The N addition may have reduced the relationship between patch moisture and N_2O production, but as the same quantity of liquid was added to each patch, a higher r^2 than 0.13 would be expected. Furthermore, at low ERM length densities, the respiration of AMF hyphae is unlikely to result in decreased O_2 availability (discussed in Chapter 2, Section 2.4.3.1). Therefore, moisture and/or O_2 availability appeared to be influencing the N_2O production in the AMA patches, but this alone is insufficient to account for the decreases in N_2O production found here. In contrast, the N_2O flux in the NAMA treatment was positively correlated with the CO_2 flux. CO_2 emission is indicative of heterotrophic activity and mineralisation (Villegas-Pangga *et al.*, 2000; Baggs *et al.*, 2003b), therefore decomposition may have been involved in controlling the N_2O production in the NAMA patches (although not solely responsible as the r^2 was only 0.15). Something other than decomposition may have been limiting the N_2O producers in AMA treatments such as pH changes, N or C availability as discussed in Chapter 3.

While N_2O fluxes and concentrations exhibited the same trend (Figure 4.6a,b), there was large variability in N_2O fluxes over a small range of low N_2O concentrations. This may be because of the variability in the organic patch texture and pathways through which N_2O could escape to the surface (Heincke & Kaupenjohann, 1999), which may have been more tortuous under higher patch moisture contents, increasing the range of fluxes. However, the patch N_2O concentrations would be expected to increase if the N_2O fluxes were limited by slow diffusion pathways (Heincke & Kaupenjohann, 1999). Alternatively, the dilution of the gas probe samples in the Exetainers (1 cm^3 in 6 cm^3 N_2) may have reduced the accuracy of measured N_2O concentrations that were close to atmospheric (ca. 324 ppb in 2011; Blasing, 2013); the higher variability in N_2O fluxes at lower N_2O concentrations supports this, thus the N_2O fluxes appear to be more accurate at lower N_2O concentrations.

4.4.3 Gas fluxes and concentrations following the severing of AMF hyphae

Decomposition of AMF hyphae resulting from severing did not appear to significantly affect either the CO_2 or N_2O production within 11 d of severing, as the ERM length densities also did not alter within 13 d of severing. Whilst viable AMF hyphae turn over their C within 5-6 days (Staddon *et al.*, 2003), decomposition rates may be considerably longer; Steinberg & Rillig (2003) found that AMF hyphae can take over 30 d to decompose, and some hyphae could still be extracted 150 d after severing. Thus, hyphal decomposition was probably not the cause for the increased N_2O fluxes measured post-severing in Chapter 2, although the ERM length densities in this experiment were lower, which may be why no differences were found.

Nonetheless, in Chapter 2, even when low ERM length densities were present and KNO_3 was added to the post-harvest soil, there was a significant increase in the N_2O flux from the AMA soils at 120 h post-harvesting (96 h post-N addition; Chapter 2, Section 2.3.3.4), which did not occur in the present study. Up to 25% of the C that AMF receive from their host plant can be transferred to the ERM (Hamel, 2004), in

structural tissue, stored as lipids or broken down to hexoses (Pfeffer *et al.*, 1999; Bago *et al.*, 2000), and severing them will probably result in the release of low molecular weight C compounds. In contrast to Chapter 2, the hyphae in the current experiment were only severed in one location, immediately next to the mesh membrane, and away from the actual organic patch. Therefore any C and/or N released may have been used by other organisms before it could reach the N_2O producers, and may explain why N_2O production was unaffected by severing in this experiment. However, upon severing, the CO_2 fluxes decreased slightly in both the AMA and NAMA treatments. As in Chapter 2, root respiration in the plant compartment was probably influencing the outer compartment CO_2 fluxes as demonstrated by the positive relationship between the AMA and NAMA CO_2 fluxes. The insertion of the blades probably reduced the CO_2 transfer from the plant to the outer compartment and a similar plant compartment influence was probably responsible for the weak positive relationship (r^2 = 0.11) between the AMA and NAMA N_2O fluxes pre-severing. However, as in Chapter 2 (Section 2.4.3), it is also possible that the flow of air over the soils in the closed loop system (CDC system) may have resulted in some purging of air from the sand/Agsorb® mix which could also have contributed to the relationship between gas fluxes in the AMA and NAMA compartments and may have reduced the possibility of finding a significant difference between the AMA and NAMA treatments.

4.4.4 The change in N_2O production following the addition of inorganic N to organic matter patches

4.4.4.1 The pathways of N_2O production in organic matter patches

The addition of $(NH_4)_2SO_4$ resulted in an increase in N_2O production from both the AMA and NAMA treatments, whereas the addition of KNO_3, K_2SO_4 or water did not (Figure 4.10). Ammonium (NH_4) is the precursor for nitrification pathways in soil (reviewed by Baggs, 2011), and although it can be converted to NO_3 via nitrification (Baggs, 2011), the separate addition of KNO_3 had no comparable effect within 48 h of N addition; consequently, NO_3 reducing pathways can be discounted for this experiment under these specific conditions. Denitrifiers are facultative anaerobes

(Wrage *et al.*, 2001), therefore if there were insufficient anaerobic sites within the patches, the conditions would not favour denitrification, although this is unlikely as the patches were moist upon harvesting (ca. 37% moisture g g^{-1} DW) and thus would be expected to contain anaerobic microsites. However, the majority of denitrifiers are also heterotrophic (Parkin, 1987; Hino *et al.*, 2010), and therefore if there was not enough available C, the NO_3 addition would not stimulate N_2O production which may have been occurring here.

The source of N_2O in the organic patches can therefore be limited to one of three different pathways; nitrifier nitrification (NN), nitrifier denitrification (ND), or chemodenitrification (CD) (Figure 4.1). While the addition of inorganic N increased the moisture content of the patches, it is probable that there were still aerobic microsites within the organic patches and therefore the N_2O may have been produced via NN. Nitrifier nitrification can be performed by a range of organisms, predominantly autotrophic ammonia oxidising bacteria (AOB; Prosser, 2007) and archaea (AOA; Leininger *et al.*, 2006), although methane oxidising bacteria (Stein, 2011) and heterotrophic fungi (Laughlin *et al.*, 2008) can also nitrify. Autotrophic nitrification can be a significant source of N_2O between 35 – 60% water filled pore space (Bateman & Baggs, 2005), although it is still relatively unclear as to how much N_2O produced by nitrifiers is produced as a by-product of the ammonia oxidation pathway (Snider *et al.*, 2012).

Alternatively, following N addition, if the availability of O_2 was quite low, particularly at the centre of soil aggregates; the source of N_2O could have been via an anaerobic pathway, ND. In a recent microcosm study, Zhu *et al*. (2013a) found that under low O_2 availability (≤ 0.5% O_2), within 36 h of $(NH_4)_2SO_4$ addition, the predominant source of N_2O was ND rather than ammonia oxidation. Furthermore, while the gross nitrification rates decreased at lower O_2, the total amount of N_2O produced from NH_3 oxidation pathways increased 81-fold as the availability of O_2 decreased from 21% to 0.5% O_2 (Zhu *et al.*, 2013a). This contrasts with the common assumption that the rate of N_2O

production via nitrification decreases at low O_2 (Zhu *et al.*, 2013a), and could partly explain the findings here. However, if the main source of N_2O was ND, a negative relationship may have been expected between the patch moisture content (a proxy for available O_2) and N_2O flux after N addition (Venterea, 2007), which was not the case here. Finally, while CD cannot be ruled out, it is thought to be less important for N_2O production than nitrification or denitrification (Bremner, 1997), and generally only occurs under acidic conditions (Mørkved *et al.*, 2007; Venterea, 2007). It is unlikely that the organic patches were very acidic (the soil added was pH 6.6), and therefore the conditions were unlikely to be conducive to this pathway. Furthermore, AMF can reduce soil pH (Li *et al*., 1991a) and thus would be expected to increase the rate of CD rather than decrease it. Thus in both the AMA and NAMA patches, the predominant N_2O producers were almost certainly nitrifiers, following nitrification and/or denitrification pathways.

4.4.4.2 The difference between AMA and NAMA patch N_2O response upon inorganic N addition

The production of N_2O in the NAMA compartments was greater following NH_4 addition than the production of N_2O in the AMA compartments, irrespective of severing treatment. This suggests that, regardless of the route through which N_2O was produced (NN, ND or CD), the nitrifiers were partially inhibited in the AMA treatments, possibly as AMF were exhibiting a long term distal control on nitrifier activity. Nitrifiers are generally autotrophic (Prosser, 2007), and slow growing, taking from 8 h up to a number of days to double in number (Belser & Schmidt, 1980; Woldendorp & Laanbroek, 1989; Prosser, 2007). It is therefore possible that the presence of AMF hyphae throughout the experimental period prevented the slow-growing nitrifiers from increasing their population size or activity. Veresoglou *et al*. (2011a) suggested that their finding of decreased potential nitrification rates in soils from AM plants compared to soils from low- or non-AM plants could have been caused by AMF-mediated allelopathic suppression of ammonia oxidisers (AO), thus ensuring that AMF had access to available NH_4. However, although some plants can exhibit allelopathic

effects by suppressing nitrification (Alsaadawi *et al.*, 1986; Mao *et al.*, 2006), it is unlikely that AMF produce or transport allelochemicals, and there is no evidence of this to date. Furthermore, as nitrifiers are slow growing (Prosser, 2007), it is probable that AMF affect them in a less complex manner.

Nitrifiers are affected by the availability of O_2, NH_4 and the pH of the soil (Prosser, 2007) and the AMF hyphae in the current experiment may have influenced any one of these factors as discussed in Chapter 3 (Section 3.4.3) and Section 4.4.2 above. Unfortunately this experiment cannot rule out these factors, but the patch moisture content appeared to only have a minor influence in pre-N addition N_2O production (discussed in Section 4.4.2) and, as discussed in Chapter 3, the evidence so far suggests that N limitation is likely to be the most important control. The availability of NH_4 in this experiment was expected to have decreased over time as the patches had been decomposing for 62 days by the point of $(NH_4)_2SO_4$ addition. AMF hyphae are thought to predominantly take up inorganic N in the form of NH_4 (Govindarajulu *et al.*, 2005; Tanaka & Yano, 2005), and AO are generally thought to be poor competitors for NH_4 (Verhagen *et al.*, 1995; Bollmann *et al.*, 2002). If the AMF hyphae were further reducing patch NH_4 content, the AMA patches would be expected to support a smaller population of active nitrifiers if the AMF hyphae are successfully outcompeting them.

Veresoglou (2012) found that nitrification rates were lower in root compartments of non-AM *P. lanceolata* than in root compartments of AM *P. lanceolata* or root free (soil only or soil + AMF hyphae) compartments, but the nitrification rates did not differ between AM root compartments (mycorrhizosphere) and root free compartments. In comparison to rhizosphere soils, there is often a reduction in root derived C compounds in the mycorrhizosphere as these are passed on to the AMF (reviewed by Jones *et al.*, 2004). Therefore, fewer heterotrophic organisms will be present, as demonstrated by Amora-Lazcano *et al*. (1998), potentially releasing other autotrophic organisms such as AO from competition for inorganic N sources. However, Veresoglou (2012) found no difference between the nitrification rates in the AMF hyphal access

compartments (hyphosphere) and soil only compartments, which is in contrast to the findings presented here (Figure 4.6a and Figure 4.11).

Veresoglou (2012) measured unusually low potential nitrification rates in their system, which they attributed to the experimental design not being ideal for the study of slow growing nitrifiers (Veresoglou, 2012), and did not provide information on the level of AMF ERM length densities in AMF hyphal access compartments. A microbial suspension, excluding predators, was reintroduced to a sterile system and left to equilibrate for 3 weeks before treatment additions, which is in contrast to the present study, in which a soil inoculum was added to a non-sterile system. Veresoglou (2012) suggested that the lack of predators in their study resulted in an unrealistic scenario in which to study the interactions between nitrifiers and AMF hyphae. A recent study has demonstrated that the presence of protozoa in organic matter to which AMF hyphae had access, improved N uptake and transport by AMF to their host plant (Koller *et al.*, 2013). It is thought that bacteria initially remove N from the system, which is then mobilized by the protozoa as part of the 'microbial loop' (Clarholm, 1985; Bonkowski, 2004; Koller *et al.*, 2013). Evidence was also presented showing increased transfer of C from the AMF hyphae to the organic patch when protozoa were present, which could help to explain the increased mineralisation rates found in previous studies (Hodge *et al.*, 2001; Atul-Nayyar *et al.*, 2009). Therefore by excluding protozoa, Veresoglou (2012) may have limited the interactions between AMF hyphae and nitrifiers in their study.

If mineralisation rates increase in the presence of AMF hyphae (Hodge *et al.*, 2001; Atul-Nayyar *et al.*, 2009), along with the availability of C (Toljander *et al.*, 2007; Koller *et al.*, 2013), heterotrophs are likely to become more competitive for N sources including NH_4. This is in addition to the increased uptake of N by the AMF hyphae (Leigh *et al.*, 2009; Hodge & Fitter, 2010; Koller *et al.*, 2013), and means that nitrifiers are likely to be outcompeted for NH_4. However, nitrification (often measured by NO_3 production) has been found to increase in the presence of protozoa (Verhagen *et al.*,

1994; Alphei *et al.*, 1996; Bonkowski *et al.*, 2000), which is thought to be caused by the combined effects of selective grazing by protozoa on other bacteria, and the release of NH_4 by protozoa which is used by the nitrifiers (Bonkowski, 2004). The response of nitrifiers seems to depend to some extent on the presence or absence of organisms more competitive for substrates. In a pot based study, the presence of protozoa increased the potential ammonia-oxidising and nitrite-oxidising activity when only soil was present, but when a plant (*P. lanceolata*) was also present, this was not the case; in fact, all evidence of both ammonia-oxidising and nitrite-oxidising activity ceased (Verhagen *et al.*, 1994). Verhagen *et al*. (1994) attributed this to the poor competitive ability of nitrifiers in competition with plant roots for NH_4. Thus, in the present study, the AMF were probably better competitors for NH_4 than nitrifiers in the presence of protozoa, and it is very likely that the reduced N_2O response to NH_4 addition in the AMA treatments was the result of these complex microbial interactions acting as a distal control on N_2O production.

Both AOA and AOB can carry out nitrification in most soils but there is relatively little known about the niche separation of these organisms (Prosser & Nicol, 2012). A laboratory incubation study by Verhamme *et al*. (2011) demonstrated that AOA grew at low, intermediate and high NH_4 concentrations, whereas AOB only grew significantly at high NH_4 concentrations. Verhamme *et al*. (2011) suggested that AOA were more adapted for growth in most soils of low to intermediate NH_4 availability, relying on mineralisation as the main NH_4 source, whereas AOB were more suited to soils with high NH_4 availability, such as agricultural systems with fertiliser N inputs. Di *et al*. (2010) reported similar findings in a soil incubation study where urea was added to soils; AOA preferred low NH_3 conditions and AOB high NH_3 conditions. In the present study, the soil originated from an agricultural site which had probably received NH_4 fertiliser in the past. Therefore, it is likely that both AOA and AOB were present in the patches, and possible that the reduction in NH_4 availability in the AMA treatments may have resulted in less activity of the AOB when NH_4 was added in relatively high quantities. However, there are various organisms that can nitrify, including bacteria (Prosser, 2007), archaea (Leininger *et al.*, 2006) and fungi (Laughlin *et al.*, 2008) making

it difficult to predict with any certainty which of these organisms is responsible. Clearly, detailed molecular analysis of the microbial populations in the organic patches may help to further unravel which N_2O producing organisms are involved, although the pathways are still not well understood (Baggs, 2011; Stein, 2011).

4.4.5 Future study

Isotopic analysis of the N_2O produced can be used to determine the specific source of N_2O (Baggs, 2008; Ostrom & Ostrom, 2011) which could help in determining why AMF hyphae were reducing N_2O production. The distribution of ^{15}N between the central (α) and outer (β) N atoms in N_2O atoms changes depending upon the pathway of N_2O production and the difference between $\delta^{15}N^{\alpha}$ and $\delta^{15}N^{\beta}$ values (called the 'site preference', SP) can be used to differentiate some sources of N_2O (e.g. Ostrom *et al.*, 2010), as can $\delta^{18}O$ values (Ostrom & Ostrom, 2011). Unfortunately, in this study the values of atmospheric $\delta^{15}N$, $\delta^{18}O$ and SP were found to be unreliable at the low concentrations measured here. Nonetheless, by using ^{15}N labelled patch material or by adding ^{15}N labelled inorganic N (e.g. Bateman & Baggs, 2005), isotopic analysis could be used in future studies to identify the N_2O source in the presence and absence of AMF and the factors which control production, although to date, there is no one method available to identify all N_2O sources in soils (Baggs, 2008; Kool *et al.*, 2011). Furthermore, while it would be interesting to compare the N_2O source between AMA and NAMA treatments, the similar response to N addition suggests that it is probably the same dominating process in both treatments. Instead, the more interesting question relates to determining the specific effect of AMF hyphae on N_2O producers; why is N_2O sourced from nitrification reduced in the presence of AMF hyphae? This will be explored further in Chapter 5.

4.4.6 Conclusions

The presence of AMF hyphae significantly reduced N_2O production in organic patches, but as the ERM length densities produced were low, there was no significant effect on

CO_2 production. The patch N_2O concentrations and fluxes were positively related, although at lower N_2O concentrations, the gas probe sampling may have been limited by the dilution of N_2O during sampling. Nonetheless, the N_2O concentrations and fluxes demonstrated the same trends throughout. Severing the AMF hyphae did not affect N_2O production, probably because of the spatial separation between the site of severing and the organic patch, which was the site of N_2O production. Insertion of the blades also reduced the CO_2 production in both AMA and NAMA compartments, indicating that the CO_2 fluxes measured previously also included root respiration from the planted compartment.

The addition of inorganic N in the form of KNO_3 did not affect the N_2O production from either the AMA or NAMA organic patches, whereas the addition of $(NH_4)_2SO_4$ resulted in significant increases in N_2O production in both the AMA and NAMA treatments regardless of severing, suggesting that the main pathway of N_2O production in these patches was nitrification. Furthermore, the production of N_2O following N addition was lower in the AMA treatment than in the NAMA treatment, suggesting that the nitrifiers could not respond to the NH_4 addition in the presence of AMF hyphae, probably due to competition for NH_4 among these key microbial groups acting as a distal control on the nitrifying community. However, the reason for the inability of the nitrifiers to respond to the added NH_4 such as competition for NH_4 or P was not determined. Therefore, in Chapter 5, the effect of and organic N and inorganic P availability on N_2O production in an organic patch will be determined in the presence and absence of AMF hyphae.

Chapter 5. The interactions between arbuscular mycorrhizal fungi (AMF) and patch N_2O production in organic matter patches with varying N and P availability

5.1 Introduction

The experiments presented in Chapters 3 and 4 clearly demonstrated that the presence of arbuscular mycorrhizal fungal (AMF) hyphae can significantly decrease the production of nitrous oxide (N_2O) from an organic matter patch, and this appears to be caused by the inhibition of a nitrification related N_2O pathway (Section 4.4.4.2). AMF hyphae are nitrogen (N) rich (Hodge & Fitter, 2010) and may have a role in N provision to their plant hosts (Blanke *et al.*, 2005; Leigh *et al.*, 2009), as well as producing glomalin which can be a significant component of soil N pools (Lovelock *et al.*, 2004). Thus, the N requirements of AMF may be high, and they do have the potential to deplete N in the hyphosphere (Johansen *et al.*, 1992; Bago *et al.*, 1996). The specific mechanism by which nitrifiers are inhibited in the presence of AMF hyphae is unknown, but, it is likely that the uptake of inorganic N by AMF hyphae results in increased competition for NH_4, with the slow growing nitrifiers losing out (Belser & Schmidt, 1980; Woldendorp & Laanbroek, 1989). However, besides N acquisition (Hodge *et al.*, 2001), AMF hyphae can have a range of effects in the hyphosphere, including C input via exudation (Toljander *et al.*, 2007) micronutrient acquisition (Liu *et al.*, 2000a), and soil structural changes (Rillig & Mummey, 2006; Wilson *et al.*, 2009; detailed further in Chapter 2, Section 2.1); perhaps the best studied being the uptake of phosphorus (P) (Cavagnaro *et al.*, 2005; Smith & Read, 2008).

The major controls on P availability in soils are often chemical processes (Plante, 2007). This includes the rapid adsorption of soluble P (predominantly orthophosphate) onto clay mineral surfaces (such as calcium (Ca), aluminium (Al) or iron (Fe)) forming secondary minerals, and the formation of precipitates including Fe, Al, or Ca

phosphates (Plante, 2007). As a result, the amount of inorganic phosphate (Pi) in soils (the form of P taken up by roots and AMF) is generally low, controlled in part by the pH of the soil; the optimal availability of orthophosphate is around pH 6.5 (Plante, 2007). Transpiration by plants creates a water potential gradient along which water and nutrients are carried towards the plant roots via mass flow (Barber, 1995; Fitter & Hay, 2002). However, as the concentration of Pi in soil solution is low, mass flow is insufficient to fulfil plant P requirements, thus Pi movement to plant roots is predominantly via diffusion (Schachtman *et al.*, 1998). Since the concentration of Pi in soils is low (Raghothama, 1999), diffusion of Pi to the plant roots is slow (Tinker & Nye, 2000; Fitter & Hay, 2002), and consequently P can become very limiting for plants, resulting in depletion zones of Pi around roots (Bagshaw *et al.*, 1972; Jackson & Caldwell, 1993; Tinker & Nye, 2000). It is for this reason that AMF are thought to have evolved the ability to uptake and transfer P to their host plants as, by producing fine hyphae, they effectively increase the volume of soil from which P can be obtained (Li *et al.*, 1991b; Smith & Read, 2008).

Other effects of AMF presence on plant P uptake have also been reported; root colonisation by AMF in the presence of P solubilising bacteria in soils can cause positive, synergistic effects on plant P uptake and growth (Azcon *et al.*, 1976; Kim *et al.*, 1997). The pH of the medium in which AMF hyphae are present has also been found to decrease, as P uptake by AMF hyphae increases, leading to the suggestion that AMF decrease the pH in order to solubilise P (Villegas & Fortin, 2001; Shi *et al.*, 2011). There is also evidence for production of phosphatases by AMF hyphae that can mineralise organic P to inorganic P (Joner & Johansen, 2000; Feng *et al.*, 2002); although the overall contribution of AMF phosphatases to plant P nutrition in soil systems is thought to be low (Joner *et al.*, 2000). Whether driven by one or all of these mechanisms, in the presence of AMF hyphae, the P content of the media decreases (Li *et al.*, 1991b; Joner & Jakobsen, 1994; Feng *et al.*, 2002) which means that AMF hyphae are competing with other organisms for P (Joner & Jakobsen, 1994).

N_2O production is predominantly driven by pH and the availability of N, O_2, and C in soils (Parton *et al.*, 1996; Gillam *et al.*, 2008), and nitrification in particular is controlled by the availability of NH_4 (Norton & Stark, 2011). Thus, if similar processes to those described above are affecting soil NH_4 availability in the presence of AMF hyphae, the production of N_2O via nitrification is also likely to decrease (Kool *et al.*, 2011). Additionally, under conditions where N availability is not limiting the activity of nitrifiers, the availability of P itself may become important to nitrifiers. Phosphorus is an essential nutrient for living organisms as it is involved in the production of essential cell compounds such as nucleic acids and DNA; after N, P is the second most limiting element for biological productivity (Plante, 2007). Nitrifiers can be limited by the availability of P in soil (Purchase, 1974; Pastor *et al.*, 1984) and groundwater systems (de Vet *et al.*, 2012), with Purchase (1974) finding that under conditions of non-limiting NH_4, the rate of nitrification was decreased with limited P availability, and nitrite oxidisers were more affected than ammonia oxidisers. If P limitation in the presence of AMF hyphae was significant for ammonia oxidisers, production of N_2O via nitrification pathways would be reduced. In contrast, if P was limiting for nitrite oxidisers, it is possible that the production of N_2O via nitrifier denitrification (ND), carried out by ammonia oxidising bacteria (AOB) (Baggs, 2011) may increase, as the availability of the ND substrate nitrite (NO_2^-) is increased.

It has also been suggested that under high N but low P conditions, the rate of P immobilisation decreases and therefore fewer active organisms are competing for N, resulting in increased N_2O production via nitrification or denitrification (Hall & Matson, 1999; Sundareshwar *et al.*, 2003). In support of this, Mori *et al*. (2013) found that N_2O produced via denitrification was reduced upon NO_3 and Pi fertilisation of soils sampled from under an *Acacia mangium* plantation. It was proposed that addition of Pi could have released denitrifiers from P limitation (Mori *et al.*, 2013). Alternatively, the activity and therefore respiration of other soil organisms may have increased, resulting in decreased O_2 availability and thus more anaerobic microsites, promoting denitrification (Mori *et al.*, 2013). However, as N_2O production from organic matter patches decreased in the presence of AMF in Chapters 3 and 4, and because this

appeared to be a result of an AMF mediated effect on nitrifier N_2O production, it may have been caused by decreased availability to nitrifiers of N, P or a combination of the two.

Nitrogen mineralisation rates of decomposing organic material are largely controlled by the N and C content of the organic matter (Killham, 2006). Similarly, microbial P mineralisation is controlled by the C and P content of the organic matter, with higher P mineralisation rates at lower C:P ratios (Plante, 2007). Therefore, by altering the N and/or P content of the *Zea mays* L. leaf material used as organic matter patches this could modify the rates of mineralisation and, consequently, the supply of mineralised N and P. The form of N available to AMF hyphae can affect the growth response of AMF. In a field study, equivalent quantities of organic N and NH_4NO_3 were added in discreet mesh bag patches to soil containing AMF hyphae in symbiosis with *Z. mays* (Aleklett & Wallander, 2012). The growth response of the AMF (quantified as relative biomass from phospholipid and neutral lipid fatty acid analyses) was only positive in the N rich organic N treatment (*Medicago sativa* L.), whereas addition of NH_4NO_3 or low N organic material (*Hordeum vulgare* L. straw) resulted in negative and neutral AMF growth responses, respectively (Aleklett & Wallander, 2012). However, Aleklett & Wallander (2012) did not report the P content of the organic material added, and as N and P content of plant material can often be related (Garten, 1976), the conclusion that N content of organic matter was the sole driver for AMF responses may be misleading, particularly as AMF are also strongly linked to P uptake (Smith & Smith, 2011b).

Thus, in the current Chapter, the interactions between the availability of N and P in organic material and the impact of this on N_2O production in the presence of AMF hyphae were assessed. *Zea mays* shoots were grown with different N and P availabilities to create four organic nutrient treatments, high N high P (HNHP), high N low P (HNLP), low N high P (LNHP), low N low P (LNLP). Unfortunately, manipulation of the P content in harvested shoot tissue proved to be unsuccessful, therefore the leaf

material from the HNLP and LNLP treatments (with similar P contents) were used and the high P treatments were created by soaking half of the organic patches in 7 cm^3 of 25 mM sodium dihydrogen phosphate (NaH_2PO_4) before addition to the microcosms. This concentration of NaH_2PO_4 is in the range of concentrations used in previous studies on root (e.g. Auken *et al.*, 1992; Duke & Caldwell, 2000) and AMF hyphal (Cui & Caldwell, 1996) responses to patchy P availability, and by adding 7 cm^3 (i.e. 5.39 mg P) to each organic patch, the P added almost doubled the quantity of P added in the organic leaf material (ca. 6 mg).

The addition of Pi was expected to increase the available P and therefore reduce competition between N_2O producers and AMF hyphae for P. However, upon addition to soils, Pi rapidly becomes fixed via immobilisation following microbial uptake, precipitation of mineral phosphates, or sorption of ions onto soil surfaces (Plante, 2007). AMF will rely on mineralisation of organic P sources (such as the milled *Z. mays* shoots added here), or solubilisation of non-labile P pools to release orthophosphate (Smith & Read, 2008), the soluble form of P thought to be taken up by AMF hyphae (Smith *et al.*, 2003b). By increasing the total P in the organic patch, the relative abundance of soluble P was expected to increase in the high P treatments, as the rate of P mineralisation can be related to P content of media (Mafongoya *et al.*, 2000), therefore competition among the organisms for P may be reduced. The organic patches were added to the outer compartments of a three-compartment microcosm that either allowed (AMA) or prevented (NAMA) AMF hyphal access as described in Chapter 2 (Section 2.2.3.2), and the CO_2 fluxes were measured to assess hyphal activity in the AMA compared to the NAMA compartments. As in Chapter 4 (Section 4.2.5), both gas probes (Chapter 3, Section 3.2.1) and a Los Gatos Isotopic N_2O analyser (Chapter 4, Section 4.2.5) were used to measure organic patch N_2O concentrations and compartment N_2O fluxes, respectively.

The hypothesis under test was that the production of N_2O in the NAMA treatments would be higher than those in the AMA treatments regardless of the nutrient

treatment (HNHP, HNLP, LNHP or LNLP) because the AMF hyphae would effectively reduce the availability of N and P to other soil organisms.

5.2 Materials and Methods

5.2.1 Experimental design

Twenty, three-compartment microcosm units (described in Chapter 2, Section 2.2.3.2) were planted in the central compartment with a *Z. mays* host plant colonised by the AMF *Glomus intraradices* (details in Section 5.2.2). In each unit, one outer compartment allowed AMF hyphal access (AMA) or prevented AMF hyphal access (NAMA) creating a paired design. The outer compartments each contained an organic patch with a gas probe inserted. Dried, milled *Z. mays* shoots were mixed with soil to make the organic matter patches, half of the organic patches contained milled *Z. mays* shoots that were high in N content, and half were low in N content. There were also two levels of P content (high and low P), created by adding NaH_2PO_4 to the organic patches in half of the treatments. This created the four nutrient treatments outlined in Section 5.2.4; high N high P (HNHP), high N low P (HNLP), low N high P (LNHP), and low N low P (LNLP), for each of the AMF access treatments (AMA, NAMA), totalling eight treatments. Gas probes and closed dynamic chamber (CDC) gas sampling were used to determine N_2O concentrations in the organic patches and N_2O and CO_2 fluxes from the outer compartments respectively (see Section 5.2.4 below).

5.2.2 Microcosm design and growth media

The three-compartment microcosms were constructed as described in Chapter 2, Section 2.2.3.2. All three compartments were filled with a 50/50 mix of washed sand and Agsorb® (v/v) as prepared in Chapter 3, Section 3.2.4. The central planted compartments had 0.25 g^{-1} bonemeal (a complex N and P source; 3.5% N, 8.7% P; Vitax, Leicestershire, UK) added along with 90 g of a well-mixed live *Glomus intraradices* inoculum (Plantworks Ltd., Kent, UK) and root mix (*P. lanceolata/Trifolium repens* L.), cut into 2 cm long fragments that had previously been growing for at least 6

months. Three pre-germinated *Z. mays* seeds were added to each plant compartment on 30th September 2012 and thinned to one seedling per pot after 13 d, as in Chapter 2, Section 2.2.3.3. At 13 d post-planting, the outer compartments were filled with a 50/50 mix of sand/Agsorb® (v/v). As in Chapter 2 (Section 2.2.3.4), a sterile 50 cm^3 centrifuge tube was added to the outer compartments to create a hole into which the organic matter patches could be added at a later date.

5.2.3 Growth conditions and harvesting

The microcosm units were placed in a temperature controlled glasshouse in a randomised block design. The mean daily temperature was 19.1°C ± 0.4 and the mean PAR (measured at plant height) between 10 am and 2 pm was 83 ± 12 µmol m^{-2} s^{-1}. There was no significant difference in PAR among blocks and supplementary lighting (high pressure sodium 400 W; Philips SON-T AGRO) was used to extend the photo-period to 16 h per day. The moisture contents of the outer compartments were equalised weekly as described in Chapter 2, Section 2.2.3.5 and the plants were watered with equal volumes of deionised water daily as required. After 13 d of plant growth, the plant compartments received 50 cm^3 of a nutrient solution modified from Thornton & Bausenwein (2000) (1/10th N and P; Appendix 1) once a week. After 6 weeks of growth (42 d post-planting) this was increased to twice a week (50 cm^3 of 1/10th N and P, 50 cm^3 of full N 1/10th P). Additionally, to avoid nutrient deficiency, the plants were provided with increasing quantities of P during the last 4 weeks of growth, by adding full N and 20, 40, 50 or 75% P twice a week at 11, 12, 13 and 14 weeks post-planting respectively. The units were destructively harvested on 11th and 12th December 2012 (at 103 to 104 d post-planting or 71 to 72 d post-organic patch addition) following the same methods as described in Chapter 2, Section 2.2.1.6 but without quantification of root length colonisation and post-harvest gas sampling.

5.2.4 Organic matter patches and treatments

At 32 d post-planting, an organic matter patch was added to each of the AMA and NAMA compartments, 2 cm away from the mesh membrane and 8 cm deep. The organic patches were made up of 2 g dried, milled *Z. mays* shoots and 13 g DW equivalent soil (details in following paragraph). This mixed 'organic patch material' was placed in a 20.0 µm mesh bag as used in Chapter 3, Section 3.2.6. As also described in Chapter 3, Section 3.2.6 each organic patch bag contained a gas probe within the 20.0 µm mesh bag. To reduce the retention of moisture in the organic patches, as had been observed in the previous experiments, the 20.0 µm mesh bags were pierced with a 0.6 mm diameter needle (BD Microlance Nr.14, Beckton, Dickinson and Company, New Jersey, USA) in a regular pattern so that each organic patch bag had 50 holes at a density of 2.4 holes per cm^2. This ensured that the organic patch material stayed within the patch bag, whilst improving drainage of water out of the organic patch.

The soil used in the organic patches was collected from the same site as in Chapter 2, Section 2.2.1.3 and was sieved through a 2 mm sieve before use. In order to produce *Z. mays* leaf material with high or low N contents, *Z. mays* seedlings (F1 Incredible, Moles Seeds (U.K.) Ltd., Essex, UK) were grown in seed trays containing a 50/50 mix (v/v) of sand/Agsorb® that had been washed three times in deionised water to reduce the availability of N. The treatments were provided with 120 cm^3 increasing to 160 cm^3 per tray of a nutrient solution daily (Thornton & Bausenwein, 2000; Appendix 1). Throughout the growing period of 4 to 5 weeks, the high N treatments received a total of 139.8 mg N and the low N treatments received 38.3 mg N. Once the plants had 6 leaves (BBCH stage 16; Lancashire *et al.*, 1991) they were harvested and the leaves were separated from the stalks. The leaf material was then dried at 70^{o}C before milling.

The milled leaf material for each of the high N (HN) and low N (LN) treatments was pooled, mixed well and a sub-sample was taken to measure the N and P content. The

N content was quantified using an elemental combustion system (Costech Analytical Technologies Inc., California, USA) and the P content was determined after triple acid digestion using the molybdenum blue method (Allen, 1974). The N and P contents of the milled leaf material are outlined in Table 5.1. Before addition to the microcosm, each high P treatment organic patch was soaked in 7 cm^3 of 25 mM NaH_2PO_4 to ensure even uptake throughout the organic patch, which equated to 5.39 mg P added per organic patch. This almost doubled the quantity of P provided by the leaf material in each organic patch, which was 6.15 ± 0.39 mg or 6.59 ± 0.62 mg in the HN and LN treatments respectively. Seven cm^3 of deionised water was added, as a control, to the low P treatments.

Table 5.1. Carbon (C), nitrogen (N) and phosphorus (P) content of *Z. mays* leaf material used in the organic patches ± standard error of the mean for the high nitrogen (HN) and low nitrogen (LN) treatments (*n* = 3). [P] = P concentration (mg g^{-1} DW). n.a = not applicable.

	Units	High nitrogen (HN)	Low nitrogen (LN)
% N	%	1.57 ± 0.01	0.85 ± 0.03
% C	%	42.30 ± 0.11	41.83 ± 0.06
Leaf [P]	mg P g^{-1} DW	3.08 ± 0.19	3.29 ± 0.31
C:N ratio	n.a	27:1	49:1

5.2.5 Gas sampling and calculations

Following the same methods as described in Chapter 4, Section 4.2.5, the organic patch N_2O concentrations and compartment N_2O, CO_2 and CH_4 fluxes were sampled at 69, 84, 91 and 98 d post-planting (37, 52, 59 and 66 d post-patch addition, pre-N gas samples), followed by an addition of $(NH_4)_2SO_4$ at 98 d post-planting (66 d post-patch addition) before a second set of gas sampling (post-N addition gas samples) at 48 and 96 h post-N addition (100 and 102 d post-planting or 68 and 70 d post-patch addition). As there was no effect of nutrient treatment or AMF hyphal presence on CH_4

production, these data are not reported. Gas concentration measurements from the CDC system were calculated as in Chapter 2, Section 2.2.3.10 followed by regression analysis to determine the fluxes as described in Chapter 4, Section 4.2.6. Cumulative fluxes were calculated by plotting the four pre-N gas sample N_2O or CO_2 fluxes, integrating the area under the curve and then dividing that value by the number of days and N_2O concentrations in the gas probes were corrected for dilution as in Chapter 3, Section 3.2.8.

5.2.6 Data analysis

Data were first tested for normality and equality of variance using Kolmogorov-Smirnov and Levene's Homogeneity of Variance tests respectively. Proportion data were arcsine transformed before analysis and all data analysis was carried out in SAS (v9.3 SAS institute Inc., North Carolina, USA). The untransformed data are shown in all figures and tables.

Where all eight treatments were compared, and fulfilled normality and equality of variance assumptions, two-way ANOVAs including block were used with Duncan's multiple range *post hoc* tests to determine the source of any significant results. Where data failed normality or equality of variance assumptions, and transformation of the data failed to improve this, a Friedman's non-parametric two-way ANOVA controlling for block with Mann Whitney U (a.k.a. Wilcoxon rank sum) *post hoc* tests and Bonferroni corrections were used on the untransformed data. Differences between AMA and NAMA treatment values were determined by taking the AMA-NAMA value and comparing to zero using either a one-sample t-test or Wilcoxon Signed ranks test depending on whether or not the data fulfilled normality assumptions. As in Chapter 4, Section 4.2.8, the pre-N addition fluxes or concentrations (at 98 d post-planting) were subtracted from the post-N addition fluxes or concentrations respectively (at 48 h or 96 h post-N addition, 100 or 102 d post-planting) to obtain the change in N_2O flux or

concentration following N addition (referred to as the ΔN_2O flux or ΔN_2O concentration, respectively).

To determine if there was significant production of CO_2 or N_2O, the flux values were compared to zero within each treatment using a one-sample t-test or Wilcoxon Signed rank test depending on fulfilment of normality assumptions. Similarly, a one-way ANOVA was used to compare the N_2O concentration data to the atmospheric N_2O concentration at 0.324 ppm (Blasing, 2013), but if data failed normality assumptions, a Wilcoxon rank sum test was used. Relationships between variables were determined using Pearson's correlations, or if variables failed normality assumptions, Spearman's correlations were used.

The extraradical mycelium (ERM) length density data failed equality of variance assumptions and were therefore $\log_{10}$ transformed prior to analysis using a two-way ANOVA. Within each nutrient treatment, the AMA – NAMA values were compared to zero using one-sample t-tests but the results did not differ from the Duncan's *post hoc* tests in this case. The sand/Agsorb® moisture content data were arcsine transformed before analysis. The cumulative CO_2 flux, cumulative N_2O flux, organic patch N_2O concentration (pre-N addition), and organic patch moisture content data failed normality assumptions, as did the ΔN_2O flux and ΔN_2O concentration data following $(NH_4)_2SO_4$ addition, even after transformation. These data were therefore analysed using a Friedman's non-parametric two-way ANOVA.

5.3 Results

5.3.1 Extraradical mycelium (ERM) length densities of AMF in organic matter patches

There were significantly higher ERM length densities present in the AMA patches for all treatments except the HNHP treatment (Figure 5.1; $F_{7,24}$ = 5.67, P < 0.0001). In the HNHP treatment the ERM length densities in the AMA patches did not differ from those in the NAMA patches or from the NAMA patches in the LNLP treatment.

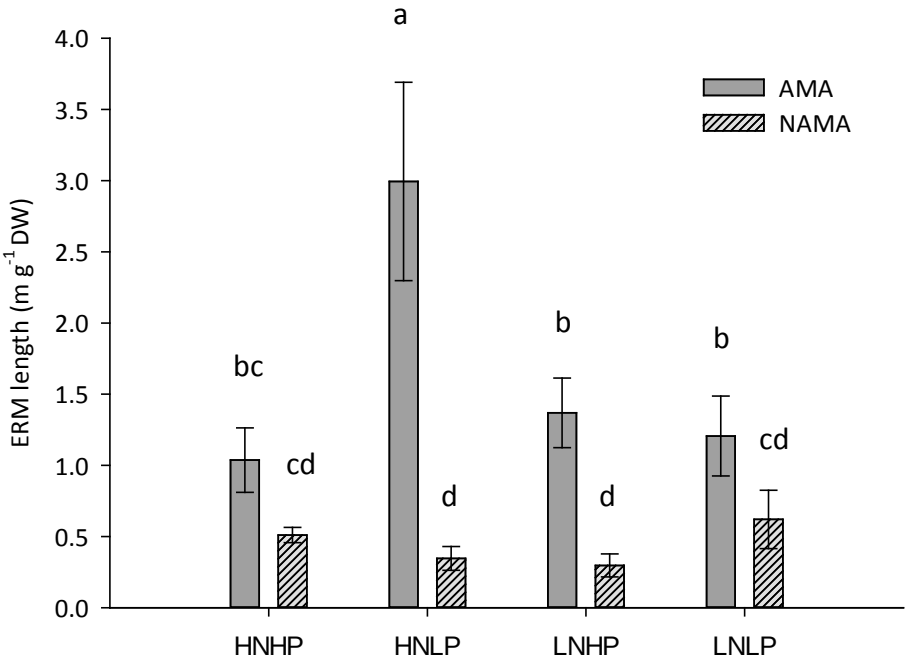


Figure 5.1. Mean extraradical mycelium (ERM) length densities (m g^{-1} DW) in AMF access (AMA; solid bars) and no AMF access (NAMA; hatched bars) patches for each nutrient treatment (high N high P: HNHP; high N low P: HNLP; low N high P: LNHP; low N low P: LNLP). Error bars represent ± standard error of the mean (n = 5). Significant differences among treatments are identified by different letters as determined using a two-way ANOVA with a Duncan's multiple range *post hoc* test on $\log_{10}$ transformed data.

5.3.2 CO_2 and N_2O production in the presence or absence of AMF hyphae

There was no significant difference in the CO_2 flux among organic patch nutrient treatments (Q_7 = 12.33, P = 0.090). However, there was a higher CO_2 flux from the AMA treatments overall (S_{19} = 84, P = 0.0009), and the AMA CO_2 fluxes were higher than the NAMA fluxes in the LNLP treatment (Figure 5.2, statistics in Table 5.2). There was also a positive relationship between the AMA and NAMA cumulative CO_2 fluxes overall (Figure 5.3; r = 0.5847, P = 0.0068), but there was no relationship between the CO_2 fluxes and ERM length densities in the AMA patches (r = 0.6368, P = 0.248).

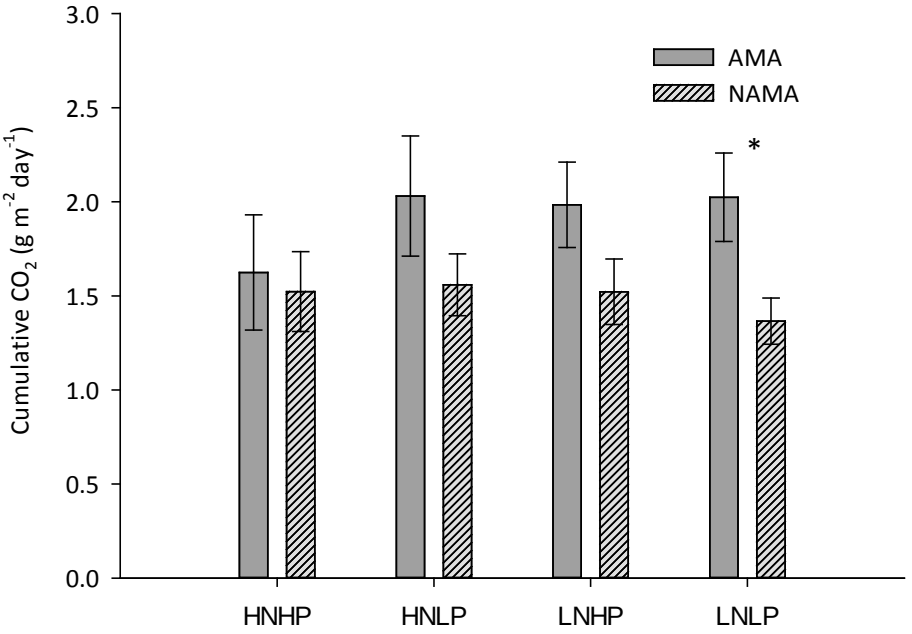


Figure 5.2. Mean cumulative CO_2 flux from 69 to 98 d post-planting (37 to 66 d post-patch addition) for each of the AMF access (AMA; solid bars), no AMF access (NAMA; hatched bars) and nutrient treatments (high N high P: HNHP; high N low P: HNLP; low N high P: LNHP; low N low P: LNLP). Error bars represent ± standard error of the mean (n = 5). Significant differences between AMA and NAMA CO_2 fluxes within each nutrient treatment are indicated by an asterisk and were determined using one-sample t-tests or Wilcoxon Signed rank tests to compare the difference to zero (*P < 0.05, Table 5.2).

Table 5.2. One-sample t-tests (*t*) or Wilcoxon signed rank tests (*S*) comparing AM access (AMA) – no AMA access (NAMA) cumulative CO_2 fluxes to zero for each of the nutrient treatments (high N high P: HNHP; high N low P: HNLP; low N high P: LNHP; low N low P: LNLP). Test statistics are either *t* or *S* values (depending on the test used). df = degrees of freedom. Significant results are shown in bold (*$P < 0.05$).

		Test statistic	df	*P*
HNHP	*t*	0.42	4	0.694
HNLP	*t*	2.48	4	0.068
LNHP	*S*	7.5	4	0.063
LNLP	*t*	2.82	4	**0.048***

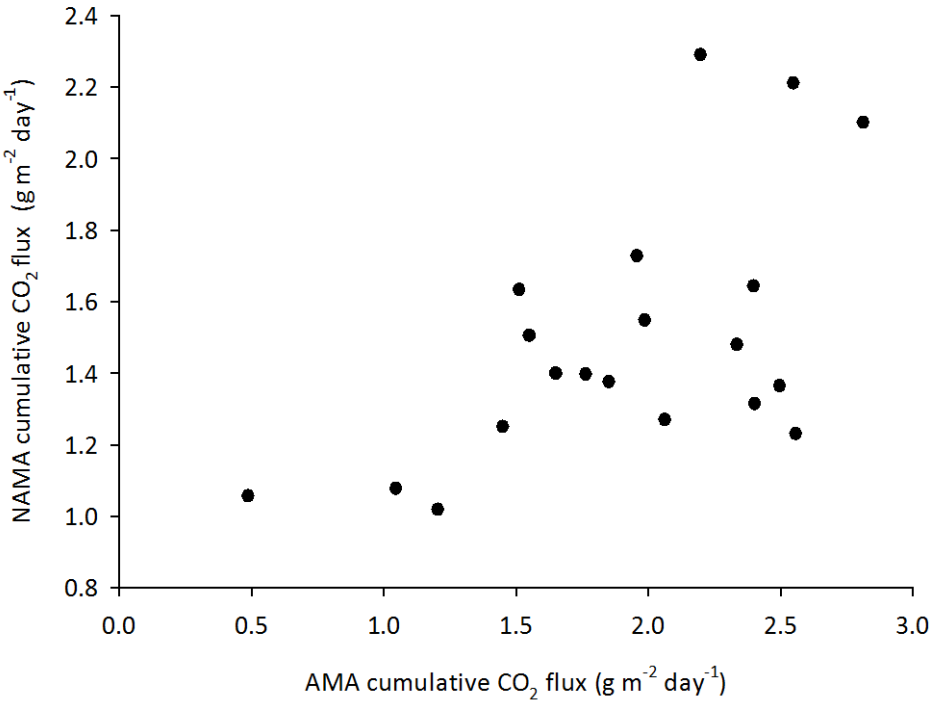


Figure 5.3. AMF access (AMA) compartment plotted against no AMF access (NAMA) compartment cumulative CO_2 fluxes from 69 to 98 d post-planting (37 to 66 d post-patch addition). The positive relationship between AMA and NAMA cumulative CO_2 fluxes was determined using a Pearson's correlation ($n = 20$; $r^2 = 0.34$).

There was no significant difference in the cumulative N_2O flux among the eight nutrient and AMF access treatments (Figure 5.4; $Q_7 = 8.87$, $P = 0.263$). There was also no significant difference between the AMA and NAMA N_2O fluxes within each nutrient treatment ($P > 0.1$ in each case), and the N_2O fluxes from the HNLP NAMA treatment was the only one that was significantly greater than zero ($t_4 = 3.37$, $P = 0.028$).

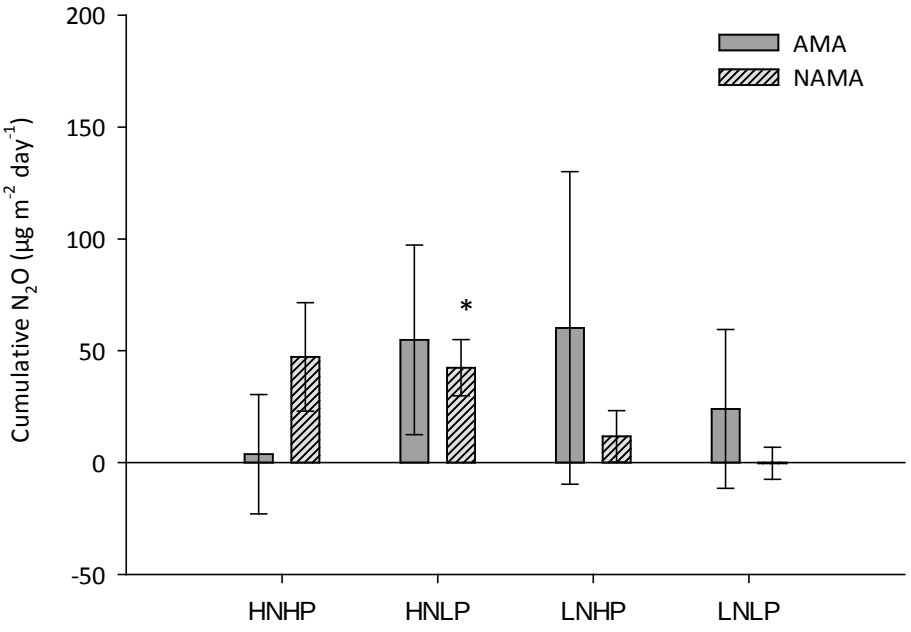


Figure 5.4. Mean cumulative N_2O flux from 69 to 98 d post-planting (37 to 66 d post-patch addition) for each of the AMF access (AMA; solid bars), no AMF access (NAMA; hatched bars) and nutrient treatments (high N high P: HNHP; high N low P: HNLP; low N high P: LNHP; low N low P: LNLP). Error bars represent ± standard error of the mean ($n = 5$). There were no significant differences among any of the treatments. Cumulative N_2O flux values that are greater than zero are indicated by an asterisk above the bar (*$P < 0.05$), as determined using a one-sample t-test.

The patch N_2O concentrations did not significantly differ among treatments (Q_7 = 1.6, P = 0.979), the N_2O concentrations of the AMA and NAMA patches also did not differ within any of the nutrient treatments (P > 0.05 in each case) and the N_2O fluxes were positively correlated with the patch N_2O concentration (r_s = 0.4993, P = 0.001). There was no relationship between the patch N_2O concentration and the compartment CO_2 flux before N addition (98 d post-planting, 66 d post-patch addition) in either the AMA or NAMA treatments (AMA: r_s = 0.3429, P = 0.139; NAMA: r = 0.0795, P = 0.739).

5.3.3 Change in N_2O production following the addition of $(NH_4)_2SO_4$

The ΔN_2O flux at 48 h post-N addition was not significantly different among treatments (Figure 5.5a; Q_7 = 5.67, P = 0.579), and only the HNLP NAMA treatment was significantly greater than zero (t_4 = 3.01, P = 0.039). There were also no differences among treatments in the ΔN_2O flux at 96 h post-N addition (Figure 5.5b; Q_7 = 7.0, P = 0.429), although again only one treatment (LNLP NAMA; t_4 = 3.04, P = 0.039) was significantly greater than zero. There were also no differences in the ΔN_2O concentrations in the organic matter patches among treatments at 48 h (Q_7 = 8.2, P = 0.315) or 96 h (Q_7 = 9.87, P = 0.196) post-N addition. However, the patch N_2O concentrations were not always greater than atmospheric N_2O (0.324 ppm; Blasing, 2013), as shown in Table 5.3.

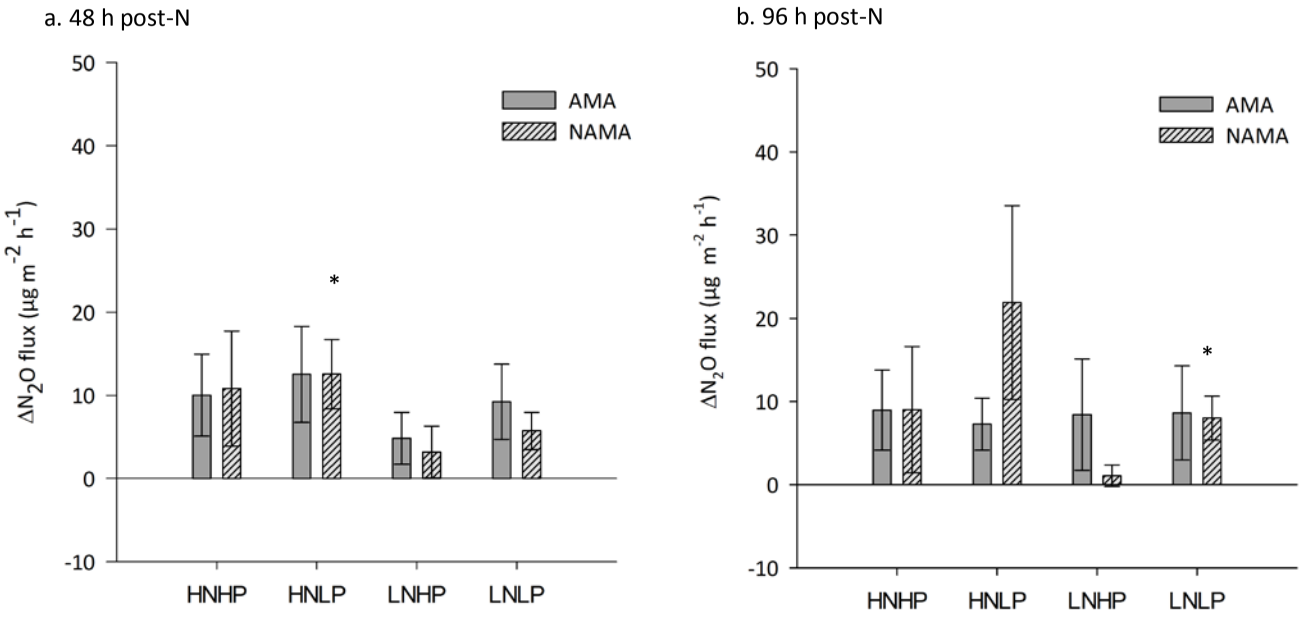


Figure 5.5. Mean change in N_2O flux from pre-N addition values (98 d post-planting) to (a) 48 h and (b) 96 h after the addition of $(NH_4)_2SO_4$ for each of the AMF access (AMA; solid bars), no AMF access (NAMA; hatched bars) and nutrient treatments (high N high P: HNHP; high N low P: HNLP; low N high P: LNHP; low N low P: LNLP). There were no significant differences among treatments at either 48 h or 96 h post-N addition (Friedman's two-way ANOVA). Any change in N_2O flux values > zero are indicated by an asterisk above the bar (*P < 0.05). Error bars represent ± standard error of the mean (n = 5).

Table 5.3. One-way ANOVA or Wilcoxon rank sum statistics comparing the N_2O concentration from within the organic patches to atmospheric N_2O at 0.324 ppm (Blasing, 2013). The comparisons were made for each treatment within each gas sample at pre-N addition (98 d post-planting, 66 d post-patch addition), 48 h and 96 h post-N addition for each of the AMF access (AMA), no AMF access (NAMA) and nutrient treatments (high N high P: HNHP; high N low P: HNLP; low N high P: LNHP; low N low P: LNLP). Asterisks show values that are significantly greater than 0.324 ppm, *P < 0.05, **P < 0.01 and ***P < 0.001. No values were significantly lower than 0.324 ppm.

Gas sample	AMF access	Nutrient treatment		Test statistic	P
Pre-N	AMA	HNHP	t_4	3.73	**0.020***
		HNLP	t_4	6.40	**0.0031****
		LNHP	S_4	2.5	0.625
		LNLP	t_4	1.21	0.291
	NAMA	HNHP	t_4	1.46	0.218
		HNLP	t_4	1.20	0.298
		LNHP	t_4	3.79	**0.019***
		LNLP	t_4	3.79	**0.019***
48 h post-N	AMA	HNHP	t_4	1.90	0.130
		HNLP	t_4	3.40	**0.027***
		LNHP	t_4	1.97	0.120
		LNLP	t_4	1.76	0.153
	NAMA	HNHP	t_4	3.79	**0.019***
		HNLP	t_4	2.60	0.060
		LNHP	S_4	6.5	0.125
		LNLP	t_4	1.99	0.118
96 h post-N	AMA	HNHP	S_4	7.5	0.063
		HNLP	t_4	2.27	0.086
		LNHP	S_4	7.5	0.063
		LNLP	S_4	7.5	0.063
	NAMA	HNHP	t_4	3.15	**0.034***
		HNLP	t_4	2.17	0.096
		LNHP	t_4	14.10	**0.0001*****
		LNLP	t_4	3.23	**0.032***

5.3.4 Organic patch and sand/Agsorb® moisture contents

There was a significant difference in the organic matter patch gravimetric moisture content (Table 5.4; Q_7 = 15.87, P = 0.026) among treatments, but when a Bonferroni correction was applied (where P = 0.00179), the source of this significance could not be identified. There was no significant difference among treatments in the sand/Agsorb® gravimetric moisture content (Table 5.4; $F_{7,28}$ = 1.29, P = 0.290).

Table 5.4. Organic matter patch and sand/Agsorb® gravimetric moisture contents (%) following the harvest at 103 d post-planting (71 d post-patch addition) for each of the AMF access (AMA), no AMF access (NAMA) and nutrient treatments (high N high P: HNHP; high N low P: HNLP; low N high P: LNHP; low N low P: LNLP) ± standard error of the mean (n = 5). There were no significant differences among treatments in either sand/Agsorb® or patch moisture contents (Wilcoxon rank sum tests; P = 0.00179).

		HNHP (%)	HNLP (%)	LNHP (%)	LNLP (%)
Organic patch	AMA	40.1 ± 3.7	46.2 ± 3.7	34.1 ± 4.8	47.0 ± 1.3
	NAMA	46.0 ± 1.7	46.2 ± 2.5	41.7 ± 6.9	44.4 ± 2.7
Sand/Agsorb®	AMA	23.1 ± 1.2	21.0 ± 1.0	22.6 ± 1.3	20.8 ± 1.2
	NAMA	23.1 ± 0.7	20.4 ± 1.5	21.6 ± 1.0	19.9 ± 0.9

5.4 Discussion

5.4.1 The density of AMF hyphae in organic matter patches

The higher CO_2 fluxes in the AMA treatments compared to the NAMA treatments demonstrated, non-invasively, that the organic matter patches appeared successfully colonised by AMF hyphae. However, when the cumulative CO_2 fluxes were split by treatment, this significant difference was only maintained in the LNLP treatment (Figure 5.2). Additionally, the ERM length densities measured in the organic matter patches were not related to the CO_2 fluxes overall, or within each nutrient treatment. This is probably because, as in the previous Chapters, the measured CO_2 flux was

derived from a combination of AMF hyphal respiration and soil microorganism respiration driven by decomposition (as discussed in Chapter 2, Section 2.4.3.1). Furthermore, since the 0.45 µm and 20.0 µm mesh membranes separating the central (planted) and outer (AMA and NAMA) compartments did not impede gaseous diffusion (Chapter 2, Section 2.3.2), the CO_2 produced via root respiration in the central compartment may have diffused into the outer compartments, potentially concealing the CO_2 produced by AMF hyphae, which is supported by the positive relationship between the AMA and NAMA CO_2 fluxes.

Root respiration from the central compartment may have also masked any effect of patch N content on AMA and NAMA CO_2 fluxes. At higher C:N ratios, the rate of decomposition decreases (Taylor *et al.*, 1989; Enríquez *et al.*, 1993; Riffaldi *et al.*, 1996). In an incubation study, Huang *et al*. (2004) found that cumulative CO_2 emissions were negatively correlated with the C:N ratio of plant residues (ranging from 8 to 118) that were incubated with soil for 21 d. Thus, in the present study, lower CO_2 fluxes may have been expected from the LN, compared to the HN, treatments; yet this was not the case. However, this may help to explain why the LNLP treatment was the only one in which the AMA compartments maintained a higher CO_2 flux than the NAMA compartments, despite not having the highest ERM length densities (Figure 5.1). Presumably, lower decomposition rates would reduce the background CO_2 flux from the organic patches, and therefore make it easier to detect the contribution of AMF hyphae to the measured CO_2 fluxes.

5.4.2 The impact of nutrient content on the density of AMF in organic matter patches

Extraradical AMF hyphae appear to have three main functions; finding, and subsequent uptake of nutrients, or searching for a new host plant from which to obtain C (Friese & Allen, 1991). Net N mineralisation from organic material generally occurs when the C:N ratio is < 25:1 with immobilisation of N occurring at C:N > 30:1 (Hodge *et al.*, 2000; Killham, 2006; Robertson & Groffman, 2007). In the present study,

the N mineralisation rates in the LN treatment were probably low as the added leaf material had a high C:N ratio (49:1), which is high when compared to other plant litters (e.g. Muhammad *et al.*, 2011), thus the availability of inorganic N was probably limiting the production of the N rich hyphae (Hodge & Fitter, 2010) regardless of the availability of P. This could explain why the hyphal lengths measured were lower at 71 d post-patch addition in the LN treatments (LNHP: 1.37 ± 0.24 m g^{-1} DW; LNLP: 1.21 ± 0.28 m g^{-1} DW) than those in Chapter 2 at 58 d post-patch addition (2.20 ± 0.84 m g^{-1} DW). In contrast, there may have been slightly higher levels of N mineralisation in the HN treatment, as the added leaf material had a C:N of 27:1, with saprotrophic fungi, but not bacteria, still being able to cause net N mineralisation (Hodge *et al.*, 2000). There may have been an increased availability of mineralised inorganic N in the HN treatment, for which the AMF hyphae could compete. This N could then be used by the AMF for growth, producing more AMF hyphae and a further increase in nutrient uptake (e.g. P), consequently producing ERM length densities at 71 d post-patch addition (HNLP: 2.99 ± 0.70 m g^{-1} DW), that were closer to those measured in Chapter 2 at 79 d post-patch addition (3.84 ± 1.57 m g^{-1} DW).

However, the ERM length densities measured in the HNLP treatment were almost double those measured in any of the other treatments (Figure 5.1), which is perhaps counter-intuitive since the highest N and P availability would have been expected in the HNHP treatment. The ERM length densities measured in the HNLP treatment were similar to those reported in other studies where the AMF hyphae have proliferated in organic material; thought to be a response to an increase in nutrient availability such as P or N (St John *et al.*, 1983; Joner & Jakobsen, 1995; Ravnskov *et al.*, 1999; Leigh *et al.*, 2009). The only difference between the HNHP and HNLP treatments was the supply of P; it may be that the addition of Pi was inhibitory for some of the microorganisms present, possibly as a result of osmotic effects (Madigan & Martinko, 2006). However, other studies have used concentrations of 25 mM and above of NaH_2PO_4 or K_2PO_4 to create 'patches' of high Pi concentration to determine both root (e.g. Auken *et al.*, 1992; Duke & Caldwell, 2000) and AMF hyphal (Cui & Caldwell, 1996) responses to patchy P availability, and these additions were not detrimental to root or hyphal

growth. Furthermore, the addition of a 25 mM solution of NaH_2PO_4 to forest soils in a field study resulted in an increase in microbial biomass (Liu *et al.*, 2012a). Since the ERM length densities measured in the LNHP treatment were in the range of those in previous experiments (Chapters 2, 3 and 4), it was therefore unlikely that the addition of 25 mM NaH_2PO_4 was negatively affecting the microorganisms present in this study.

The uptake of P by external hyphae can be up to 10x slower than the rate of P transfer at the symbiotic interface with the host plant (Sukarno *et al.*, 1996), therefore the AMF hyphae are usually expected to increase the surface area over which P is absorbed. However, in the HNHP treatment, the ERM length densities were not as high as in the HNLP treatment. Even in the HN treatments used here, N was probably limiting, as the C:N ratios of the organic matter patches were > 20:1. Thus, in the HNHP treatment, rather than produce more N rich hyphae, it may have been more efficient for the AMF to increase the number of hyphal Pi transporters or Pi uptake rates in response to the higher levels of P. This could also explain why the HNHP treatment exhibited lower ERM length densities than the HNLP treatment. In a microcosm study, Cavagnaro *et al*. (2005) used fine meshes to prevent root, but allow AMF hyphae, access to an attached compartment in which there were three different levels of added KH_2PO_4, equivalent to bicarbonate extractable P concentrations of 9, 29, and 68 μg g^{-1} soil (P0, P1 and P2 respectively). Hyphae from *G. intraradices* proliferated in the P1 treatment, but less so in the P2 treatment, even though the acquisition of P remained the same. Cavagnaro *et al*. (2005) suggested that the AMF may have increased the rate of Pi uptake or number of Pi transporters in response to encountering a higher concentration of Pi, rather than responding through increased hyphal production.

Evidence from studies using both split-plate (transformed 'hairy' root cultures) and whole plant (non-transformed) systems show that high affinity Pi transporters can be up-regulated in AMF hyphae when they are exposed to an increased Pi supply (in the range of 1-320 μM; Maldonado-Mendoza *et al.*, 2001; Fiorilli *et al.*, 2013), which could be occurring in the HNHP treatment here. However, these high-affinity transporters

are down-regulated at unusually high Pi concentrations (3.5 mM Pi; Maldonado-Mendoza *et al.*, 2001; Fiorilli *et al.*, 2013). Therefore, up-regulation of high affinity Pi transporters would only be relevant in this study if the soluble Pi concentration that the hyphae were exposed to was well below 3.5 mM. However, there is some evidence for passive low affinity Pi transporters in AMF (Thomson *et al.*, 1990), which could be more important under higher P availability, but their role in nutrient uptake is poorly understood (Smith & Read, 2008). There is also evidence that active H^+-ATPases (capable of driving H^+-phosphate co-transport; Lei *et al.*, 1991) exist in AMF and may energize Pi uptake across an electrochemical gradient in AMF hyphae (Smith & Smith, 2011b).

Alternatively, if significant quantities of P leached out of the organic patch bag along with N in the HNHP treatment, the AMF hyphae may have proliferated outside the patch and therefore were not accounted for here. High levels of fertiliser addition can result in P leaching (Withers *et al.*, 2001), and as the P release in the HNLP treatment would be lower, this could also account for why this did not occur in the HNLP treatment. The P may also have reached the plant compartment and thus reduced the P limitation of the host plant. Generally fertilisation of soils by adding inorganic N and P will reduce colonisation of plant roots by AMF and production of AMF hyphae (Liu *et al.*, 2012b) but in sites that are heavily polluted with P, AMF can still be present and are thought to provide the host plants with N when it is limiting (Blanke *et al.*, 2005; Blanke *et al.*, 2011). In this case as the plants were both N and P limited, the hyphae would still be expected to respond to the higher N availability in the organic matter patches and the response of the HNLP treatment indicates that N was clearly limiting. Furthermore, it is felt that significant P leaching is unlikely as the majority of P would have been rapidly fixed following addition to the patches (Section 5.1).

5.4.3 The effect of AMF presence on N_2O production in organic matter patches of varying N and P content

The organic patches did not produce any N_2O between 37 to 66 d post-patch addition (Figure 5.4) which is in contrast to the results in Chapters 2, 3 & 4, and therefore any impact of AMF presence on N_2O production could not be assessed. This was probably because the milled leaf material in the patches had considerably higher initial C:N ratios (27:1 and 49:1 in the HN and LN treatments respectively) than in the previous Chapters (where the C:N ratios ranged from 11:1 to 16:1). As discussed in Section 5.4.2, mineralisation rates are reduced in material with a C:N ratio < 30:1 (Hodge *et al.*, 2000; Killham, 2006; Robertson & Groffman, 2007). Ernfors *et al*. (2008) found that 88% of the estimated total N_2O emissions from drained organic forest soils in Sweden was from soils with a C:N ratio below 25:1, and other studies have shown the C:N ratios of both organic material (Khalil *et al.*, 2002) and plant residues (Huang *et al.*, 2004; Toma & Hatano, 2007) were negatively correlated with the N_2O production during incubation. Nitrification rates are also decreased in the presence of residues with higher C:N ratios (Eiland *et al.*, 2001; Robertson & Groffman, 2007). Thus, the C:N ratio of decomposing material is clearly an important control on N_2O production, and in the current experiment was probably the main factor limiting the production of N_2O. In the HN treatment, it is possible that low levels of N mineralisation may have occurred (Section 5.4.2), but following the results in Chapter 4, the N_2O producers were expected to be predominantly nitrifiers, which are poor competitors for NH_4 (e.g. Verhagen *et al.*, 1995; Bollmann *et al.*, 2002), and were therefore probably N limited in both HN and LN treatments regardless of AMF hyphal presence.

Generally, the decomposition of organic materials with a C:P ratio < 200:1 will result in P mineralisation, whereas those with C:P > 300:1 will result in immobilization of P and material with a C:P of 200:1 to 300:1 will result in no net change in P availability (Plante, 2007). The C:P of the plant material before any inorganic P addition here was 137:1 (HN) or 127:1 (LN), therefore as these were well below the 200:1 threshold, net mineralisation of P should have occurred, thus P should not have been limiting to N_2O

producers in the NAMA treatments, yet no N_2O was produced. It would appear, therefore, that it was N rather than P that was limiting to the N_2O producers here.

5.4.4 The effect of $(NH_4)_2SO_4$ addition on N_2O production in organic patches of varying N and P content

While the N_2O fluxes did not increase following the NH_4 addition, the concentration of N_2O in the patches did marginally increase at 96 h post-NH_4 addition, but only very slightly (Table 5.3). Thus, dilution of the N_2O produced from the patches in the headspace of the outer compartments was probably preventing the N_2O from being detected in the N_2O fluxes. The source of N_2O in Chapter 4 was likely to have been via nitrification (see Chapter 4, Section 4.4.4.1) and, as the experimental design was very similar here, using the same soil and similar leaf material amendments in the organic patches (Section 5.2.4), a nitrification pathway was predicted to be the main N_2O source here too. As discussed above, there were probably few nitrifiers present in the patches preceding $(NH_4)_2SO_4$ addition and nitrifiers can take from 8 h up to a number of days to double in number (Belser & Schmidt, 1980; Woldendorp & Laanbroek, 1989; Prosser, 2007). Therefore, the slow growing nitrifiers were probably simply outcompeted by faster growing organisms for any available NH_4, immobilizing the majority of it before the nitrifiers could respond, regardless of the presence of AMF hyphae (Killham, 2006).

5.4.5 Conclusions

AMF hyphae successfully colonised the organic matter patches in this experiment, as indicated by the increased CO_2 fluxes from the AMA treatments, although CO_2 fluxes were not related to the ERM length densities measured. This may be because the CO_2 measured was derived from a combination of root, microbial and hyphal respiration. The ERM length densities were three times higher in the HNLP treatment compared to the other three nutrient treatments, possibly because the higher N availability allowed the AMF hyphae to proliferate in search of nutrients. In contrast, the ERM length

densities were much lower in the HNHP treatment. The AMF hyphae may have responded to the increased P availability by increasing Pi uptake or the number of Pi transporters rather than investing in more hyphal structures when N was limiting.

The patches were producing very little or no N_2O in all four nutrient treatments, regardless of AMF hyphal presence and this was probably because the organic patch C:N ratios were too high for significant levels of mineralisation to occur, demonstrating that the availability of N was very important to the N_2O production in these patches. Addition of NH_4 did not increase the N_2O fluxes, and only slightly increased the N_2O concentration measured in some of the NAMA patches, probably because the slow growing nitrifiers were being outcompeted for NH_4. Therefore, it was not possible to demonstrate any interactions between AMF hyphae, N or P availability and N_2O production in the organic matter patches in this study. Clearly, the experiment needs to be repeated, but with patches that have lower C:N ratios (e.g. below 20:1), or using LNLP patch material with a high C:N ratio together with additions of NH_4 and/or inorganic NaH_2PO_4 at regular intervals to create the four nutrient treatments; current evidence suggests it is the available N in the soil which dominates any interactions between N_2O production and AMF hyphae.

Chapter 6. General Discussion

6.1 Summary of the initial aims

Arbuscular mycorrhizal fungi (AMF) can influence both carbon (C) (Jones *et al.*, 2009) and nitrogen (N) cycling (Veresoglou *et al.*, 2012b) in soils. Therefore it was predicted that the gaseous outputs of these cycles, including CO_2, CH_4 and N_2O, were likely to be affected by the presence of AMF hyphae in soils, and could have major implications for our understanding of the soil-atmosphere fluxes of these important greenhouse gases (GHG). In order to determine if this was the case, a microcosm was designed to allow measurement of CO_2, CH_4 and N_2O fluxes in the presence or absence of AMF hyphae using either closed static chamber (CSC) or closed dynamic chamber (CDC) methods (described in Chapter 2, Section 2.1). The microcosms had an AMF access (AMA) and/or a no AMF access (NAMA) compartment that either allowed or prevented AMF hyphal access, respectively (Chapter 2, Section 2.2.3.2). Gas probes were also designed to enable gas sampling from within the organic matter patches, with subsequent measurement of the concentrations of N_2O and CO_2 (Chapter 3, Section 3.2.1). The gas probes were intended to enable quantification of N_2O production in the organic matter patches, as this was found to be the main site of N_2O production (Chapter 2, Section 2.4.4). Using these methods the following questions were addressed:

- Can the compartment CO_2 fluxes be used as a non-invasive indicator of AMF hyphal presence in microcosm studies?
- Do the fluxes of CH_4 or N_2O differ when AMF hyphae are present compared to when they are absent?
- Which pathway(s) of N_2O production are affected by the presence of AMF hyphae?
- Does the relative availability of N and/or P affect the production of N_2O when AMF hyphae are present?

6.2 The interactions between AMF hyphae and CO_2 or CH_4 fluxes

In two of the three experiments in which CO_2 fluxes were measured (Chapters 2, 4 and 5), the CO_2 flux increased in the presence of AMF hyphae, enabling their presence and activity in AMF hyphal access (AMA) compartments to be detected without destructive sampling. Where the CO_2 fluxes did not differ between the AMA and no AMF hyphal access (NAMA) compartments, the extraradical mycelium (ERM) length densities were found to be uncharacteristically low (Chapter 4). It is therefore probable that there was a threshold ERM length density that was required in the AMA compartment before the CO_2 fluxes were sufficient to indicate AMF hyphal presence. In Chapters 2 and 5, the ERM length densities measured in the organic matter patches exceeded 1 m g^{-1} DW before CO_2 fluxes could be relied upon to detect the presence of AMF hyphae. In contrast, in Chapter 4, the organic patch ERM length densities were only 0.88 ± 0.08 m g^{-1} DW (at 72 d post-patch addition) and there was no difference in the CO_2 fluxes, probably because the ERM length densities were too low to affect the CO_2 fluxes. The CO_2 concentrations within the organic matter patches were also not informative with regards to AMF hyphal presence (Chapters 3 and 4), probably because the concentration of CO_2 within the patches was very high, including large background respiration contributions from sources other than AMF hyphae. Also, when measuring CO_2 production within the organic patch alone, the AMF hyphal respiration from outside the patch (in the surrounding sand/Agsorb®) is not included. Therefore, the presence of AMF hyphae would be difficult to determine using patch CO_2 concentrations, as AMF respiration would be a relatively low contribution to a larger respiratory flux.

Existing methods for assessing AMF hyphal presence often limit the number of replicates possible in experiments and require destructive samples to quantify hyphal presence, either via hyphal extractions (e.g. Hodge & Fitter, 2010) or phospholipid fatty acid analysis (PLFA; e.g. Li *et al.*, 2013). Destructive sampling can disturb the soil, and soil disturbance can affect the fluxes of CO_2 (Omonode *et al.*, 2007), CH_4 (Kessavalou *et al.*, 1998) and N_2O (MacDonald *et al.*, 2011). Therefore, there is a

technical conflict between minimising disturbance when quantifying GHG fluxes, and checking that units are sufficiently colonised by AMF hyphae before treatment application. The microcosm based studies in this thesis have demonstrated that, while not necessarily directly related to measured ERM length densities (Chapter 2, 4 and 5, also see Chapter 4, Section 4.4.1), the CO_2 flux from AMA and NAMA compartments can be compared and used to determine whether AMF hyphae are present at ERM length densities exceeding 1 m g^{-1} DW in the AMA compartments. Hyphal lengths of 1 m g^{-1} DW and above are often reported in microcosm (Leigh *et al.*, 2009; Li *et al.*, 2013) and field (Treseder & Allen, 2002; Mummey & Rillig, 2006) studies, and therefore it may also be possible to use CO_2 as a non-invasive indicator of AMF hyphal presence in other microcosm and field systems.

The presence of AMF hyphae had no significant effect on the CH_4 fluxes reported in Chapter 2 (Section 2.3.3.4), and it was decided to focus attention on the observed interactions with N_2O; consequently, CH_4 was not further discussed in Chapters 3 to 5. However, CH_4 fluxes were measured using the CDC system in Chapters 4 and 5 (Chapter 4, Section 4.2.5 and Chapter 5, Section 5.2.5), and, consistent with the findings described in Chapter 2, there was also no effect of AMF hyphae on CH_4 fluxes in these experiments. In Chapter 2, the pre-harvest CH_4 oxidation rates were not affected by the presence of AMF hyphae, and following the harvest, even under optimal conditions for CH_4 oxidation (ca. 20 ppm CH_4), there was also no effect of AMF hyphae on CH_4 oxidation. There are various ways in which AMF hyphae could potentially interact with CH_4 production or consumption (described in Chapter 2, Section 2.1), the most likely being the effect of AMF hyphae on soil O_2 availability, either via AMF hyphal respiration, increased decomposition rates in the presence of AMF (Hodge *et al.*, 2001; Atul-Nayyar *et al.*, 2009) or changes in soil structure (Rillig & Mummey, 2006) and water retention (Augé *et al.*, 2001); despite these possible effects, no such impacts were observed in Chapters 2, 4 or 5. It is therefore likely that either the influence of AMF hyphae on soil O_2 availability or other controlling factors (e.g. C availability or pH) were not sufficient to impact on the activity of CH_4 producers and consumers under the conditions used here.

6.3 AMF hyphae significantly affect N_2O production in soils and organic matter patches

The presence of AMF hyphae has been shown here to reduce the production of N_2O and, in Chapter 4, the N_2O was probably produced via a nitrification pathway as this effect was exaggerated by NH_4 addition (Table 6.1; Chapters 3 & 4). However, AMF hyphae have also been shown to increase the production of N_2O when the hyphae are severed (Table 6.1; Chapter 2). Only one other study, to date, has investigated the impact of AMF hyphae on N_2O production, finding no interaction, but this was under quite contrasting conditions (Cavagnaro *et al.*, 2012). Cavagnaro *et al*. (2012) worked in an organic tomato production system, using either wild type AM tomatoes or non-AM mutant tomatoes, with the production of $^{14+15}N_2O$ or $^{15}N_2O$ from mycorrhizosphere soils following the application of $^{15}N\text{-}KNO_3$ not differing when the plants present were either AM or non-AM. Unfortunately, N_2O fluxes before KNO_3 addition were not reported by Cavagnaro *et al*. (2012), therefore it is possible that any AMF mediated effects preceding KNO_3 addition could have been masked by the addition of KNO_3. A similar effect was found in Chapter 3, where there was no longer a difference in the N_2O concentration of AMA and NAMA patches within 48 h of NH_4NO_3 addition, although by 96 h the N_2O concentration in the AMA treatment dropped below that of the NAMA treatment again; Cavagnaro *et al*. (2012) did not measure the N_2O fluxes beyond 48 h post-KNO_3 addition. It is therefore possible that Cavagnaro *et al*. (2012) missed any AMF mediated effects on nitrifiers, by stimulating the denitrifier activity beyond that of the nitrifiers and failing to quantify N_2O production in the absence of fertiliser addition.

Table 6.1. The observed changes in N_2O production in the presence of AMF hyphae found in this thesis. The increase (↑), decrease (↓) or no significant difference (n.s) in production of N_2O in AMF access (AMA) compared to no AMF access (NAMA) treatments are shown (n.a = not applicable). Hypothesised sources of N_2O are listed, and those in bold are supported by additional information outlined in the relevant Chapter. Experiments are listed in order of whether or not N_2O was produced, and if so, how the AMA treatment differed from the NAMA treatment; dashed lines group the data by the effect of AMF hyphae on N_2O production. The timing (pre- or post- harvest), source of N_2O measurement (patch, soil or compartment which includes both patch and soil or sand/Agsorb® fluxes) and additional treatments are detailed.

Chapter	Was N_2O produced?	AMA vs NAMA N_2O production	Hypothesised N_2O source	Timing	Measured N_2O	Additional Treatment
2	Yes	↑	Denitrification	Post	Patch	n.a
2	Yes	↑	**Denitrification**	Post	Soil	+ KNO_3
3	Yes	↓	Nitrification	Pre	Patch	n.a
4	Yes	↓	Nitrification	Pre	Compartment, Patch	n.a
4	Yes	↓	**Nitrification**	Pre	Compartment, Patch	Patch + NH_4
2	Yes	n.s	Unknown	Pre	Compartment	n.a
2	Yes	n.s	**Denitrification**	Post	Soil	+ glucose
3	Yes	n.s	Nitrification & Denitrification	Pre	Patch	+ NH_4NO_3
4	Yes	n.s	Nitrification	Pre	Patch	Severed hyphae
2	No	n.a	n.a	Post	Soil	n.a
4	No	n.a	n.a	Pre	Compartment, patch	Patch + NO_3
5	No	n.a	n.a	Pre	Compartment, Patch	Patch N and P content

6.3.1 Interactions between AMF and denitrification pathways

In this thesis, the addition of KNO_3 to soils containing AMF hyphae resulted in an increase in N_2O production, but only after the hyphae had been severed (Table 6.1; Chapter 2). In contrast, when KNO_3 was added to soils containing intact AMF hyphae (Table 6.1; Chapter 4), there was no significant difference in N_2O produced between AMA and NAMA treatments, suggesting that in the intact hyphosphere, the interactions between AMF hyphae and N_2O produced via denitrification were minimal.

Cavagnaro *et al*. (2012) compared rhizosphere soils to mycorrhizosphere soils, in which one may expect a reduction in C available to denitrifiers as result of decreased root exudation (Bansal & Mukerji, 1994). This could partly explain the decrease in the number (Amora-Lazcano *et al.*, 1998) or changes in the community structure (Veresoglou *et al.*, 2012a) of denitrifiers reported in other studies (Table 6.2). However, there was no difference in the production of N_2O from mycorrhizosphere and rhizosphere soils measured by Cavagnaro *et al*. (2012), and in the study of Veresoglou *et al*. (2012a), plant age was the main explanatory variable for the changes in denitrifying community, while AM status was secondary. However, the population or community of denitrifiers are not necessarily good indicators of N_2O production in response to changing environmental conditions (Attard *et al.*, 2011), as different species of denitrifiers can have different potential to denitrify (Ka *et al.*, 1997). Nonetheless, there is some evidence for AMF hyphae interacting with N_2O produced through the process of denitrification in this thesis, probably as a result of a peak in C availability acting as a proximal control (*sensu* Wallenstein *et al.*, 2006) on the activity of the denitrifiers present when the AMF hyphae were severed at harvest (Table 6.1; Chapter 2), but this is only under certain conditions; this interaction could be very different in the mycorrhizosphere, which was not studied here.

6.3.2 Interactions between AMF and nitrification pathways

As N_2O can be a side product of nitrification (Hooper & Terry, 1979; Stein, 2011), N_2O production can be linearly related to the rate of nitrification (Khalil *et al.*, 2004). Veresoglou *et al*. (2011a) reported a series of experiments (three mesocosm and one field based) in which the potential nitrification rate (PNR) was lower in soils surrounding plants that were AM, compared to those that were weakly AM. While the method used by Veresoglou *et al*. (2011a) for determining the mycorrhizal treatments was not ideal (they compared the percentage colonisation of plant roots by AMF, which may not necessarily represent AMF activity (Hart & Reader, 2002; Smith & Smith, 2011a), the findings of Veresoglou *et al*. (2011a) are in agreement with the results obtained here in Chapters 3 and 4 (Tables 6.1 and 6.2). In Chapters 3 and 4, the production of N_2O decreased in the AMA treatments compared to the NAMA treatments, thus, decreased PNR rates in the presence of AMF (Veresoglou *et al.*, 2011a) could explain the reduced N_2O production in the AMA treatments following the NH_4 addition (Chapter 4), the substrate for nitrification (Prosser, 2007).

In a further study by Veresoglou (2012), actual nitrification rates did not differ in hyphosphere compared to soils in which AMF hyphae were denied access, yet, in the mycorrhizosphere, the actual nitrification rates increased when AMF were present (compared to non-AM rhizosphere soils). This latter study did not include protozoa, which may have impacted upon the competitive interactions between AMF and nitrifiers in the hyphosphere (see Chapter 4, Section 4.4.4.2). A reduction in C exudation in the mycorrhizosphere, compared to non-AM rhizosphere soils, can result in fewer saprotrophic microorganisms being present (Christensen & Jakobsen, 1993; Bansal & Mukerji, 1994), which may have caused less competition from heterotrophs for NH_4. Furthermore, their use of sterilised soils that were re-inoculated with bacteria was probably not appropriate for studying slow growing nitrifiers alongside AMF hyphae (Veresoglou, 2012).

Table 6.2. Reported effects of AMF presence on N_2O producers or N_2O producing pathways in either mycorrhizosphere (vs. non-AM or low-AM rhizosphere), or hyphosphere (vs. soil only) soils from the published literature. The effect of AMF presence on the measured variable is shown here as an increase (↑), decrease (↓) or no significant change (n.s) in the measured variable. Ammonia oxidisers = AO.

Reference	Location of interactions	Measured variable	Effect of AMF	Limitation of the study
Veresoglou *et al*. (2011)	Mycorrhizosphere	Potential nitrification rate	↓	AMF treatment may be misleading
Amora-Lazcano *et al*. (1998)	Mycorrhizosphere	Number of:		
		◦ Ammonia oxidisers (AO)	↑	Doesn't include AO activity and AO numbers in control were not stable
		◦ Ammonifiers	↓	
		◦ Denitrifiers	↓	
Cavagnaro *et al*. (2007)	Mycorrhizosphere	AO bacteria:		Doesn't include AO activity, short duration (2 months), AOB only
		◦ Community composition	n.s	
		◦ Population density	n.s	
Veresoglou (2012)	◦ Hyphosphere	Actual nitrification rate	n.s	Excluded predators & re-inoculation of sterilised soil with microbes not suitable for slow growing nitrifiers
	◦ Mycorrhizosphere		↑	
Cavagnaro *et al*. (2012)	Mycorrhizosphere	$^{14+15}N_2O$ and $^{15}N_2O$ production following $^{15}N\text{-}KNO_3$ addition	n.s	High variability in N_2O fluxes, focus on denitrification as only measured N_2O after KNO_3 addition
Veresoglou *et al*. (2012a)	Mycorrhizosphere	Denitrifying community	Community change	Response mainly driven by plant age

Other studies (summarised in Table 6.2) have considered the effect of AMF presence on the number and communities of nitrifying organisms i.e. the organisms which carry out the pathways which potentially lead to N_2O production. However, the number of nitrifiers are not necessarily related to the rate of nitrification (Klopatek & Klopatek, 1997), and the methods used to quantify the ammonia oxidizers (AO) differ between studies, making direct comparisons difficult (Smith *et al.*, 2001; Cavagnaro *et al.*, 2007). These published studies have been inconclusive, with an increase (Amora-Lazcano *et al.*, 1998) or no change (Cavagnaro *et al.*, 2007) in numbers of ammonia oxidisers (AO) reported in the presence of AMF. There are also many other N_2O producing organisms including heterotrophic nitrifiers (e.g. Laughlin *et al.*, 2008), and ammonia oxidising archaea (AOA; e.g. Leininger *et al.*, 2006) that have not been studied alongside AMF. Despite this, there is increasing evidence for AMF interacting with nitrification in both the published literature (Table 6.2) and this thesis (Table 6.1), which may result in a reduction in the production of N_2O in the hyphosphere.

6.3.3 Specifying the N_2O pathways that are affected by AMF hyphae

There are a range of methods involving stable isotopes that could be used to further specify the source of N_2O production that is affected by the presence of AMF hyphae, but to date no single method can identify every N_2O source in soils. Using ^{15}N or ^{18}O enrichment alone cannot distinguish between nitrifier nitrification and nitrifer denitrification pathways (Bateman & Baggs, 2005), or account for the exchange of oxygen between nitrogen oxides and water (Kool *et al.*, 2011). Alternatively, source partitioning (quantifying the distribution of naturally abundant ^{15}N between the central and outer ($\delta^{15}N^{\alpha}$ and $\delta^{15}N^{\beta}$) N atoms in N_2O) can help to broadly identify the source of N_2O without the need for ^{15}N enrichment (Ostrom & Ostrom, 2011), but it is only useful in discriminating between pathways which use different enzymes, and therefore doesn't separate N_2O produced via nitrifier or denitrifier denitrification. Kool *et al*. (2011) described a method of dual labelling using ^{15}N and ^{18}O which can identify between N_2O produced from main pathways in soils. However, this method does not account for all N_2O producing pathways (e.g. codenitrification and DNRA) and so far

has only been demonstrated under laboratory conditions using soil incubations (Kool *et al.*, 2011). The main controls on N_2O production (i.e. N, C or O_2 availability) can be split by nitrification and denitrification pathways (Baggs & Philippot, 2010). Therefore, the techniques that are currently available to identify the source of N_2O will probably not tell us much more about the interactions between AMF hyphae and N_2O production than was achieved by additions of NH_4 and/or NO_3 as in Chapters 2 and 4. Instead, it would be more interesting to determine how the functions of N_2O producers change in the presence of AMF hyphae.

6.3.4 The drivers of the interactions between AMF hyphae and N_2O production

Evidence in Chapter 4 suggested that nitrifiers producing N_2O could not respond to an NH_4 addition in the presence of AMF hyphae, but the reason for this was not clear (Table 6.1). Possible explanations include a short term proximal effect, such as competitive exclusion of nitrifiers by AMF hyphae, or longer term distal effects of AMF hyphal presence on the nitrifying community composition (described in Chapter 3, Section 3.4.4), via N, P or O_2 limitation, or pH changes. The hyphae of AMF are N-rich (Hodge & Fitter, 2010), and can also transfer N to their host plant (Nuccio *et al.*, 2013), thus AMF have a high N requirement, and consequently may be able to outcompete other soil organisms for N (see Chapter 4, Section 4.4.4.2). Ectomycorrhizal fungi have been found to outcompete other soil microorganisms (e.g. saprotrophs) and therefore decrease decomposition rates (the 'Gadgil effect'; Gadgil & Gadgil, 1971; Gadgil & Gadgil, 1975). One suggested mechanism is that ECM have a competitive advantage over other soil microorganisms for nutrient uptake (e.g. N) as they are not C limited (Bending, 2003). Similarly, AMF obtain C from their host plants (Paul & Kucey, 1981) and, may also have a competitive advantage over other soil microorganisms. However, unlike ECM, AMF are not thought to have saprotrophic capabilities (Smith & Read, 2008) and so may actually compete more intensely for nutrients with the surrounding microbial community (Leigh *et al.*, 2011). Alternatively, it could be argued that soil N transforming organisms have a spatial advantage over AMF hyphae (typically 2 - 20 µm

diameter; Friese & Allen, 1991) since they can be smaller. However, although less mobile than NO_3, NH_4 is still mobile in soils, with a diffusion coefficient in the range of 4×10^{-12} to 1×10^{-10} m^2s^{-1} (Fitter & Hay, 2002), and thus, at that scale, diffusion gradients of NH_4 and NO_3 are probably not large enough to exclude AMF.

While AMF hyphae may influence soils in the hyphosphere in various ways (including P or copper limitation, pH changes, changes in O_2 availability or production of allelochemicals; outlined in Chapter 3, Section 3.1), competition for N alone is likely to be great enough to reduce rates of nitrification as AO are thought to be poor competitors for NH_4 (Verhagen *et al.*, 1995; Bollmann *et al.*, 2002). Therefore, the observed decreases in N_2O production in the presence AMF hyphae are probably a side-effect of AMF outcompeting nitrifiers for NH_4. In comparison, an alternative AMF driven mechanism to reduce the competition from nitrifiers for NH_4 such as increased production or transport of allelochemicals to inhibit nitrifiers, as proposed by Veresoglou *et al*. (2011a), would require more 'effort' from the AMF to achieve the same goal, and therefore seems less likely.

Veresoglou *et al*. (2011a) tried to determine if nitrogen (N) limitation was the main cause for the change in PNR. Three forb species were grown in upland grassland soil in pot-based monocultures for six months. While a single N addition as NH_4NO_3 at the beginning of the experiment increased the PNR in soils from all three forbs (one AM, two non-AM), the soils from the AM forb still exhibited a lower PNR than soils from the two non-AM species. Similarly, in Chapter 4, the change in N_2O production 48 h after NH_4 addition was lower in the AMA than in the NAMA treatment. The application of a single dose of N is unlikely to release the slow growing nitrifiers (Woldendorp & Laanbroek, 1989) from N limitation given they may not be able to respond as quickly as other organisms to a sudden peak in available N, and would therefore be outcompeted by other organisms (Verhagen *et al.*, 1995). Thus, long term N limitation of nitrifiers was not sufficiently assessed in Chapter 4 or in the study by Veresoglou *et al*. (2011a). In contrast, the experiment described in Chapter 5 was designed to identify the

relative importance of N and P availability on N_2O production in the presence of AMF hyphae. However, because the C:N ratios (above 20:1) of the milled leaf material used in the organic patches was probably too high for N mineralisation (Robertson & Groffman, 2007) or N_2O production (Ernfors *et al.*, 2008), there was no N_2O produced. Thus, If the experiment in Chapter 5 was repeated with organic patches of higher N content (lower C:N ratios), the relative important of N and P availability on N_2O producers in the presence of AMF hyphae may be revealed.

Alternatively, since it is hypothesised from the results in Chapters 3 and 4 that AMF hyphae reduce the availability of NH_4 in the patches via competition, inorganic N additions could be used to determine the relative importance of long versus short term availability of NH_4 on N_2O production in the presence of AMF hyphae. This could be achieved by adding inorganic N at regular intervals to organic patches to create non-limiting conditions of N. Therefore, N could be added as NH_4 or NO_3 to determine the relative importance of each of these forms of N. However, since NH_4 is converted to NO_3 via nitrification (Wrage *et al.*, 2001), it would be necessary to include a nitrification inhibitor. Bateman and Baggs (2005) used a nitrification inhibitor to determine the relative contribution of nitrification and denitrification to N_2O production in soils with different water filled pore spaces. Since non-invasive isotopic methods are still under development (Baggs, 2008; Butterbach-Bahl *et al.*, 2013), a nitrification inhibitor may be the most effective method at present for separating these pathways. If there was no effect of AMF hyphae on N_2O production in the presence of increased NH_4 or NO_3, then N limitation could be assumed. If this was not the case, further study manipulating each of the proposed drivers (such as O_2, pH etc.) in turn would then be appropriate to determine the driver.

As N_2O production can be affected by the availability of O_2 (Khalil *et al.*, 2004), which in turn can be controlled by the water content of the medium (Franzluebbers, 1999; Moyano *et al.*, 2013), in all but one of the experiments outlined here (Chapters 2, 4 and 5) the moisture content of the outer compartments was equalised weekly. Nonetheless, the patch moisture content was higher in the AMA treatment by the final

(105 d post-planting) harvest in Chapter 2 and the positive correlations between patch moisture content and N_2O concentration (regardless of AMF hyphal presence) in the water addition treatments (but not the NH_4NO_3 addition treatments) demonstrated the importance of moisture content for N_2O production in Chapter 3 (Section 3.3.4).

An increase in the water content of soils can change conditions for microorganisms in one of two ways. Firstly, it may increase the mobility of nutrients (such as NH_4 or NO_3) via diffusion (Moyano *et al.*, 2013), which is particularly important in dry soils, and consequently was unlikely to have had an impact in the experiments in this thesis as the patches were quite wet, regardless of presence of AMF hyphae. Secondly, because O_2 diffusion through water is slower than in air (Schlesinger, 1997), an increase in water content can reduce the availability of O_2 for nitrifiers, potentially reducing N_2O production via aerobic nitrifier nitrification (Wrage *et al.*, 2001). There was a negative correlation between organic patch moisture content and pre-harvest N_2O flux in the AMA treatment, but not NAMA treatment in Chapter 2 (r^2 = 0.34) and Chapter 4 *(*r^2 = 0.13), but a negative relationship between organic patch moisture content and N_2O concentration was found in the NAMA (r^2 = 0.46), but not the AMA treatment in Chapter 3 (Section 3.3.4). There was also no relationship between organic patch moisture content and post-harvest patch N_2O production in Chapter 2. Similarly, the only evidence for a negative relationship between N_2O and CO_2 production (a proxy for respiration and therefore O_2 availability) was found in the AMA treatment in Chapter 3. These variable results for relationships and effects of moisture contents on patch N_2O production may be a result of Type 1 error, especially considering the low statistical significance in some cases. Taken together, this suggests that water content and/or O_2 availability is unlikely to be the only driver of the observed changes in N_2O production in the presence of AMF hyphae, with N and C availability also being as, if not more important in determining the rate of N_2O production under these non-saturated conditions.

Whatever the cause is for the observed changes in N_2O production in the presence of AMF hyphae, there is very little evidence in the experiments outlined in this thesis for AMF hyphal length densities being related to N_2O production (but see Chapter 2, Section 2.3.3.3). While the AMF hyphal length densities can be positively related to the plant N captured from hyphal only compartments (Ames *et al.*, 1983; Hodge *et al.*, 2001), this is not always the case (Leigh *et al.*, 2009). This is probably because the methods used to quantify the ERM length densities do not identify which hyphae are viable. However, in part, it may also be because AMF species differ in their own N requirements, but very little is known about why different AMF species produce different ERM length densities under the same conditions (e.g. Cavagnaro *et al.*, 2005; Smith & Smith, 2011b). In a microcosm study comparing the hyphal lengths produced by three different AMF species, Jakobsen *et al*. (1992) found that whilst the P uptake of the symbiosis between *Trifolium subterraneum* L. and *Acualospora laevis* (Gerdemann and Trappe) was twice that of the symbiosis with *Scutellospora calospora* (Nicolson and Gerdemann), the total ERM lengths produced were very similar; clearly the rate of hyphal spread or ability to uptake P per unit hypha may be more important than the total ERM length in some cases. This diversity among species probably allows them to fulfil different and complementary but, as yet, poorly understood niches (Koide, 2000). The variability in ERM length densities for the same AMF species has been demonstrated in Chapter 5, where the highest ERM length densities were measured in the high N, low P treatment, possibly indicating an up-regulation of Pi transporters in the high P but N limiting conditions. Thus, it is perhaps not surprising that the ERM length densities were generally not related to the rate of N_2O production in the presence of AMF hyphae in the current work.

6.4 Proposed mechanisms for interactions between AMF hyphae and microbial N_2O production

Following the results from the experiments described in this thesis (summarised in Table 6.1), three predominant mechanisms are proposed by which AMF hyphae interact with N_2O production pathways (outlined in Figure 6.1). Firstly, AMF hyphae

increase competition for NH_4 in the hyphosphere (1; Chapters 3 & 4), this distal control will limit slow-growing nitrifiers, and thus reduce N_2O production via nitrification (dashed orange pathways in Figure 6.1). Secondly, the severing of AMF hyphae may release low molecular weight (LMW) C compounds (2; Chapter 2), a short term proximal control that could increase the rate of heterotrophic denitrification when NO_3 is not limiting (solid blue pathways in Figure 6.1), but hyphal turnover and exudation are proposed to have less impact on denitrification, since severing the connections between AMF hyphae and their host plant did not affect the N_2O production after 11 d in Chapter 4 (Section 4.3.4). Finally, AMF hyphae might increase the water content of organic matter patches (Chapter 2) thus reducing the availability of O_2 (a long term, distal control) and subsequently the rate of N_2O production via aerobic nitrification (3), or increase N_2O production via anaerobic denitrification. Other factors including changes in pH, allelopathy and reduced Cu or P availability in the presence of AMF hyphae have been included (4) but are considered less likely to be the drivers of changes in N_2O production compared to N, C and O_2 availability (Chapters 2, 3, 4 and 5). While beyond the scope of this thesis, ideally, further microcosm based studies could be used to sequentially determine the relative importance of each of these proposed drivers in defining the interactions between AMF hyphae and N_2O production.

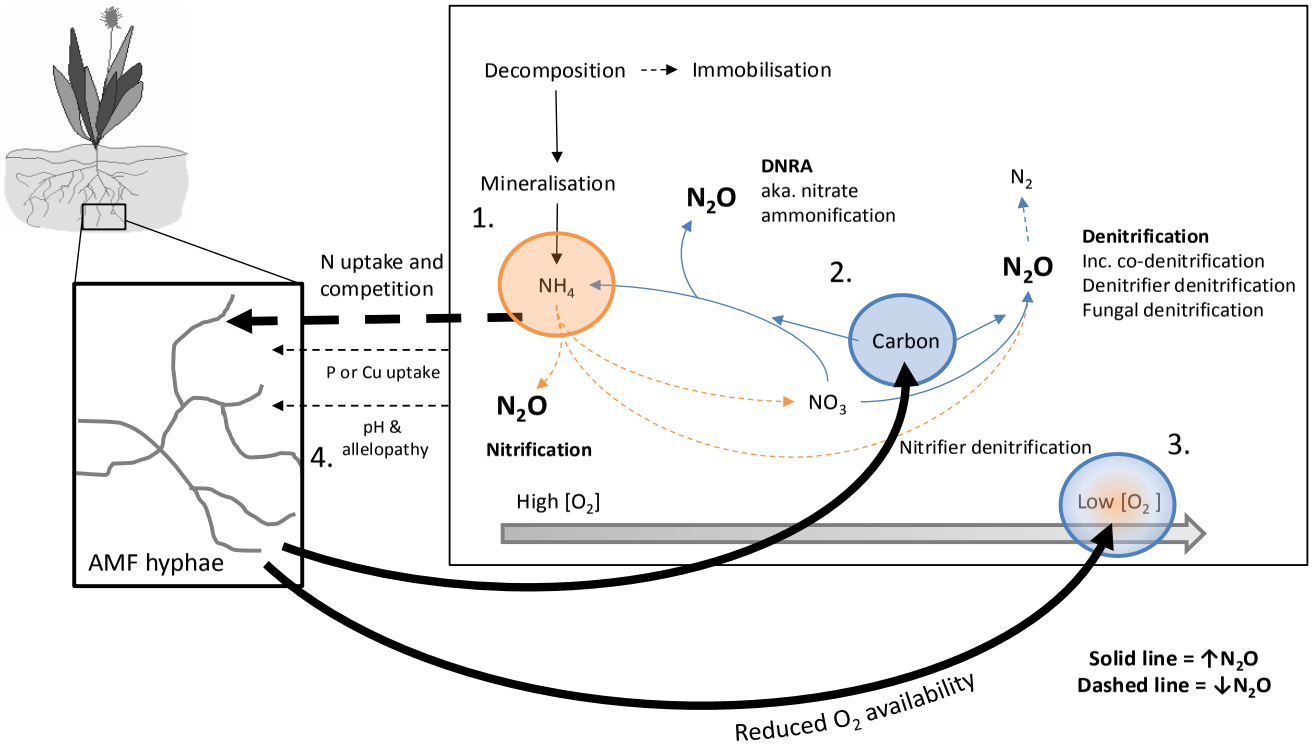


Figure 6.1. Proposed interactions between AMF hyphae and N_2O production in organic matter patches as described in this thesis. The orange lines represent nitrification pathways and the blue lines represent denitrification pathways. Solid lines indicate increases in N_2O production whereas dashed lines indicate a reduction in N_2O production. DNRA: dissimilatory nitrate reduction to ammonium. The factors proposed to be the main influences of AMF hyphae on N_2O production are indicated by the three circles and the colours represent which pathways are likely to be affected (orange: nitrification, blue: denitrification). As nitrifier denitrification is carried out by nitrifiers, it is classed under nitrification pathways here.

6.5 The organisms involved in interactions between AMF and N_2O production

Recent developments in metagenomics and metaproteomics are making previously impossible insights into the organisms and processes underpinning functional activities in soil microbial communities (Zhang *et al.*, 2010). When combined with stable isotope probing (SIP) fresh insights into the functional response of some nitrifying communities are now possible. For example, by using ^{18}O-water Adair & Schwartz (2011) found that the growth of AOB was stimulated by the addition of NH_4 whereas the growth of AOA was not. However, SIP techniques are relatively targeted since the organisms under study need to assimilate the substrate, and SIP methods are not yet available for denitrifiers (Butterbach-Bahl *et al.*, 2013). N_2O can be produced via a variety of pathways and organisms that may be using the same enzymes (Baggs, 2011; Stein, 2011), or may have the ability to carry out more than one pathway of N_2O production, and there may still be some organisms capable of N_2O production that we are not yet aware of (Baggs & Philippot, 2010). Furthermore, N_2O is measured at a much broader scale than 'omics' or SIP approaches, information is generally only available on the net N_2O release and it is not yet possible to separate all N_2O pathways. Therefore, targeting specific functional responses would be premature for research on AMF and N_2O producers at this stage. However, if the drivers of interactions between AMF hyphae and N_2O production were further understood, and the techniques available for assessing functional responses were linked to N_2O production pathways in the future, this could help to explain and predict the response of N_2O producers to changes in environmental conditions.

6.6 The implications of interactions between AMF hyphae and N_2O production

The experiments presented in this thesis have further supported the role of AMF in N cycling (Hodge & Fitter, 2010), by demonstrating that AMF hyphae significantly influence the output of the GHG N_2O (Chapters 2, 3 and 4). Microcosm systems are

ideal for determining mechanistic interactions between organisms under controlled conditions (Drake & Kramer, 2012), and it is under these conditions that the mechanisms underlying the interactions between AMF and N_2O production can be further explored (Section 6.3.3). Equally, it is essential to determine the real-world *in situ* relevance of these interactions. There is microcosm based evidence of the increased transfer of N to host plants and increased residue decomposition when a mix of AMF species are present (Tu *et al.*, 2006), or when hyphae have access to organic N along with earthworms (Li *et al.*, 2013). In field systems other factors, such as plant nutrient status, can affect plant colonisation by AMF (Blanke *et al.*, 2005). These are examples of factors that could affect the interactions of AMF with N_2O production in a field system and it is necessary to compare rhizosphere, mycorrhizosphere and hyphosphere interactions of AMF with N_2O producers under field scenarios, in order to determine how relevant the potential implications for field estimates of AMF influences on N_2O fluxes are and, subsequently, to determine the relevance of any mechanism controls discovered under laboratory conditions (Section 6.3.3).

Studying the interactions between AMF hyphae and N_2O production in field systems is not a simple task. The creation of hyphal only treatments can be difficult in the field, since it usually requires the use of fine mesh membranes to either allow (20.0 µm) or prevent (0.45 µm) AMF hyphal access, and these can be easily damaged. However, it is possible, Johnson *et al*. (2001) designed soil cores that allowed the study of AMF hyphae in field soils with minimal disturbance to the soil system. These cores had four windows that were covered by a 35.0 µm mesh membrane, allowing AMF hyphal access, but preventing root access. In a subsequent field study, Johnson *et al*. (2002a) used these cores and found that AMF hyphae represent a significant route via which plant C can enter soils. However, creating a non-AMF access control is difficult, and Johnson *et al*. (2002a) rotated the cores to sever the AMF hyphae from their host plant, but the evidence presented in this thesis show that in some circumstances severing AMF hyphae would affect the N_2O flux (Chapter 2). Therefore the use of finer mesh membranes may be more appropriate to create the no-AMF access treatment when studying N_2O, but whether these fine membranes (0.45 µm) will remain intact

under field conditions is yet to be determined. Furthermore, the cores designed by Johnson *et al*. (2001) were small (18 mm inner diameter), thus gas sampling with a CDC system would not be feasible without significantly increasing the core size, although a gas probe (Chapter 3, Section 3.2.1) could be designed to fit inside small cores.

The recent development of instruments that are capable of rapid N_2O measurement greatly reduce the methodological limitations that have in the past added to the variation in N_2O measurements in laboratory (e.g. Chapter 2) and field systems (e.g. Cavagnaro *et al.*, 2012), and it would now be possible to use cores that can exclude roots but allow AMF hyphal access in field studies (Heinemeyer *et al.*, 2012b; Johnson *et al.*, 2002a). Combining exclusion cores with an automated multiplexed chamber system (e.g. Heinemeyer *et al.*, 2012b) that included an isotopic N_2O analyser (e.g. Los Gatos Isotopic N_2O analyser, as used in Chapter 4, Section 4.2.5) would allow high frequency measurement of the N_2O fluxes in the presence and absence of AMF hyphae; we now know that CO_2 fluxes can (in certain cases) be used as a non-invasive indicator of AMF hyphal presence or absence (Section 6.2). An understanding of factors and interactions that affect the rate of N_2O production in field systems will be vital to improve the models that are under development to help predict future N_2O emissions and the influence of changes in environmental conditions on the production of N_2O (Reay *et al.*, 2012). Therefore, while understanding the basic mechanisms controlling the interactions between AMF and N_2O production are essential, their relevance in real-world scenarios will be equally important if models are to sufficiently predict responses of N_2O fluxes to environmental change.

AMF can form a symbiosis with over 2/3 of all land plants (Smith & Read, 2008), and AMF hyphae can make up a significant proportion of the soil biomass (Olsson *et al.*, 1999). If, as in the microcosm systems used here (Chapters 3 and 4), AMF hyphae reduce the production of N_2O in field systems, they could be important globally for our understanding of soil N_2O production, particularly in agricultural systems. In conventional agricultural systems, the colonisation of plants by AMF can be reduced

when compared to low-input agricultural systems (Mäder *et al.*, 2000b), and the diversity of AMF in agricultural systems is much lower than in woodland systems (Helgason *et al.*, 1998). The production of N_2O in agricultural systems is also generally high, mainly because of high N inputs (Bouwman *et al.*, 2010), and therefore a key target for sustainable agriculture is to improve N use efficiency and reduce N_2O outputs (Reay *et al.*, 2012). The use of AMF in sustainable or low-input agriculture is often discussed (Hart & Trevors, 2005; Gosling *et al.*, 2006; Fitter *et al.*, 2011) since these symbionts can improve the nutritional status of their host plants (Mäder *et al.*, 2000a; van der Heijden *et al.*, 2006) as well as providing other benefits, including improved soil structure (Rillig & Mummey, 2006; Purin & Rillig, 2007), and pathogen resistance (Cameron *et al.*, 2013; Vos *et al.*, 2013). The reduction of N_2O production may therefore be an additional benefit of encouraging AMF in sustainable agriculture, but evidence of similar effects on N_2O in the mycorrhizosphere and in field systems need to be tested.

Agricultural management could also be important in controlling field AMF-N_2O interactions, with evidence in this thesis (Chapter 2) suggesting that the presence of AMF hyphae may actually result in a burst of N_2O production upon tilling, as severed hyphae resulted in increased N_2O production. Fitter *et al*. (2011) argued in favour of no- or low-till agriculture, as tilling can have detrimental effects on AMF growth (Kabir *et al.*, 1997; Fitter *et al.*, 2011), and no-till agriculture can reduce CO_2 emissions and improve C storage (e.g. Fuentes *et al.*, 2012). Therefore, the evidence that N_2O fluxes may increase if AMF are severed (Chapter 2), but decrease in the presence of intact AMF hyphae (Chapters 3 and 4) supports the use of low- or no-till management. However, within the first 10 years of no-till management there are often increases in N_2O production (Six *et al.*, 2004), possibly because the low-tilled soils retain more water and therefore improve conditions for anaerobic denitrification (e.g. Beheydt *et al.*, 2008).

Furthermore, tilling occurs after the plants have been harvested, when the AMF hyphae are no longer connected to their host plants, and the host plant C supply will no longer be present, therefore loss of C from the AMF hyphae via 'leaking' or exudation (see discussion in Chapter 4, Section 4.4.3) may not be as important as the decomposition of AMF hyphae with regards to supplying additional C to the N_2O producers. In Chapter 4 (Section 4.3.4), after 11 d, the decomposition of AMF hyphae did not significantly affect N_2O production. However, since AMF hyphae can take 30 to 150 d to decompose (Steinberg & Rillig, 2003), decomposition of AMF hyphae may affect N_2O production over a longer time period than measured in the experiments described here. The role of AMF in no-till or low-till agriculture in N_2O production has not been considered to date. Additionally, harvesting root crops will inevitably sever AMF hyphae, and any mechanical disturbance of the hyphae during agricultural management may result in a burst of N_2O via denitrification. If this were to occur, the N_2O produced during or after harvesting might counteract any AMF mediated reduction in N_2O production, via nitrification, during the growth of the crop. Once the underlying mechanisms driving the interactions between AMF and N_2O production are better understood, it would be interesting to consider the role of AMF in N_2O production from agricultural systems throughout the annual cycle of land management.

6.7 Conclusions

In recent years, there has been growing evidence suggesting that AMF interact with soil N cycling (Hodge & Fitter, 2010; reviewed by Veresoglou *et al.*, 2012b). The series of microcosm based studies outlined in this thesis provide further evidence of this by demonstrating AMF hyphae can significantly affect the loss of N_2O from soils and organic matter patches. The hyphae of AMF can reduce the loss of N_2O from decomposing organic matter via nitrification (Chapters 3 and 4), whereas the presence of severed AMF hyphae can result in an increase in N_2O loss under conditions that favour denitrification (Chapter 2). In undisturbed systems, the severing of AMF hyphae

will be negligible, and therefore AMF hyphae may have the potential to significantly decrease soil N_2O losses.

Possible interactions between AMF hyphae and N_2O pathways under the specific microcosm conditions used throughout this thesis have been described (Figure 6.1). It is proposed that the uptake of NH_4 by AMF hyphae results in less N_2O produced from nitrification as the nitrifiers are outcompeted in the long term for NH_4 (Chapter 4), whereas a release of C upon severing of AMF hyphae can stimulate N_2O production in the short term via denitrification (Chapter 2). However, future work is needed to elucidate the main mechanisms that are driving the observed increases and decreases in N_2O production in the presence of AMF hyphae. The roles of N and P in these interactions were not clear from the current studies although, for N_2O production in general, N availability appeared more important than P availability in this system (Chapter 5). As the work presented here was microcosm based, further work should also determine the relevance of these findings under field conditions, but the indication is that AMF may have a significant, previously unappreciated role in reducing soil-atmosphere N_2O losses.

Appendix

Appendix 1. Modified nutrient solution after Thornton & Bausenwein (2000). The concentration of NH_4NO_3 and NaH_2PO_4 have been reduced to $1/10^{th}$ of the original concentrations and $(NH_4)_6Mo_7O_{24}.4H_2O$ was added. The concentrations are included for both stock solutions and final concentrations in the solution used on the plants. The salt and concentration from the original are listed on the left, and on the right the salts used in this thesis are included.

Stock No.	Salt	Concentration (mol m^{-3})	Salt used in solution	Concentration in stock solution (g L^{-1})	Final concentration in solution (mg L^{-1})
1	NH_4NO_3	0.15	NH_4NO_3	1.201	12.01
2	$CaCl_2$	2.1	$CaCl_2$	23.31	233.1
3	$MgSO_4$	0.75	$MgSO_4.7H_2O$	18.49	184.85
4	K_2SO_4	0.5	K_2SO_4	8.71	87.129
5	NaH_2PO_4	0.0307	$NaH_2PO_4.2H_2O$	0.48	4.789
6	$FeC_6H_5O_7$	0.01	$FeC_6H_5O_7$	0.245	2.449
7	H_3BO_3	0.05	H_3BO_3	0.31	3.091
	$MnSO_4$	0.0086	$MnSO_4.4H_2O$	0.19	1.918
	$ZnSO_4$	0.002	$ZnSO_4.7H_2O$	0.058	0.575
	$CuSO_4$	0.001	$CuSO_4.5H_2O$	0.025	0.249
	$(NH_4)_6Mo_7O_{24}.4H_2O$	0.0000712	$(NH_4)_6Mo_7O_{24}.4H_2O$	0.0088	0.088

List of References

Abbasi MK, Adams WA. **1999.** Assessment of the contribution of denitrification to N losses from compacted grassland soil by NO_3^- disappearance and N_2O production during anaerobic incubation. *Canadian Journal of Soil Science* **79:** 57-64.

Abbasi MK, Adams WA. **2000.** Gaseous N emission during simultaneous nitrification-denitrification associated with mineral N fertilization to a grassland soil under field conditions. *Soil Biology and Biochemistry* **32:** 1251-1259.

Adair K, Schwartz E. **2011.** Stable isotope probing with ^{18}O-water to investigate growth and mortality of ammonia oxidizing bacteria and archaea in soil. *Methods in Enzymology* **486:** 155-169.

Albanito F, Saunders M, Jones MB. **2009.** Automated diffusion chambers to monitor diurnal and seasonal dynamics of the soil CO_2 concentration profile. *European Journal of Soil Science* **60:** 507-514.

Albertsen A, Ravnskov S, Green H, Jensen DF, Larsen J. **2006.** Interactions between the external mycelium of the mycorrhizal fungus *Glomus intraradices* and other soil microorganisms as affected by organic matter. *Soil Biology and Biochemistry* **38:** 1008-1014.

Aleklett K, Wallander H. **2012.** Effects of organic amendments with various nitrogen levels on arbuscular mycorrhizal fungal growth. *Applied Soil Ecology* **60:** 71-76.

Allen SE. **1974.** *Chemical analysis of ecological materials*. Oxford, UK: Blackwell Scientific Publications.

Alphei J, Bonkowski M, Scheu S. **1996.** Protozoa, Nematoda and Lumbricidae in the rhizosphere of *Hordelymus europaeus* (Poaceae): faunal interactions, response of microorganisms and effects on plant growth. *Oecologia* **106:** 111-126.

Alsaadawi IS, Al-Uqaili JK, Alrubeaa AJ, Al-Hadithy SM. **1986.** Allelopathic suppression of weed and nitrification by selected cultivars of *Sorghum bicolor* (L.) moench. *Journal of chemical ecology* **12:** 209-219.

Ames RN, Reid CPP, Porter LK, Cambardella C. **1983.** Hyphal uptake and transport of nitrogen from two ^{15}N-labelled sources by *Glomus mosseae*, a vesicular-arbuscular mycorrhizal fungus. *New Phytologist* **95:** 381-396.

Amora-Lazcano E, Vázquez MM, Azcón R. **1998.** Response of nitrogen-transforming microorganisms to arbuscular mycorrhizal fungi. *Biology and Fertility of Soils* **27:** 65-70.

Anderson-Teixeira KJ, DeLucia EH. **2011.** The greenhouse gas value of ecosystems. *Global Change Biology* **17:** 425-438.

Andrade G, Mihara KL, Linderman RG, Bethlenfalvay GJ. **1998.** Soil aggregation status and rhizobacteria in the mycorrhizosphere. *Plant and Soil* **202:** 89-96.

Angel R, Claus P, Conrad R. **2012.** Methanogenic archaea are globally ubiquitous in aerated soils and become active under wet anoxic conditions. *The ISME journal* **6:** 847-862.

Antil RS, Sharma T, Inubushi K. **2013.** Laboratory and greenhouse assessment of plant-available N in organic materials. *Archives of Agronomy and Soil Science* **59:** 411-422.

Arnaout CL, Gunsch CK. **2012.** Impacts of silver nanoparticle coating on the nitrification potential of *Nitrosomonas europaea*. *Environmental Science and Technology* **46:** 5387-5395.

Aronson EL, Helliker BR. **2010.** Methane flux in non-wetland soils in response to nitrogen addition: a meta-analysis. *Ecology* **91:** 3242-3251.

Artursson V, Finlay RD, Jansson JK. **2006.** Interactions between arbuscular mycorrhizal fungi and bacteria and their potential for stimulating plant growth. *Environmental Microbiology* **8:** 1-10.

Asghari HR, Cavagnaro TR. **2011.** Arbuscular mycorrhizas enhance plant interception of leached nutrients. *Functional Plant Biology* **38:** 219-226.

Asghari HR, Cavagnaro TR. **2012.** Arbuscular mycorrhizas reduce nitrogen loss via leaching. *PLoS ONE* **7:** e29825.

Attard E, Recous S, Chabbi A, De Berranger C, Guillaumaud N, Labreuches J, Philippot L, Schmid B, Le Roux X. **2011.** Soil environmental conditions rather than denitrifier abundance and diversity drive potential denitrification after changes in land uses. *Global Change Biology* **17:** 1975-1989.

Atul-Nayyar A, Hamel C, Hanson K, Germida J. **2009.** The arbuscular mycorrhizal symbiosis links N mineralization to plant demand. *Mycorrhiza* **19:** 239-246.

Augé RM. **2001.** Water relations, drought and vesicular-arbuscular mycorrhizal symbiosis. *Mycorrhiza* **11:** 3-42.

Augé RM, Stodola AJW, Tims JE, Saxton AM. **2001.** Moisture retention properties of a mycorrhizal soil. *Plant and Soil* **230:** 87-97.

Auken OWV, Manwaring JH, Caldwell MM. **1992.** Effectiveness of phosphate acquisition by juvenile cold-desert perennials from different patterns of fertile-soil microsites. *Oecologia* **91:** 1-6.

Azam F, Gill S, Farooq S, Lodhi A. **2004.** Effect of CO_2 on nitrification and immobilization of NH_4^+-N. *Biology and Fertility of Soils* **40:** 427-431.

Azcon R, Barea JM, Hayman DS. **1976.** Utilization of rock phosphate in alkaline soils by plants inoculated with mycorrhizal fungi and phosphate-solubilizing bacteria. *Soil Biology and Biochemistry* **8:** 135-138.

Baggs EM. **2008.** A review of stable isotope techniques for N_2O source partitioning in soils: recent progress, remaining challenges and future considerations. *Rapid Communications in Mass Spectrometry* **22:** 1664-1672.

Baggs EM. **2011.** Soil microbial sources of nitrous oxide: recent advances in knowledge, emerging challenges and future direction. *Current Opinion in Environmental Sustainability* **3:** 321-327.

Baggs EM, Blum H. **2004.** CH_4 oxidation and emissions of CH_4 and N_2O from *Lolium perenne* swards under elevated atmospheric CO_2. *Soil Biology and Biochemistry* **36:** 713-723.

Baggs EM, Chebii J, Ndufa JK. **2006.** A short-term investigation of trace gas emissions following tillage and no-tillage of agroforestry residues in western Kenya. *Soil and Tillage Research* **90:** 69-76.

Baggs EM, Philippot L. **2010.** Microbial terrestrial pathways to nitrous oxide. In: Smith KA, ed. *Nitrous oxide and climate change*. London, UK: Earthscan Ltd., 4-35.

Baggs EM, Richter M, Cadisch G, Hartwig UA. **2003a.** Denitrification in grass swards is increased under elevated atmospheric CO_2. *Soil Biology and Biochemistry* **35:** 729-732.

Baggs EM, Smales CL, Bateman EJ. **2010.** Changing pH shifts the microbial source as well as the magnitude of N_2O emission from soil. *Biology and Fertility of Soils* **46:** 793-805.

Baggs EM, Stevenson M, Pihlatie M, Regar A, Cook H, Cadisch G. **2003b.** Nitrous oxide emissions following application of residues and fertiliser under zero and conventional tillage. *Plant and Soil* **254:** 361-370.

Bago B, Azcón-Aguilar C. **1997.** Changes in the rhizospheric pH induced by arbuscular mycorrhiza formation in onion (*Allium cepa* L.). *Zeitschrift für Pflanzenernährung und Bodenkunde* **160:** 333-339.

Bago B, Vierheilig H, Piché Y, Azcón-Aguilar C. **1996.** Nitrate depletion and pH changes induced by the extraradical mycelium of the arbuscular mycorrhizal fungus *Glomus intraradices* grown in monoxenic culture. *New Phytologist* **133:** 273-280.

Bago B, Pfeffer PE, Shachar-Hill Y. **2000.** Carbon metabolism and transport in arbuscular mycorrhizas. *Plant Physiology* **124:** 949-958.

Bagshaw R, Vaidyana LV, Nye PH. **1972.** Supply of nutrient ions by diffusion to plant roots in soil .5. Direct determination of labile phosphate concentration gradients in a sandy soil induced by plant uptake. *Plant and Soil* **37:** 617-626.

Bansal M, Mukerji KG. **1994.** Positive correlation between VAM-induced changes in root exudation and mycorrhizosphere mycoflora. *Mycorrhiza* **5:** 39-44.

Barber SA. **1995.** *Soil nutrient bioavailability: a mechanistic approach*. New York, USA: John Wiley.

Barrett G, Campbell CD, Fitter AH, Hodge A. **2011.** The arbuscular mycorrhizal fungus *Glomus hoi* can capture and transfer nitrogen from organic patches to its associated host plant at low temperature. *Applied Soil Ecology* **48:** 102-105.

Bateman EJ, Baggs EM. **2005.** Contributions of nitrification and denitrification to N_2O emissions from soils at different water-filled pore space. *Biology and Fertility of Soils* **41:** 379-388.

Beheydt D, Boeckx P, Ahmed HP, Van Cleemput O. **2008.** N_2O emission from conventional and minimum-tilled soils. *Biology and Fertility of Soils* **44:** 863-873.

Belser LW, Schmidt EL. **1980.** Growth and oxidation kinetics of three genera of ammonia oxidising nitrifiers. *FEMS Microbiology Letters* **7:** 213-216.

Bender M, Conrad R. **1992.** Kinetics of CH_4 oxidation in oxic soils exposed to ambient air or high CH_4 mixing ratios. *FEMS Microbiology Ecology* **101:** 261-270.

Bender M, Conrad R. **1995.** Effect of CH_4 concentrations and soil conditions on the induction of CH_4 oxidation activity. *Soil Biology and Biochemistry* **27:** 1517-1527.

Bending GD. **2003.** Litter decomposition, ectomycorrhizal roots and the 'Gadgil' effect. *New Phytologist* **158:** 228-229.

Bengtsson G, Fronæus S, Bengtsson-Kloo L. **2002.** The kinetics and mechanism of oxidation of hydroxylamine by iron(iii). *Journal of the Chemical Society, Dalton Transactions* 2548-2552.

Bergstermann A, Cárdenas L, Bol R, Gilliam L, Goulding K, Meijide A, Scholefield D, Vallejo A, Well R. **2011.** Effect of antecedent soil moisture conditions on emissions and isotopologue distribution of N_2O during denitrification. *Soil Biology and Biochemistry* **43:** 240-250.

Bergstrom DW, Tenuta M, Beauchamp EG. **2001.** Nitrous oxide production and flux from soil under sod following application of different nitrogen fertilizers. *Communications in Soil Science and Plant Analysis* **32:** 553-570.

Bianciotto V, Bonfante P. **2002.** Arbuscular mycorrhizal fungi: a specialised niche for rhizospheric and endocellular bacteria. *Antonie Van Leeuwenhoek* **81:** 365-371.

Bianciotto V, Lumini E, Lanfranco L, Minerdi D, Bonfante P, Perotto S. **2000.** Detection and identification of bacterial endosymbionts in arbuscular mycorrhizal fungi belonging to the family Gigasporaceae. *Applied and Environmental Microbiology* **66:** 4503-4509.

Blanke V, Renker C, Wagner M, Füllner K, Held M, Kuhn AJ, Buscot F. **2005.** Nitrogen supply affects arbuscular mycorrhizal colonization of *Artemisia vulgaris* in a phosphate-polluted field site. *New Phytologist* **166:** 981-992.

Blanke V, Wagner M, Renker C, Lippert H, Michulitz M, Kuhn AJ, Buscot F. **2011.** Arbuscular mycorrhizas in phosphate-polluted soil: interrelations between root colonization and nitrogen. *Plant and Soil* **343:** 379-392.

Blasing TJ. 2013. *Recent greenhouse gas concentrations.* Carbon dioxide information and analysis centre, Oak Ridge National Laboratory. [WWW document] URL http://cdiac.ornl.gov/pns/current_ghg.html [accessed 5 September 2013] doi: 10.3334/CDIAC/atg.032.

Bollmann A, Bär-Gilissen MJ, Laanbroek HJ. **2002.** Growth at low ammonium concentrations and starvation response as potential factors involved in niche differentiation among ammonia-oxidizing bacteria. *Applied and Environmental Microbiology* **68:** 4751-4757.

Bollmann A, Conrad R. **1998.** Influence of O_2 availability on NO and N_2O release by nitrification and denitrification in soils. *Global Change Biology* **4:** 387-396.

Bonfante P, Anca IA. **2009.** Plants, mycorrhizal fungi, and bacteria: A network of interactions. *Annual Review of Microbiology* **63:** 363-383.

Bonkowski M. **2004.** Protozoa and plant growth: the microbial loop in soil revisited. *New Phytologist* **162:** 617-631.

Bonkowski M, Griffiths B, Scrimgeour C. **2000.** Substrate heterogeneity and microfauna in soil organic 'hotspots' as determinants of nitrogen capture and growth of ryegrass. *Applied Soil Ecology* **14:** 37-53.

Boomsma CR, Vyn TJ. **2008.** Maize drought tolerance: Potential improvements through arbuscular mycorrhizal symbiosis? *Field Crops Research* **108:** 14-31.

Booth MS, Stark JM, Rastetter E. **2005.** Controls on nitrogen cycling in terrestrial ecosystems: a synthetic analysis of literature data. *Ecological Monographs* **75:** 139-157.

Bouwman L, Stehfest E, van Kessel C. **2010.** Nitrous oxide emissions from the nitrogen cycle in arable agriculture: estimation and mitigation. In: Smith KA, ed. *Nitrous oxide and climate change*. London, UK: Earthscan Ltd., 85-106.

Bowden RD, Newkirk KM, Rullo GM. **1998.** Carbon dioxide and methane fluxes by a forest soil under laboratory-controlled moisture and temperature conditions. *Soil Biology and Biochemistry* **30:** 1591-1597.

Bremner J. **1997.** Sources of nitrous oxide in soils. *Nutrient Cycling in Agroecosystems* **49:** 7-16.

Brierley EDR, Wood M. **2001.** Heterotrophic nitrification in an acid forest soil: isolation and characterisation of a nitrifying bacterium. *Soil Biology and Biochemistry* **33:** 1403-1409.

Bronick CJ, Lal R. **2005.** Soil structure and management: a review. *Geoderma* **124:** 3-22.

Bryla DR, Eissenstat DM. **2005.** Respiratory costs of mycorrhizal associations. In: Lambers H, Ribas-Carbo M, eds. *Plant Respiration*. Springer Netherlands, 207-224.

Bull ID, Parekh NR, Hall GH, Ineson P, Evershed RP. **2000.** Detection and classification of atmospheric methane oxidizing bacteria in soil. *Nature* **405:** 175-178.

Butterbach-Bahl K, Baggs EM, Dannenmann M, Kiese R, Zechmeister-Boltenstern S. **2013.** Nitrous oxide emissions from soils: how well do we understand the processes and their controls? *Philosophical Transactions of the Royal Society B: Biological Sciences* **368:** 20130122.

Cabello P, Roldán MD, Moreno-Vivián C. **2004.** Nitrate reduction and the nitrogen cycle in archaea. *Microbiology* **150:** 3527-3546.

Camenzind T, Rillig MC. **2013.** Extraradical arbuscular mycorrhizal fungal hyphae in an organic tropical montane forest soil. *Soil Biology and Biochemistry* **64:** 96-102.

Cameron DD, Neal AL, van Wees SCM, Ton J. **2013.** Mycorrhiza-induced resistance: more than the sum of its parts? *Trends in Plant Science*. doi: 10.1016/j.tplants.2013.06.004.

Canfield DE, Glazer AN, Falkowski PG. **2010.** The evolution and future of Earth's nitrogen cycle. *Science* **330:** 192-196.

Caris C, Hördt W, Hawkins HJ, Römheld V, George E. **1998.** Studies of iron transport by arbuscular mycorrhizal hyphae from soil to peanut and sorghum plants. *Mycorrhiza* **8:** 35-39.

Castro MS, Melillo JM, Steudler PA, Chapman JW. **1994.** Soil moisture as a predictor of methane uptake by temperate forest soils. *Canadian Journal of Forest Research* **24:** 1805-1810.

Cavagnaro TR, Barrios-Masias FH, Jackson LE. **2012.** Arbuscular mycorrhizas and their role in plant growth, nitrogen interception and soil gas efflux in an organic production system. *Plant and Soil* **353:** 181-194.

Cavagnaro TR, Jackson LE, Scow KM, Hristova KR. **2007.** Effects of arbuscular mycorrhizas on ammonia oxidizing bacteria in an organic farm soil. *Microbial Ecology* **54:** 618-626.

Cavagnaro TR, Smith FA, Smith SE, Jakobsen I. **2005.** Functional diversity in arbuscular mycorrhizas: exploitation of soil patches with different phosphate enrichment differs among fungal species. *Plant, Cell and Environment* **28:** 642-650.

Cavagnaro TR. **2008.** The role of arbuscular mycorrhizas in improving plant zinc nutrition under low soil zinc concentrations: a review. *Plant and Soil* **304:** 315-325.

Cavagnaro TR, Langley AJ, Jackson LE, Smukler SM, Koch GW. **2008.** Growth, nutrition, and soil respiration of a mycorrhiza-defective tomato mutant and its mycorrhizal wild-type progenitor. *Functional Plant Biology* **35:** 228-235.

Chan ASK, Parkin TB. **2001.** Effect of land use on methane flux from soil. *Journal of Environmental Quality* **30:** 786-797.

Chen J, Strous M. **2013.** Denitrification and aerobic respiration, hybrid electron transport chains and co-evolution. *Biochimica et Biophysica Acta (BBA) - Bioenergetics* **1827:** 136-144.

Chèneby, Philippot L, Hartmann A, Hénault C, Germon JC. **2000.** 16S rDNA analysis for characterization of denitrifying bacteria isolated from three agricultural soils. *FEMS Microbiology Ecology* **34:** 121-128.

Cheng L, Booker FL, Tu C, Burkey KO, Zhou L, Shew HD, Rufty TW, Hu S. **2012.** Arbuscular mycorrhizal fungi increase organic carbon decomposition under elevated CO_2. *Science* **337:** 1084-1087.

Cheng WEUX, Kuzyakov Y. **2005.** Root effects on soil organic matter decomposition. In: Zobel RW, Wright SF, eds. Roots and soil management: interactions between roots and the soil. Agronomy Monograph No. 48. Wisconsin, USA: American Society of Agronomy, Inc. 119-143.

Cheng X, Baumgartner K. **2006.** Effects of mycorrhizal roots and extraradical hyphae on ^{15}N uptake from vineyard cover crop litter and the soil microbial community. *Soil Biology and Biochemistry* **38:** 2665-2675.

Cheng Y, Wang J, Mary B, Zhang JB, Cai ZC, Chang SX. **2013.** Soil pH has contrasting effects on gross and net nitrogen mineralizations in adjacent forest and grassland soils in central Alberta, Canada. *Soil Biology and Biochemistry* **57:** 848-857.

Chirinda N, Kracher D, Lægdsmand M, Porter JR, Olesen JE, Petersen BM, Doltra J, Kiese R, Butterbach-Bahl K. **2011.** Simulating soil N_2O emissions and heterotrophic CO_2 respiration in arable systems using FASSET and MoBiLE-DNDC. *Plant and Soil* **343:** 139-160.

Christensen H, Jakobsen I. **1993.** Reduction of bacterial growth by a vesicular-arbuscular mycorrhizal fungus in the rhizosphere of cucumber (*Cucumis-Sativus* L.). *Biology and Fertility of Soils* **15:** 253-258.

Clarholm M. **1985.** Interactions of bacteria, protozoa and plants leading to mineralization of soil-nitrogen. *Soil Biology and Biochemistry* **17:** 181-187.

Claus G, Kutzner HJ. **1985.** Physiology and kinetics of autotrophic denitrification by *Thiobacillus denitrificans*. *Applied Microbiology and Biotechnology* **22:** 283-288.

Conrad R, Rothfuss F. **1991.** Methane oxidation in the soil surface layer of a flooded rice field and the effect of ammonium. *Biology and Fertility of Soils* **12:** 28-32.

Conrad R. **2009.** The global methane cycle: recent advances in understanding the microbial processes involved. *Environmental Microbiology Reports* **1:** 285-292.

Cotrufo MF, Ineson P, Rowland AP. **1994.** Decomposition of tree leaf litters grown under elevated CO_2: effect of litter quality. *Plant and Soil* **163:** 121-130.

Cox PM, Betts RA, Jones CD, Spall SA, Totterdell IJ. **2000.** Acceleration of global warming due to carbon-cycle feedbacks in a coupled climate model. *Nature* **408:** 184-187.

Cruz AF, Ishii T. **2012.** Arbuscular mycorrhizal fungal spores host bacteria that affect nutrient biodynamics and biocontrol of soil-borne plant pathogens. *Biology Open* **1:** 52-57.

Cruz C, Egsgaard H, Trujillo C, Ambus P, Requena N, Martins-Loução MA, Jakobsen I. **2007.** Enzymatic evidence for the key role of arginine in nitrogen translocation by arbuscular mycorrhizal fungi. *Plant Physiology* **144:** 782-792.

Čuhel J, Šimek M, Laughlin RJ, Bru D, Chèneby D, Watson CJ, Philippot L. **2010.** Insights into the effect of soil pH on N_2O and N_2 emissions and denitrifier community size and activity. *Applied and Environmental Microbiology* **76:** 1870-1878.

Cui MUYI, Caldwell MM. **1996.** Facilitation of plant phosphate acquisition by arbuscular mycotrhizas from enriched soil patches. I. Roots and hyphae exploring the same soil volume. *New Phytologist* **133:** 453-460.

Cui MUYI, Caldwell MM. **1996.** Facilitation of plant phosphate acquisition by arbuscular mycorrhizas from enriched soil patches. II. Hyphae exploiting root-free soil. *New Phytologist* **133:** 461-467.

Curtin D, Beare MH, Hernandez-Ramirez G. **2012.** Temperature and moisture effects on microbial biomass and soil organic matter mineralization. *Soil Science Society of America Journal* **76:** 2055-2067.

Dandie CE, Burton DL, Zebarth BJ, Henderson SL, Trevors JT, Goyer C. **2008.** Changes in bacterial denitrifier community abundance over time in an agricultural field and their relationship with denitrification activity. *Applied and Environmental Microbiology* **74:** 5997-6005.

De Boer W, Kowalchuk GA. **2001.** Nitrification in acid soils: Micro-organisms and mechanisms. *Soil Biology and Biochemistry* **33:** 853-866.

de Graaff MA, Classen AT, Castro HF, Schadt CW. **2010.** Labile soil carbon inputs mediate the soil microbial community composition and plant residue decomposition rates. *New Phytologist* **188:** 1055-1064.

de Vet WWJM, van Loosdrecht MCM, Rietveld LC. **2012.** Phosphorus limitation in nitrifying groundwater filters. *Water Research* **46:** 1061-1069.

DeSutter TM, Sauer TJ, Parkin TB. **2006.** Porous tubing for use in monitoring soil CO_2 concentrations. *Soil Biology and Biochemistry* **38:** 2676-2681.

Di HJ, Cameron KC, Shen JP, Winefield CS, O'Callaghan M, Bowatte S, He JZ. **2010.** Ammonia-oxidizing bacteria and archaea grow under contrasting soil nitrogen conditions. *FEMS Microbiology Ecology* **72:** 386-394.

Ding X, Fu L, Liu C, Chen F, Hoffland E, Shen J, Zhang F, Feng G. **2011.** Positive feedback between acidification and organic phosphate mineralization in the rhizosphere of maize (*Zea mays* L.). *Plant and Soil* **349:** 13-24.

Dobbie KE, Smith KA. **1996.** Comparison of CH_4 oxidation rates in woodland, arable and set aside soils. *Soil Biology and Biochemistry* **28:** 1357-1365.

Drake JM, Kramer AM. **2012.** Mechanistic analogy: how microcosms explain nature. *Theoretical Ecology* **5:** 433-444.

Drew EA, Murray RS, Smith SE, Jakobsen I. **2003.** Beyond the rhizosphere: growth and function of arbuscular mycorrhizal external hyphae in sands of varying pore sizes. *Plant and Soil* **251:** 105-114.

Drigo B, Piji AS, Duyts H, Kielak AM, Gamper HA, Houtekamer MJ, Boschker HTS, Bodelier PLE, Whiteley AS, van Veen JA, Kowalchuk GA. 2010. Shifting carbon flow from roots into associated microbial communities in response to elevated atmospheric CO_2. *Proceedings of the National Academy of Sciences of the United States of America* **107:** 10938-10942.

Drigo B, van Veen JA, Kowalchuk GA. 2009. Specific rhizosphere bacterial and fungal groups respond differently to elevated atmospheric CO_2. *The ISME Journal* **3:** 1204-1217.

Driver JD, Holben WE, Rillig MC. **2005.** Characterization of glomalin as a hyphal wall component of arbuscular mycorrhizal fungi. *Soil Biology and Biochemistry* **37:** 101-106.

Duckmanton L, Widden P. **1994.** Effect of ozone on the development of vesicular-arbuscular mycorrhizae in sugar maple saplings. *Mycologia* **86:** 181-186.

Duke SE, Caldwell MM. **2000.** Phosphate uptake kinetics of *Artemisia tridentata* roots exposed to multiple soil enriched-nutrient patches. *FLORA* **195:** 154-163.

Duxbury JM, Bouldin DR, Terry RE, Tate RL. **1982.** Emissions of nitrous oxide from soils. *Nature* **298:** 462-464.

Eiland F, Klamer M, Lind AM, Leth M, Bååth E. **2001.** Influence of initial C/N ratio on chemical and microbial composition during long term composting of straw. *Microbial Ecology* **41:** 272-280.

Enríquez S, Duarte CM, Sand-Jensen K. **1993.** Patterns in decomposition rates among photosynthetic organisms: the importance of detritus C:N:P content. *Oecologia* **94:** 457-471.

Enwall K, Throbäck IN, Stenberg M, Söderström M, Hallin S. **2010.** Soil resources influence spatial patterns of denitrifying communities at scales compatible with land management. *Applied and Environmental Microbiology* **76:** 2243-2250.

Ernfors M, von Arnold K, Stendahl J, Olsson M, Klemedtsson L. **2008.** Nitrous oxide emissions from drained organic forest soils – an up-scaling based on C:N ratios. *Biogeochemistry* **89:** 29-41.

Ernfors M, Rutting T, Klemedtsson L. 2011. Increased nitrous oxide emissions from a drained organic forest soil after exclusion of ectomycorrhizal mycelia. *Plant and Soil* **343:**161-170.

Ettwig KF, Butler MK, Le Paslier D, Pelletier E, Mangenot S, Kuypers MMM, Schreiber F, Dutilh BE, Zedelius J, de Beer D. ***et al.*** **2010.** Nitrite-driven anaerobic methane oxidation by oxygenic bacteria. *Nature* **464:** 543-548.

FAO. **2012.** *The state of food and agriculture*. Rome, Italy: Food and Agriculture Organisation of the United Nations (FAO).

Farzaneh M, Vierheilig H, Lössl A, Kaul HP. **2011.** Arbuscular mycorrhiza enhances nutrient uptake in chickpea. *Plant, Soil and Environment* **57:** 465-470.

Fazzolari E, Nicolardot B, Germon JC. **1998.** Simultaneous effects of increasing levels of glucose and oxygen partial pressures on denitrification and dissimilatory nitrate reduction to ammonium in repacked soil cores. *European Journal of Soil Biology* **34:** 47-52.

Fellbaum CR, Gachomo EW, Beesetty Y, Choudhari S, Strahan GD, Pfeffer PE, Kiers ET, Bücking H. **2012.** Carbon availability triggers fungal nitrogen uptake and transport in arbuscular mycorrhizal symbiosis. *Proceedings of the National Academy of Sciences* **109:** 2666-2671.

Feng G, Su Y, Li X, Wang H, Zhang F, Tang C, Rengel Z. **2002.** Histochemical visualization of phosphatase released by arbuscular mycorrhizal fungi in soil. *Journal of Plant Nutrition* **25:** 969-980.

Fester T, Sawers R. **2011.** Progress and challenges in agricultural applications of arbuscular mycorrhizal fungi. *Critical Reviews in Plant Sciences* **30:** 459-470.

Field A, Miles J. **2010.** *Discovering statistics using SAS*. London, UK: SAGE Publications.

Finlay RD. **2008.** Ecological aspects of mycorrhizal symbiosis: with special emphasis on the functional diversity of interactions involving the extraradical mycelium. *Journal of Experimental Botany* **59:** 1115-1126.

Fiorilli V, Lanfranco L, Bonfante P. **2013.** The expression of *GintPT*, the phosphate transporter of *Rhizophagus irregularis*, depends on the symbiotic status and phosphate availability. *Planta* **237:** 1267-1277.

Firestone MK, Davidson EA. **1989.** Microbiological basis of NO and N_2O production and consumption in soil. In: Andreae MO, Schimel DS, eds. *Exchange of trace gases between terrestrial ecosystems and the atmosphere.* Chichester: John Wiley and Sons, 7-21.

Fitter A, Hay RKM. **2002.** *Environmental physiology of plants*. London, UK: Academic Press.

Fitter AH. **2006.** What is the link between carbon and phosphorus fluxes in arbuscular mycorrhizas? A null hypothesis for symbiotic function. *New Phytologist* **172:** 3-6.

Fitter AH, Heinemeyer A, Staddon PL. **2000.** The impact of elevated CO_2 and global climate change on arbuscular mycorrhizas: a mycocentric approach. *New Phytologist* **147:** 179-187.

Fitter AH, Helgason T, Hodge A. **2011.** Nutritional exchanges in the arbuscular mycorrhizal symbiosis: Implications for sustainable agriculture. *Fungal Biology Reviews* **25:** 68-72.

Forster P, Ramaswamy V, Artaxo P, Berntsen T, Betts R, Fahey DW, Haywood J, Lean J, Lowe DC, Myhre G *et al*. 2007. Changes in atmospheric constituents and in radiative forcing. Solomon S, Qin D, Manning M, Chen Z, Marquis M, Averyt KB, Tignor M, Miller HL, eds. Climate change 2007: the physical science basis. Contribution of Working Group I to the fourth assessment report of the Intergovernmental Panel on Climate Change. Cambridge, UK & New York, NY, USA: Cambridge University Press.

Fortin JA, Bécard G, Declerck S, Dalpé Y, St-Arnaud M, Coughlan AP, Piché Y. **2002.** Arbuscular mycorrhiza on root-organ cultures. *Canadian Journal of Botany* **80:** 1-20.

Franzluebbers AJ. **1999.** Microbial activity in response to water-filled pore space of variably eroded southern Piedmont soils. *Applied Soil Ecology* **11:** 91-101.

Friese CF, Allen MF. **1991.** The spread of VA mycorrhizal fungal hyphae in the soil: inoculum types and external hyphal architecture. *Mycologia* **83:** 409-418.

Fritz M, Jakobsen I, Lyngkjær MF, Thordal-Christensen H, Pons-Kühnemann J. **2006.** Arbuscular mycorrhiza reduces susceptibility of tomato to *Alternaria solani*. *Mycorrhiza* **16:** 413-419.

Fuentes M, Hidalgo C, Etchevers J, De León F, Guerrero A, Dendooven L, Verhulst N, Govaerts B. **2012.** Conservation agriculture, increased organic carbon in the top-soil macro-aggregates and reduced soil CO_2 emissions. *Plant and Soil* **355:** 183-197.

Gadgil RL, Gadgil PD. **1971.** Mycorrhiza and litter decomposition. *Nature* **233:** 133.

Gadgil RL, Gadgil PD. **1975.** Suppression of litter decomposition by mycorrhizal roots of *Pinus radiata*. *New Zealand Journal of Forest Science* **5:** 33-41.

Gadkar V, Rillig MC. **2006.** The arbuscular mycorrhizal fungal protein glomalin is a putative homolog of heat shock protein 60. *FEMS Microbiology Letters* **263:** 93-101.

Gange AC, West HM. **1994.** Interactions between arbuscular mycorrhizal fungi and foliar-feeding insects in *Plantago lanceolata* L. *New Phytologist* **128:** 79-87.

Garten CT. **1976.** Correlations between concentrations of elements in plants. *Nature* **261:** 686-688.

Ghignone S, Salvioli A, Anca I, Lumini E, Ortu G, Petiti L, Cruveiller S, Bianciotto V, Piffanelli P, Lanfranco L, Bonfante P. **2012.** The genome of the obligate endobacterium of an AM fungus reveals an interphylum network of nutritional interactions. *The ISME Journal* **6:** 136-145.

Giles M, Morley N, Baggs EM, Daniell TJ. **2013.** Soil nitrate reducing processes - drivers, mechanisms for spatial variation, and significance for nitrous oxide production. *Frontiers in Microbiology* **3:** 1-16.

Gillam KM, Zebarth BJ, Burton DL. **2008.** Nitrous oxide emissions from denitrification and the partitioning of gaseous losses as affected by nitrate and carbon addition and soil aeration. *Canadian Journal of Soil Science* **88:** 133-143.

Godbold DL, Hoosbeek MR, Lukac M, Cotrufo MF, Janssens IA, Ceulemans R, Polle A, Velthorst EJ, Scarascia-Mugnozza G, De Angelis P *et al*. 2006. Mycorrhizal hyphal turnover as a dominant process for carbon input into soil organic matter. *Plant and Soil* **281:** 15-24.

Gödde M, Conrad R. **2000.** Influence of soil properties on the turnover of nitric oxide and nitrous oxide by nitrification and denitrification at constant temperature and moisture. *Biology and Fertility of Soils* **32:** 120-128.

Godfray HCJ, Beddington JR, Crute IR, Haddad L, Lawrence D, Muir JF, Pretty J, Robinson S, Thomas SM, Toulmin C. **2010.** Food security: the challenge of feeding 9 billion people. *Science* **327:** 812-818.

Göhre V, Paszkowski U. **2006.** Contribution of the arbuscular mycorrhizal symbiosis to heavy metal phytoremediation. *Planta* **223:** 1115-1122.

Gosling P, Hodge A, Goodlass G, Bending GD. **2006.** Arbuscular mycorrhizal fungi and organic farming. *Agriculture Ecosystems and Environment* **113:** 17-35.

Govindarajulu M, Pfeffer PE, Jin HR, Abubaker J, Douds DD, Allen JW, Bücking H, Lammers PJ, Shachar-Hill Y. **2005.** Nitrogen transfer in the arbuscular mycorrhizal symbiosis. *Nature* **435:** 819-823.

Grace C, Stribley DP. **1991.** A safer procedure for routine staining of vesicular-arbuscular mycorrhizal fungi. *Mycological Research* **95:** 1160-1162.

Groffman PM, Altabet MA, Böhlke JK, Butterbach-Bahl K, David MB, Firestone MK, Giblin AE, Kana TM, Nielsen LP, Voytek MA. **2006.** Methods for measuring denitrification: diverse approaches to a difficult problem. *Ecological Applications* **16:** 2091-2122.

Guo Y, George E, Marschner H. **1996.** Contribution of an arbuscular mycorrhizal fungus to the uptake of cadmium and nickel in bean and maize plants. *Plant and Soil* **184:** 195-205.

Gupta V, Smemo KA, Yavitt JB, Fowle D, Branfireun B, Basiliko N. **2013.** Stable isotopes reveal widespread anaerobic methane oxidation across latitude and peatland type. *Environmental Science and Technology* **47**: 8273-8279.

Hall SJ, Matson PA. **1999.** Nitrogen oxide emissions after nitrogen additions in tropical forests. *Nature* **400:** 152-155.

Hamel C. **2004.** Impact of arbuscular mycorrhizal fungi on N and P cycling in the root zone. *Canadian Journal of Soil Science* **84:** 383-395.

Harrison MJ, van Buuren ML. **1995.** A phosphate transporter from the mycorrhizal fungus *Glomus versiforme*. *Nature* **378**: 626-629.

Hart MM, Trevors JT. **2005.** Microbe management: application of mycorrhyzal fungi in sustainable agriculture. *Frontiers in Ecology and the Environment* **3:** 533-539.

Hart MM, Reader RJ. **2002.** Does percent root length colonization and soil hyphal length reflect the extent of colonization for all AMF? *Mycorrhiza* **12:** 297-301.

Hawkes CV, Hartley IP, Ineson P, Fitter AH. **2008.** Soil temperature affects carbon allocation within arbuscular mycorrhizal networks and carbon transport from plant to fungus. *Global Change Biology* **14:** 1181-1190.

Hawkins HJ, Johansen A, George E. **2000.** Uptake and transport of organic and inorganic nitrogen by arbuscular mycorrhizal fungi. *Plant and Soil* **226:** 275-285.

Heincke M, Kaupenjohann M. **1999.** Effects of soil solution on the dynamics of N_2O emissions: a review. *Nutrient Cycling in Agroecosystems* **55:** 133-157.

Heinemeyer A, Gruber V, Bahn M. **2012a.** The 'Gas-Snake': Design and validation of a versatile membrane-based gas flux measurement system in a grassland soil respiration study. *Agricultural and Forest Meteorology* **154-155:** 166-173.

Heinemeyer A, Hartley IP, Evans SP, De la Fuente JAC, Ineson P. **2007.** Forest soil CO_2 flux: uncovering the contribution and environmental responses of ectomycorrhizas. *Global Change Biology* **13:** 1786-1797.

Heinemeyer A, Ineson P, Ostle N, Fitter AH. **2006.** Respiration of the external mycelium in the arbuscular mycorrhizal symbiosis shows strong dependence on recent photosynthates and acclimation to temperature. *New Phytologist* **171:** 159-170.

Heinemeyer A, McNamara NP. **2011.** Comparing the closed static versus the closed dynamic chamber flux methodology: Implications for soil respiration studies. *Plant and Soil* **346:** 145-151.

Heinemeyer A, Tortorella D, Petrovičová B, Gelsomino A. **2012b.** Partitioning of soil CO_2 flux components in a temperate grassland ecosystem. *European Journal of Soil Science* **63:** 249-260.

Helgason T, Daniell TJ, Husband R, Fitter AH, Young JPW. **1998.** Ploughing up the wood-wide web? *Nature* **394:** 431.

Henry S, Texier S, Hallet S, Bru D, Dambreville C, Chèneby D, Bizouard F, Germon JC, Philippot L. **2008.** Disentangling the rhizosphere effect on nitrate reducers and denitrifiers: insight into the role of root exudates. *Environmental Microbiology* **10:** 3082-3092.

Herman DJ, Firestone MK, Nuccio E, Hodge A. **2012.** Interactions between an arbuscular mycorrhizal fungus and a soil microbial community mediating litter decomposition. *FEMS Microbiology Ecology* **80:** 236-247.

Herold MB, Baggs EM, Daniell TJ. **2012.** Fungal and bacterial denitrification are differently affected by long-term pH amendment and cultivation of arable soil. *Soil Biology and Biochemistry* **54:** 25-35.

Hijikata N, Murase M, Tani C, Ohtomo R, Osaki M, Ezawa T. **2010.** Polyphosphate has a central role in the rapid and massive accumulation of phosphorus in extraradical mycelium of an arbuscular mycorrhizal fungus. *New Phytologist* **186:** 285-289.

Hiltbrunner D, Zimmermann S, Karbin S, Hagedorn F, Niklaus PA. **2012.** Increasing soil methane sink along a 120-year afforestation chronosequence is driven by soil moisture. *Global Change Biology* **18:** 3664-3671.

Hino T, Matsumoto Y, Nagano S, Sugimoto H, Fukumori Y, Murata T, Iwata S, Shiro Y. **2010.** Structural basis of biological N_2O generation by bacterial nitric oxide reductase. *Science* **330:** 1666-1670.

Hodge A. **2001.** Arbuscular mycorrhizal fungi influence decomposition of, but not plant nutrient capture from, glycine patches in soil. *New Phytologist* **151:** 725-734.

Hodge A, Campbell CD, Fitter AH. **2001.** An arbuscular mycorrhizal fungus accelerates decomposition and acquires nitrogen directly from organic material. *Nature* **413:** 297-299.

Hodge A, Fitter AH. **2010.** Substantial nitrogen acquisition by arbuscular mycorrhizal fungi from organic material has implications for N cycling. *Proceedings of the National Academy of Sciences of the United States of America* **107:** 13754-13759.

Hodge A, Helgason T, Fitter AH. **2010.** Nutritional ecology of arbuscular mycorrhizal fungi. *Fungal Ecology* **3:** 267-273.

Hodge A, Robinson D, Griffiths BS, Fitter AH. **1999.** Nitrogen capture by plants grown in N-rich organic patches of contrasting size and strength. *Journal of Experimental Botany* **50:** 1243-1252.

Hodge A, Robinson D, Fitter A. **2000.** Are microorganisms more effective than plants at competing for nitrogen? *Trends in Plant Science* **5:** 304-308.

Hooper AB, Terry KR. **1979.** Hydroxylamine oxidoreductase of *Nitrosomonas*: Production of nitric oxide from hydroxylamine. *Biochimica et Biophysica Acta (BBA) - Enzymology* **571:** 12-20.

Horn R, Smucker A. **2005.** Structure formation and its consequences for gas and water transport in unsaturated arable and forest soils. *Soil and Tillage Research* **82:** 5-14.

Hu ZB, Du M. **2006.** Hairy root and its application in plant genetic engineering. *Journal of Integrative Plant Biology* **48:** 121-127.

Huang Y, Zou J, Zheng X, Wang Y, Xu X. **2004.** Nitrous oxide emissions as influenced by amendment of plant residues with different C:N ratios. *Soil Biology and Biochemistry* **36:** 973-981.

Hütsch BW. **1996.** Methane oxidation in soils of two long-term fertilization experiments in Germany. *Soil Biology and Biochemistry* **28:** 773-782.

Islas-Lima S, Thalasso F, Gómez-Hernandez J. **2004.** Evidence of anoxic methane oxidation coupled to denitrification. *Water Research* **38:** 13-16.

Jacinthe PA, Dick WA. **1996.** Use of silicone tubing to sample nitrous oxide in the soil atmosphere. *Soil Biology and Biochemistry* **28:** 721-726.

Jackson RB, Caldwell MM. **1993.** Geostatistical patterns of soil heterogeneity around individual perennial plants. *Journal of Ecology* **81:** 683-692.

Jakobsen I, Abbott LK, Robson AD. **1992.** External hyphae of vesicular-arbuscular mycorrhizal fungi associated with *Trifolium subterraneum* L. 1. Spread of hyphae and phosphorus inflow into roots. *New Phytologist* **120:** 371-380.

Jakobsen I, Rosendahl L. **1990.** Carbon flow into soil and external hyphae from roots of mycorrhizal cucumber plants. *New Phytologist* **115:** 77-83.

Janssen BH. **1996.** Nitrogen mineralization in relation to C:N ratio and decomposability of organic materials. *Plant and Soil* **181:** 39-45.

Javot H, Penmetsa RV, Terzaghi N, Cook DR, Harrison MJ. **2007.** A *Medicago truncatula* phosphate transporter indispensable for the arbuscular mycorrhizal symbiosis. *Proceedings of the National Academy of Sciences of the United States of America* **104:** 1720-1725.

Jiang QQ, Bakken LR. **1999.** Nitrous oxide production and methane oxidation by different ammonia-oxidizing bacteria. *Applied and Environmental Microbiology* **65:** 2679-2684.

Jin H, Pfeffer PE, Douds DD, Piotrowski E, Lammers PJ, Shachar-Hill Y. **2005.** The uptake, metabolism, transport and transfer of nitrogen in an arbuscular mycorrhizal symbiosis. *New Phytologist* **168:** 687-696.

Johansen A, Jakobsen I, Jensen ES. **1992.** Hyphal transport of ^{15}N-labeled nitrogen by a vesicular-arbuscular mycorrhizal fungus and its effect on depletion of inorganic soil-N. *New Phytologist* **122:** 281-288.

Johansson JF, Paul LR, Finlay RD. **2004.** Microbial interactions in the mycorrhizosphere and their significance for sustainable agriculture. *FEMS Microbiology Ecology* **48:** 1-13.

Johnson D, Leake JR, Ostle N, Ineson P, Read DJ. **2002a.** *In situ* $^{13}CO_2$ pulse-labelling of upland grassland demonstrates a rapid pathway of carbon flux from arbuscular mycorrhizal mycelia to the soil. *New Phytologist* **153:** 327-334.

Johnson D, Leake JR, Read DJ. **2001.** Novel in-growth core system enables functional studies of grassland mycorrhizal mycelial networks. *New Phytologist* **152:** 555-562.

Johnson D, Leake JR, Read DJ. **2002b.** Transfer of recent photosynthate into mycorrhizal mycelium of an upland grassland: short-term respiratory losses and accumulation of ^{14}C. *Soil Biology and Biochemistry* **34:** 1521-1524.

Joner EJ, Jakobsen I. **1995.** Growth and extracellular phosphatase activity of arbuscular mycorrhizal hyphae as influenced by soil organic matter. *Soil Biology and Biochemistry* **27:** 1153-1159.

Joner EJ, Johansen A. **2000.** Phosphatase activity of external hyphae of two arbuscular mycorrhizal fungi. *Mycological Research* **104:** 81-86.

Joner EJ, Jakobsen I. **1994.** Contribution by two arbuscular mycorrhizal fungi to P uptake by cucumber (*Cucumis sativus* L.) from ^{32}P-labelled organic matter during mineralization in soil. *Plant and Soil* **163:** 203-209.

Joner EJ, van Aarle IM, Vosatka M. **2000.** Phosphatase activity of extra-radical arbuscular mycorrhizal hyphae: a review. *Plant and Soil* **226:** 199-210.

Jones DL, Hodge A, Kuzyakov Y. **2004.** Plant and mycorrhizal regulation of rhizodeposition. *New Phytologist* **163:** 459-480.

Jones DL, Nguyen C, Finlay RD. **2009.** Carbon flow in the rhizosphere: carbon trading at the soil-root interface. *Plant and Soil* **321:** 5-33.

Jung SC, Martinez-Medina A, Lopez-Raez JA, Pozo MJ. **2012.** Mycorrhiza-induced resistance and priming of plant defenses. *Journal of Chemical Ecology* **38:** 651-664.

Ka JO, Urbance J, Ye RW, Ahn TY, Tiedje JM. **1997.** Diversity of oxygen and N-oxide regulation of nitrite reductases in denitrifying bacteria. *FEMS Microbiology Letters* **156:** 55-60.

Kabir Z, O'Halloran IP, Fyles JW, Hamel C. **1997.** Seasonal changes of arbuscular mycorrhizal fungi as affected by tillage practices and fertilization: hyphal density and mycorrhizal root colonization. *Plant and Soil* **192:** 285-293.

Kammann C, Grünhage L, Jäger HJ. **2001.** A new sampling technique to monitor concentrations of CH_4, N_2O and CO_2 in air at well-defined depths in soils with varied water potential. *European Journal of Soil Science* **52:** 297-303.

Karasawa T, Hodge A, Fitter AH. **2012.** Growth, respiration and nutrient acquisition by the arbuscular mycorrhizal fungus *Glomus mosseae* and its host plant *Plantago lanceolata* in cooled soil. *Plant, Cell and Environment* **35:** 819-828.

Kaschuk G, Leffelaar PA, Giller KE, Alberton O, Hungria M, Kuyper TW. **2010.** Responses of legumes to rhizobia and arbuscular mycorrhizal fungi: a meta-analysis of potential photosynthate limitation of symbioses. *Soil Biology and Biochemistry* **42:** 125-127.

Kelso BHL, Smith RV, Laughlin RJ, Lennox SD. **1997.** Dissimilatory nitrate reduction in anaerobic sediments leading to river nitrite accumulation. *Applied and Environmental Microbiology* **63:** 4679-4685.

Kessavalou A, Mosier AR, Doran JW, Drijber RA, Lyon DJ, Heinemeyer O. **1998.** Fluxes of carbon dioxide, nitrous oxide, and methane in grass sod and winter wheat-fallow tillage management. *Journal of Environmental Quality* **27:** 1094-1104.

Khahil MI, Baggs EM. **2005.** CH_4 oxidation and N_2O emissions at varied soil water-filled pore spaces and headspace CH_4 concentrations. *Soil Biology and Biochemistry* **37:** 1785-1794.

Khalil K, Mary B, Renault P. **2004.** Nitrous oxide production by nitrification and denitrification in soil aggregates as affected by O_2 concentration. *Soil Biology and Biochemistry* **36:** 687-699.

Khalil MI, Rosenani AB, Van Cleemput O, Boeckx P, Shamshuddin J, Fauziah CI. **2002.** Nitrous oxide production from an ultisol of the humid tropics treated with different nitrogen sources and moisture regimes. *Biology and Fertility of Soils* **36:** 59-65.

Kiers ET, Duhamel M, Beesetty Y, Mensah JA, Franken O, Verbruggen E, Fellbaum CR, Kowalchuk GA, Hart MM, Bago A *et al*. 2011. Reciprocal rewards stabilize cooperation in the mycorrhizal symbiosis. *Science* **333:** 880-882.

Killham K. **2006.** *Soil ecology*. Cambridge, UK: Cambridge University Press.

Kim KY, Jordan D, McDonald GA. **1997.** Effect of phosphate-solubilizing bacteria and vesicular-arbuscular mycorrhizae on tomato growth and soil microbial activity. *Biology and Fertility of Soils* **26:** 79-87.

Kim SK, Sohn EY, Kang SM, Lee IJ. **2009.** Quantification of endogenous gibberellins in two flax (*Linum usitatissimum* L.) cultivars during seed development. *Journal of Crop Science and Biotechnology* **12:** 43-46.

Klironomos JN, Kendrick WB. **1996.** Palatability of microfungi to soil arthropods in relation to the functioning of arbuscular mycorrhizae. *Biology and Fertility of Soils* **21:** 43-52.

Klopatek CC, Klopatek JM. **1997.** Nitrifiers and mycorrhizae in pristine and grazed pinyon-juniper ecosystems. *Arid Soil Research and Rehabilitation* **11:** 331-342.

Knowles R. **2005.** Denitrifiers associated with methanotrophs and their potential impact on the nitrogen cycle. *Ecological Engineering* **24:** 441-446.

Koide RT. **2000.** Functional complementarity in the arbuscular mycorrhizal symbiosis. *New Phytologist* **147:** 233-235.

Koller R, Rodriguez A, Robin C, Scheu S, Bonkowski M. **2013.** Protozoa enhance foraging efficiency of arbuscular mycorrhizal fungi for mineral nitrogen from organic matter in soil to the benefit of host plants. *New Phytologist* **199:** 203-211.

Kool DM, Van Groenigen JW, Wrage N. **2011.** Source determination of nitrous oxide based on nitrogen and oxygen isotope tracing: dealing with oxygen exchange. *Methods in Enzymology* **496**: 139-160.

Koops JG, Oenema O, van Beusichem ML. **1996.** Denitrification in the top and sub soil of grassland on peat soils. *Plant and Soil* **184:** 1-10.

Koske RE, Gemma JN. **1989.** A modified procedure for staining roots to detect VA-mycorrhizas. *Mycological Research* **92:** 486-505.

Kowalchuk GA. **2012.** Bad news for soil carbon sequestration? *Science* **337:** 1049-1050.

Krüger M, Krüger C, Walker C, Stockinger H, Schüßler A. **2012.** Phylogenetic reference data for systematics and phylotaxonomy of arbuscular mycorrhizal fungi from phylum to species level. *New Phytologist* **193:** 970-984.

Kusa K, Sawamoto T, Hu R, Hatano R. **2010.** Comparison of N_2O and CO_2 concentrations and fluxes in the soil profile between a Gray Lowland soil and an Andosol. *Soil Science and Plant Nutrition* **56:** 186-199.

Kutsch WL, Bahn M, Heinemeyer A. 2010. *Soil carbon dynamics: An integrated methodology.* Cambridge University Press, Cambridge UK.

Lancashire PD, Bleiholder H, Van Den Boom T, Langelüddeke P, Stauss R, Weber E, Witzenberger A. **1991.** A uniform decimal code for growth stages of crops and weeds. *Annals of Applied Biology* **119:** 561-601.

Langley JA, Hungate BA. **2003.** Mycorrhizal controls on belowground litter quality. *Ecology* **84:** 2302-2312.

Laughlin RJ, Stevens RJ, Müller C, Watson CJ. **2008.** Evidence that fungi can oxidize NH_4^+ to NO_3^- in a grassland soil. *European Journal of Soil Science* **59:** 285-291.

Le Mer J, Roger P. **2001.** Production, oxidation, emission and consumption of methane by soils: a review. *European Journal of Soil Biology* **37:** 25-50.

Leake JR, Johnson D, Donnelly D, Muckle G, Boddy L, Read DJ. **2004.** Networks of power and influence: the role of mycorrhizal mycelium in controlling plant communities and agroecosystem functioning. *Canadian Journal of Botany* **82:** 1016-1045.

Lee SW, Im J, DiSpirito AA, Bodrossy L, Barcelona MJ, Semrau JD. **2009.** Effect of nutrient and selective inhibitor amendments on methane oxidation, nitrous oxide production, and key gene presence and expression in landfill cover soils: characterization of the role of methanotrophs, nitrifiers, and denitrifiers. *Applied Microbiology and Biotechnology* **85:** 389-403.

Lei J, Bécard G, Catford JG, Piché Y. **1991.** Root factors stimulate ^{32}P uptake and plasmalemma ATPase activity in vesicular-arbuscular mycorrhizal fungus, *Gigaspora margarita*. *New Phytologist* **118:** 289-294.

Leigh J, Fitter AH, Hodge A. **2011.** Growth and symbiotic effectiveness of an arbuscular mycorrhizal fungus in organic matter in competition with soil bacteria. *FEMS Microbiology Ecology* **76:** 428-438.

Leigh J, Hodge A, Fitter AH. **2009.** Arbuscular mycorrhizal fungi can transfer substantial amounts of nitrogen to their host plant from organic material. *New Phytologist* **181:** 199-207.

Leininger S, Urich T, Schloter M, Schwark L, Qi J, Nicol GW, Prosser JI, Schuster SC, Schleper C. **2006.** Archaea predominate among ammonia-oxidizing prokaryotes in soils. *Nature* **442:** 806-809.

Lenhart K, Bunge M, Ratering S, Neu TR, Schüttmann I, Greule M, Kammann C, Schnell S, Müller C, Zorn H, Keppler F. **2012.** Evidence for methane production by saprotrophic fungi. *Nature Communications* **3:** 1-8.

Li H, Wang C, Li X, Christie P, Dou Z, Zhang J, Xiang D. **2013.** Impact of the earthworm *Aporrectodea trapezoides* and the arbuscular mycorrhizal fungus *Glomus intraradices* on ^{15}N uptake by maize from wheat straw. *Biology and Fertility of Soils* **49:** 263-271.

Li XL, George E, Marschner H. **1991a.** Phosphorus depletion and pH decrease at the root-soil and hyphae-soil interfaces of VA mycorrhizal white clover fertilized with ammonium. *New Phytologist* **119:** 397-404.

Li XL, George E, Marschner H. **1991b.** Extension of the phosphorus depletion zone in VA-mycorrhizal white clover in a calcareous soil. *Plant and Soil* **136:** 41-48.

Li XL, Marschner H, George E. **1991c.** Acquisition of phosphorus and copper by VA-mycorrhizal hyphae and root-to-shoot transport in white clover. *Plant and Soil* **136:** 49-57.

Li ZA, Zou B, Xia HP, Ding YZ, Tan WN, Ma ZR. **2005.** Effect of fertilizer and water content on N_2O emission from three plantation soils in south China. *Journal of Environmental Sciences-China* **17:** 970-976.

Liang N, Nakadai T, Hirano T, Qu L, Koike T, Fujinuma Y, Inoue G. **2004.** *In situ* comparison of four approaches to estimating soil CO_2 efflux in a northern larch (*Larix kaempferi* Sarg.) forest. *Agricultural and Forest Meteorology* **123:** 97-117.

Liu A, Hamel C, Hamilton RI, Ma BL, Smith DL. **2000a.** Acquisition of Cu, Zn, Mn and Fe by mycorrhizal maize (*Zea mays* L.) grown in soil at different P and micronutrient levels. *Mycorrhiza* **9:** 331-336.

Liu A, Hamel C, Hamilton RI, Smith DL. **2000b.** Mycorrhizae formation and nutrient uptake of new corn (*Zea mays* L.) hybrids with extreme canopy and leaf architecture as influenced by soil N and P levels. *Plant and Soil* **221:** 157-166.

Liu L, Gundersen P, Zhang T, Mo J. **2012a.** Effects of phosphorus addition on soil microbial biomass and community composition in three forest types in tropical China. *Soil Biology and Biochemistry* **44:** 31-38.

Liu Y, Shi G, Mao L, Cheng G, Jiang S, Ma X, An L, Du G, Johnson NC, Feng H. **2012b.** Direct and indirect influences of 8 yr of nitrogen and phosphorus fertilization on Glomeromycota in an alpine meadow ecosystem. *New Phytologist* **194:** 523-535.

López-Pedrosa A, González-Guerrero M, Valderas A, Azcón-Aguilar C, Ferrol N. **2006.** *GintAMT1* encodes a functional high-affinity ammonium transporter that is expressed in the extraradical mycelium of *Glomus intraradices*. *Fungal Genetics and Biology* **43:** 102-110.

Lovelock CE, Wright SF, Clark DA, Ruess RW. **2004.** Soil stocks of glomalin produced by arbuscular mycorrhizal fungi across a tropical rain forest landscape. *Journal of Ecology* **92:** 278-287.

MacDonald JD, Rochette P, Chantigny MH, Angers DA, Royer I, Gasser MO. **2011.** Ploughing a poorly drained grassland reduced N_2O emissions compared to chemical fallow. *Soil and Tillage Research* **111:** 123-132.

MacFarling Meure C, Etheridge D, Trudinger C, Steele P, Langenfelds R, van Ommen T, Smith A, Elkins J. **2006.** Law Dome CO_2, CH_4 and N_2O ice core records extended to 2000 years BP. *Geophysical Research Letters* **33:** L14810.

Mäder P, Vierheilig H, Streitwolf-Engel R, Boller T, Frey B, Christie P, Wiemken A. **2000a.** Transport of ^{15}N from a soil compartment separated by a polytetrafluoroethylene membrane to plant roots via the hyphae of arbuscular mycorrhizal fungi. *New Phytologist* **146:** 155-161.

Mäder P, Edenhofer S, Boller T, Wiemken A, Niggli U. **2000b.** Arbuscular mycorrhizae in a long-term field trial comparing low-input (organic, biological) and high-input (conventional) farming systems in a crop rotation. *Biology and Fertility of Soils* **31:** 150-156.

Madigan MT, Martinko JM. **2006.** *Brock Biology of Microorganisms*. 11th Edition. Lebanon, Indiana, USA: Pearson Prentice Hall, Pearson Education, Inc.

Maeda D, Ashida K, Iguchi K, Chechetka SA, Hijikata A, Okusako Y, Deguchi Y, Izui K, Hata S. **2006.** Knockdown of an arbuscular mycorrhiza-inducible phosphate transporter gene of *Lotus japonicus* suppresses mutualistic symbiosis. *Plant and Cell Physiology* **47:** 807-817.

Mafongoya PL, Barak P, Reed JD. **2000.** Carbon, nitrogen and phosphorus mineralization of tree leaves and manure. *Biology and Fertility of Soils* **30:** 298-305.

Mahmood T, Ali R, Malik KA, Shamsi SRA. **1997.** Denitrification with and without maize plants (*Zea mays* L.) under irrigated field conditions. *Biology and Fertility of Soils* **24:** 323-328.

Maldonado-Mendoza IE, Dewbre GR, Harrison MJ. **2001.** A phosphate transporter gene from the extra-radical mycelium of an arbuscular mycorrhizal fungus *Glomus intraradices* is regulated in response to phosphate in the environment. *Molecular Plant-Microbe Interactions* **14:** 1140-1148.

Manzoni S, Jackson RB, Trofymow JA, Porporato A. **2008.** The global stoichiometry of litter nitrogen mineralization. *Science* **321:** 684-686.

Mao J, Yang L, Shi Y, Hu J, Piao Z, Mei L, Yin S. **2006.** Crude extract of *Astragalus mongholicus* root inhibits crop seed germination and soil nitrifying activity. *Soil Biology and Biochemistry* **38:** 201-208.

Marschner H, Römheld V, Horst WJ, Martin P. **1986.** Root-induced changes in the rhizosphere: importance for the mineral nutrition of plants. *Zeitschrift für Pflanzenernährung und Bodenkunde* **149:** 441-456.

Martens-Habbena W, Berube PM, Urakawa H, de La Torre JR, Stahl DA. **2009.** Ammonia oxidation kinetics determine niche separation of nitrifying Archaea and Bacteria. *Nature* **461:** 976-979.

Mastepanov M, Christensen TR. **2008.** Bimembrane diffusion probe for continuous recording of dissolved and entrapped bubble gas concentrations in peat. *Soil Biology and Biochemistry* **40:** 2992-3003.

McGonigle TP, Miller MH, Evans DG, Fairchild GL, Swan JA. **1990.** A new method which gives an objective measure of colonization of roots by vesicular-arbuscular mycorrhizal fungi. *New Phytologist* **115:** 495-501.

McLain JET, Martens DA. **2005.** Nitrous oxide flux from soil amino acid mineralization. *Soil Biology and Biochemistry* **37:** 289-299.

McNamara NP, Black HIJ, Piearce TG, Reay DS, Ineson P. **2008.** The influence of afforestation and tree species on soil methane fluxes from shallow organic soils at the UK Gisburn Forest Experiment. *Soil Use and Management* **24:** 1-7.

Mengel K, Kirkby EA. **2001.** *Principles of plant nutrition*. Dordrecht, The Netherlands: Kluwer Academic Publishers.

Mergel A, Schmitz O, Mallmann T, Bothe H. **2001.** Relative abundance of denitrifying and dinitrogen-fixing bacteria in layers of a forest soil. *FEMS Microbiology Ecology* **36:** 33-42.

Merryweather JW, Fitter AH. **1991.** A modified method for elucidating the structure of the fungal partner in a vesicular-arbuscular mycorrhiza. *Mycological Research* **95:** 1435-1437.

Millar N, Ndufa JK, Cadisch G, Baggs EM. **2004.** Nitrous oxide emissions following incorporation of improved□fallow residues in the humid tropics. *Global Biogeochemical Cycles* **18:** 1-9.

Miller MN, Dandie CE, Zebarth BJ, Burton DL, Goyer C, Trevors JT. **2012.** Influence of carbon amendments on soil denitrifier abundance in soil microcosms. *Geoderma* **170:** 48-55.

Miller MN, Zebarth BJ, Dandie CE, Burton DL, Goyer C, Trevors JT. **2008.** Crop residue influence on denitrification, N_2O emissions and denitrifier community abundance in soil. *Soil Biology and Biochemistry* **40:** 2553-2562.

Miller RM, Miller SP, Jastrow JD, Rivetta CB. **2002.** Mycorrhizal mediated feedbacks influence net carbon gain and nutrient uptake in *Andropogon gerardii*. *New Phytologist* **155:** 149-162.

Minerdi D, Fani R, Gallo R, Boarino A, Bonfante P. **2001.** Nitrogen fixation genes in an endosymbiotic *Burkholderia* strain. *Applied and Environmental Microbiology* **67:** 725-732.

Mori T, Ohta S, Ishizuka S, Konda R, Wicaksono A, Heriyanto J, Hardjono A. **2013.** Effects of phosphorus addition with and without ammonium, nitrate, or glucose on N_2O and NO emissions from soil sampled under *Acacia mangium* plantation and incubated at 100 % of the water-filled pore space. *Biology and Fertility of Soils* **49:** 13-21.

Mørkved PT, Dörsch P, Bakken LR. **2007.** The N_2O product ratio of nitrification and its dependence on long-term changes in soil pH. *Soil Biology and Biochemistry* **39:** 2048-2057.

Mortimer PE, Le Roux MR, Pérez-Fernández MA, Benedito VA, Kleinert A, Xu J, Valentine AJ. **2013.** The dual symbiosis between arbuscular mycorrhiza and nitrogen fixing bacteria benefits the growth and nutrition of the woody invasive legume *Acacia cyclops* under nutrient limiting conditions. *Plant and Soil* **366:** 229-241.

Mounier E, Hallet S, Chèneby D, Benizri E, Gruet Y, Nguyen C, Piutti S, Robin C, Slezack-Deschaumes S, Martin-Laurent F *et al*. 2004. Influence of maize mucilage on the diversity and activity of the denitrifying community. *Environmental Microbiology* **6:** 301-312.

Moyano FE, Manzoni S, Chenu C. **2013.** Responses of soil heterotrophic respiration to moisture availability: an exploration of processes and models. *Soil Biology and Biochemistry* **59:** 72-85.

Muhammad W, Vaughan SM, Dalal RC, Menzies NW. **2011.** Crop residues and fertilizer nitrogen influence residue decomposition and nitrous oxide emission from a Vertisol. *Biology and Fertility of Soils* **47:** 15-23.

Mummey DL, Rillig MC. **2006.** The invasive plant species *Centaurea maculosa* alters arbuscular mycorrhizal fungal communities in the field. *Plant and Soil* **288:** 81-90.

Murray PJ, Hatch DJ, Dixon ER, Stevens RJ, Laughlin RJ, Jarvis SC. **2004.** Denitrification potential in a grassland subsoil: effect of carbon substrates. *Soil Biology and Biochemistry* **36:** 545-547.

Nazaries L, Murrell JC, Millard P, Baggs L, Singh BK. **2013a.** Methane, microbes and models: fundamental understanding of the soil methane cycle for future predictions. *Environmental Microbiology* **15**: 2395-2417.

Nazaries L, Pan Y, Bodrossy L, Baggs EM, Millard P, Murrell JC, Singh BK. **2013b.** Evidence of microbial regulation of biogeochemical cycles from a study on methane flux and land use change. *Applied and Environmental Microbiology* **79:** 4031-4040.

Nicolardot B, Recous S, Mary B. **2001.** Simulation of C and N mineralisation during crop residue decomposition: a simple dynamic model based on the C:N ratio of the residues. *Plant and Soil* **228:** 83-103.

Norton JM, Stark JM. **2011.** Regulation and measurement of nitrification in terrestrial systems. *Methods in Enzymology* **486**: 343-368.

Nuccio EE, Hodge A, Pett-Ridge J, Herman DJ, Weber PK, Firestone MK. **2013.** An arbuscular mycorrhizal fungus significantly modifies the soil bacterial community and nitrogen cycling during litter decomposition. *Environmental Microbiology* **15:** 1870-1881.

Nugroho RA, Röling WFM, Laverman AM, Verhoef HA. **2007.** Low nitrification rates in acid Scots pine forest soils are due to pH-related factors. *Microbial Ecology* **53:** 89-97.

Ojanen P, Minkkinen K, Alm J, Penttilä T. **2010.** Soil-atmosphere CO_2, CH_4 and N_2O fluxes in boreal forestry-drained peatlands. *Forest Ecology and Management* **260:** 411-421.

Olsson PA, Jakobsen I, Wallander H. **2003.** Foraging and resource allocation strategies of mycorrhizal fungi in a patchy environment. In: Heijden MGA, Sanders IR eds. *Mycorrhizal Ecology.* Berlin: Springer-Verlag, 93-115.

Olsson PA, Thingstrup I, Jakobsen I, Bååth E. **1999.** Estimation of the biomass of arbuscular mycorrhizal fungi in a linseed field. *Soil Biology and Biochemistry* **31:** 1879-1887.

Omonode RA, Vyn TJ, Smith DR, Hegymegi P, Gál A. **2007.** Soil carbon dioxide and methane fluxes from long-term tillage systems in continuous corn and corn-soybean rotations. *Soil and Tillage Research* **95:** 182-195.

Ostrom NE, Ostrom PH. **2011.** The isotopomers of nitrous oxide: analytical considerations and application to resolution of microbial production pathways. In: Baskaran M, ed. *Handbook of environmental isotope geochemistry*. Berlin: Springer Heidelberg.

Ostrom NE, Sutka R, Ostrom PH, Grandy AS, Huizinga KM, Gandhi H, von Fischer JC, Robertson GP. **2010.** Isotopologue data reveal bacterial denitrification as the primary source of N_2O during a high flux event following cultivation of a native temperate grassland. *Soil Biology and Biochemistry* **42:** 499-506.

Panikov NS, Mastepanov MA, Christensen TR. **2007.** Membrane probe array: technique development and observation of CO_2 and CH_4 diurnal oscillations in peat profile. *Soil Biology and Biochemistry* **39:** 1712-1723.

Park S, Croteau P, Boering KA, Etheridge DM, Ferretti D, Fraser PJ, Kim KR, Krummel PB, Langenfelds RL, van Ommen TD. ***et al.*** **2012.** Trends and seasonal cycles in the isotopic composition of nitrous oxide since 1940. *Nature Geoscience* **5:** 261-265.

Parkin TB. **1987.** Soil microsites as a source of denitrification variability. *Soil Science Society of America Journal* **51:** 1194-1199.

Parmesan C. **2006.** Ecological and evolutionary responses to recent climate change. *Annual Review of Ecology, Evolution, and Systematics* **37:** 637-669.

Parniske M. **2008.** Arbuscular mycorrhiza: the mother of plant root endosymbioses. *Nature Reviews Microbiology* **6:** 763-775.

Parton WJ, Mosier AR, Ojima DS, Valentine DW, Schimel DS, Weier K, Kulmala AE. **1996.** Generalized model for N_2 and N_2O production from nitrification and denitrification. *Global Biogeochemical Cycles* **10:** 401-412.

Pastor J, Aber JD, McClaugherty CA, Melillo JM. **1984.** Aboveground production and N and P cycling along a nitrogen mineralization gradient on Blackhawk Island, Wisconsin. *Ecology* **65:** 256-268.

Patz JA, Campbell-Lendrum D, Holloway T, Foley JA. **2005.** Impact of regional climate change on human health. *Nature* **438:** 310-317.

Paul EA, Kucey RMN. **1981.** Carbon flow in plant microbial associations. *Science* **213:** 473-474.

Pérez-Tienda J, Valderas A, Camañes G, García-Agustín P, Ferrol N. **2012.** Kinetics of NH_4^+ uptake by the arbuscular mycorrhizal fungus *Rhizophagus irregularis*. *Mycorrhiza* **22:** 485-491.

Pérez-Tienda J, Testillano PS, Balestrini R, Fiorilli V, Azcón-Aguilar C, Ferrol N. **2011.** GintAMT2, a new member of the ammonium transporter family in the arbuscular mycorrhizal fungus *Glomus intraradices*. *Fungal Genetics and Biology* **48:** 1044-1055.

Pfeffer PE, Douds DD, Bécard G, Shachar-Hill Y. **1999.** Carbon uptake and the metabolism and transport of lipids in an arbuscular mycorrhiza. *Plant Physiology* **120:** 587-598.

Philippot L, Hallin S, Börjesson G, Baggs EM. **2009.** Biochemical cycling in the rhizosphere having an impact on global change. *Plant and Soil* **321:** 61-81.

Philippot L, Hallin S, Schloter M. **2007.** Ecology of denitrifying prokaryotes in agricultural soil. *Advances in Agronomy* **96:** 249-305.

Phillips DA, Fox TC, Six J. **2006.** Root exudation (net efflux of amino acids) may increase rhizodeposition under elevated CO_2. *Global Change Biology* **12:** 561-567.

Plante AF. **2007.** Soil biogeochemical cycling of inorganic nutrients and metals. In: Paul EA, ed. *Soil Microbiology, Ecology and Biochemistry*. New York, USA: Springer, 339-432.

Prendergast-Miller MT, Baggs EM, Johnson D. **2011.** Nitrous oxide production by the ectomycorrhizal fungi *Paxillus involutus* and *Tylospora fibrillosa*. *FEMS Microbiology Letters* **316:** 31-35.

Priemé A, Christensen S, Dobbie KE, Smith KA. **1997.** Slow increase in rate of methane oxidation in soils with time following land use change from arable agriculture to woodland. *Soil Biology and Biochemistry* **29:** 1269-1273.

Prosser JI. **2007.** The ecology of nitrifying bacteria. In: Bothe H, Ferguson S, Newton WE, eds. *Biology of the nitrogen cycle*. Oxford, UK: Elsevier Science, 223-243.

Prosser JI, Nicol GW. **2012.** Archaeal and bacterial ammonia-oxidisers in soil: the quest for niche specialisation and differentiation. *Trends in Microbiology* **20:** 523-531.

Pumpanen J, Ilvesniemi H, Perämäki M, Hari P. **2003.** Seasonal patterns of soil CO_2 efflux and soil air CO_2 concentration in a Scots pine forest: comparison of two chamber techniques. *Global Change Biology* **9:** 371-382.

Purchase BS. **1974.** Influence of phosphate deficiency on nitrification. *Plant and Soil* **41:** 541-547.

Purin S, Rillig MC. **2007.** The arbuscular mycorrhizal fungal protein glomalin: Limitations, progress, and a new hypothesis for its function. *Pedobiologia* **51:** 123-130.

Raghoebarsing AA, Pol A, van de Pas-Schoonen KT, Smolders AJP, Ettwig KF, Rijpstra WIC, Schouten S, Damsté JSS, Op den Camp HJM, Jetten MSM, Strous M. **2006.** A microbial consortium couples anaerobic methane oxidation to denitrification. *Nature* **440:** 918-921.

Raghothama KG. **1999.** Phosphate acquisition. *Annual Review of Plant Physiology and Plant Molecular Biology* **50:** 665-693.

Ravishankara AR, Daniel JS, Portmann RW. **2009.** Nitrous Oxide (N_2O): The dominant ozone-depleting substance emitted in the 21st century. *Science* **326:** 123-125.

Ravnskov S, Larsen J, Olsson PA, Jakobsen I. **1999.** Effects of various organic compounds on growth and phosphorus uptake of an arbuscular mycorrhizal fungus. *New Phytologist* **141:** 517-524.

Reay DS, Davidson EA, Smith KA, Smith P, Melillo JM, Dentener F, Crutzen PJ. **2012.** Global agriculture and nitrous oxide emissions. *Nature Climate Change* **2:** 410-416.

Reay DS, Nedwell DB. **2004.** Methane oxidation in temperate soils: effects of inorganic N. *Soil Biology and Biochemistry* **36:** 2059-2065.

Redecker D, Kodner R, Graham LE. **2000.** Glomalean fungi from the Ordovician. *Science* **289:** 1920-1921.

Remy W, Taylor TN, Hass H, Kerp H. **1994.** Four hundred-million-year-old vesicular arbuscular mycorrhizae. *Proceedings of the National Academy of Sciences of the United States of America* **91:** 11841-11843.

Reynolds HL, Hartley AE, Vogelsang KM, Bever JD, Schultz PA. **2005.** Arbuscular mycorrhizal fungi do not enhance nitrogen acquisition and growth of old-field perennials under low nitrogen supply in glasshouse culture. *New Phytologist* **167:** 869-880.

Riffaldi R, Saviozzi A, Levi-Minzi R. **1996.** Carbon mineralization kinetics as influenced by soil properties. *Biology and Fertility of Soils* **22:** 293-298.

Riley WJ, Subin ZM, Lawrence DM, Swenson SC, Torn MS, Meng L, Mahowald NM, Hess P. **2011.** Barriers to predicting changes in global terrestrial methane fluxes: analyses using CLM4Me, a methane biogeochemistry model integrated in CESM. *Biogeosciences* **8:** 1733-1807.

Rillig MC, Hernández GY, Newton PCD. **2000.** Arbuscular mycorrhizae respond to elevated atmospheric CO_2 after long-term exposure: evidence from a CO_2 spring in New Zealand supports the resource balance model. *Ecology Letters* **3:** 475-478.

Rillig MC, Mummey DL. **2006.** Mycorrhizas and soil structure. *New Phytologist* **171:** 41-53.

Rillig MC, Wright SF, Nichols KA, Schmidt WF, Torn MS. **2001.** Large contribution of arbuscular mycorrhizal fungi to soil carbon pools in tropical forest soils. *Plant and Soil* **233:** 167-177.

Rillig MC, Wright SF, Eviner VT. **2002.** The role of arbuscular mycorrhizal fungi and glomalin in soil aggregation: comparing effects of five plant species. *Plant and Soil* **238:** 325-333.

Robertson GP, Groffman PM. **2007.** Nitrogen transformations. In: Paul EA, ed. *Soil Microbiology, Ecology and Biochemistry*. New York, USA: Springer 341-364.

Rochette P, Ellert B, Gregorich EG, Desjardins RL, Pattey E, Lessard R, Johnson BG. 1997. Description of a dynamic closed chamber for measuring soil respiration and its comparison with other techniques. *Canadian Journal of Soil Science*. **77**:195-203.

Ruiz-Lozano JM, Collados C, Barea JM, Azcón R. **2001.** Arbuscular mycorrhizal symbiosis can alleviate drought-induced nodule senescence in soybean plants. *New Phytologist* **151:** 493-502.

SAS Institute Inc. **2008.** *SAS/STAT® 9.1 User's Guide: The Freq Procedure (Book Excerpt).* Cary, NC, USA: SAS Institute, Incorporated.

Schachtman DP, Reid RJ, Ayling SM. **1998.** Phosphorus uptake by plants: from soil to cell. *Plant Physiology* **116:** 447-453.

Schaufler G, Kitzler B, Schindlbacher A, Skiba U, Sutton MA, Zechmeister-Boltenstern S. **2010.** Greenhouse gas emissions from European soils under different land use: effects of soil moisture and temperature. *European Journal of Soil Science* **61:** 683-696.

Schimel DS. **1995.** Terrestrial Ecosystems and the carbon cycle. *Global Change Biology* **1:** 77-91.

Schlesinger WH. **1997.** *Biogeochemistry: An analysis of global change*. Oxford, UK: Academic Press.

Schlesinger WH, Bernhardt ES. **2013.** *Biogeochemistry: An analysis of global change*. Oxford, UK: Academic Press.

Segers R. **1998.** Methane production and methane consumption: a review of processes underlying wetland methane fluxes. *Biogeochemistry* **41:** 23-51.

Shi Z, Wang F, Zhang C, Yang Z. **2011.** Exploitation of phosphorus patches with different phosphorus enrichment by three arbuscular mycorrhizal fungi. *Journal of Plant Nutrition* **34:** 1096-1106.

Šimek M, Cooper JE. **2002.** The influence of soil pH on denitrification: progress towards the understanding of this interaction over the last 50 years. *European Journal of Soil Science* **53:** 345-354.

Singh BK, Bardgett RD, Smith P, Reay DS. **2010.** Microorganisms and climate change: terrestrial feedbacks and mitigation options. *Nature Reviews Microbiology* **8:** 779-790.

Six J, Ogle SM, Breidt FJ, Conant RT, Mosier AR, Paustian K. **2004.** The potential to mitigate global warming with no-tillage management is only realized when practised in the long term. *Global Change Biology* **10:** 155-160.

Smith FA, Smith SE. **2011a.** What is the significance of the arbuscular mycorrhizal colonisation of many economically important crop plants? *Plant and Soil* **348:** 63-79.

Smith KA, Ball T, Conen F, Dobbie KE, Massheder J, Rey A. **2003a.** Exchange of greenhouse gases between soil and atmosphere: interactions of soil physical factors and biological processes. *European Journal of Soil Science* **54:** 779-791.

Smith SE, Read DJ. **2008.** *Mycorrhizal Symbiosis*. Cambridge, UK: Academic Press.

Smith SE, Smith FA. **2011b.** Roles of arbuscular mycorrhizas in plant nutrition and growth: new paradigms from cellular to ecosystem scales. *Annual Review of Plant Biology* **62:** 227-250.

Smith SE, Smith FA, Jakobsen I. **2003b.** Mycorrhizal fungi can dominate phosphate supply to plants irrespective of growth responses. *Plant Physiology* **133:** 16-20.

Smith SE, Smith FA, Jakobsen I. **2004.** Functional diversity in arbuscular mycorrhizal (AM) symbioses: the contribution of the mycorrhizal P uptake pathway is not correlated with mycorrhizal responses in growth or total P uptake. *New Phytologist* **162:** 511-524.

Smith Z, McCaig AE, Stephen JR, Embley TM, Prosser JI. **2001.** Species diversity of uncultured and cultured populations of soil and marine ammonia oxidizing bacteria. *Microbial Ecology* **42:** 228-237.

Snider DM, Venkiteswaran JJ, Schiff SL, Spoelstra J. **2012.** Deciphering the oxygen isotope composition of nitrous oxide produced by nitrification. *Global Change Biology* **18:** 356-370.

Spahni R, Chappellaz J, Stocker TF, Loulergue L, Hausammann G, Kawamura K, Flückiger J, Schwander J, Raynaud D, Masson-Delmotte V, Jouzel J. **2005.** Atmospheric methane and nitrous oxide of the late pleistocene from Antarctic ice cores. *Science* **310:** 1317-1321.

Spiro S. **2012.** Nitrous oxide production and consumption: regulation of gene expression by gas-sensitive transcription factors. *Philosophical Transactions of the Royal Society B: Biological Sciences* **367:** 1213-1225.

St John TV, Coleman DC, Reid CPP. **1983.** Association of vesicular-arbuscular mycorrhizal hyphae with soil organic particles. *Ecology* **64:** 957-959.

Staddon PL, Fitter AH. **1998.** Does elevated atmospheric carbon dioxide affect arbuscular mycorrhizas? *Trends in Ecology and Evolution* **13:** 455-458.

Staddon PL, Jakobsen I, Blum H. **2004.** Nitrogen input mediates the effect of free-air CO_2 enrichment on mycorrhizal fungal abundance. *Global Change Biology* **10:** 1678-1688.

Staddon PL, Ramsey CB, Ostle N, Ineson P, Fitter AH. **2003.** Rapid turnover of hyphae of mycorrhizal fungi determined by AMS microanalysis of ^{14}C. *Science* **300:** 1138-1140.

Stanford G, Vander Pol RA, Dzienia S. **1975.** Denitrification rates in relation to total and extractable soil carbon. *Soil Science Society of America Journal* **39:** 284-289.

Stanier RY. **1987.** *General microbiology*. Basingstoke, UK: Macmillan Education.

Stein LY. **2011.** Surveying N_2O-Producing Pathways in Bacteria. *Methods in Enzymology* **486**: 131-152.

Steinberg PD, Rillig MC. **2003.** Differential decomposition of arbuscular mycorrhizal fungal hyphae and glomalin. *Soil Biology and Biochemistry* **35:** 191-194.

Stern NH. **2007.** *The economics of climate change: the stern review*. London, Great Britain: Cambridge University Press.

Straub KL, Benz M, Schink B, Widdel F. **1996.** Anaerobic, nitrate-dependent microbial oxidation of ferrous iron. *Applied and Environmental Microbiology* **62:** 1458-1460.

Stremińska MA, Felgate H, Rowley G, Richardson DJ, Baggs EM. **2012.** Nitrous oxide production in soil isolates of nitrate-ammonifying bacteria. *Environmental Microbiology Reports* **4:** 66-71.

Su F, Takaya N, Shoun H. **2004.** Nitrous oxide-forming codenitrification catalyzed by cytochrome P450nor. *Bioscience, Biotechnology, and Biochemistry* **68:** 473-475.

Sukarno N, Smith FA, Smith SE, Scott ES. **1996.** The effect of fungicides on vesicular-arbuscular mycorrhizal symbiosis. II. The effects on area of interface and efficiency of P uptake and transfer to plant. *New Phytologist* **132:** 583-592.

Sundareshwar PV, Morris JT, Koepfler EK, Fornwalt B. **2003.** Phosphorus limitation of coastal ecosystem processes. *Science* **299:** 563-565.

Suzuki S, Kataoka K, Yamaguchi K. **2000.** Metal coordination and mechanism of multicopper nitrite reductase. *Accounts of Chemical Research* **33:** 728-735.

Tajini F, Trabelsi M, Drevon JJ. **2011.** Co-inoculation with *Glomus intraradices* and *Rhizobium tropici* CIAT899 increases P use efficiency for N_2 fixation in the common bean (*Phaseolus vulgaris* L.) under P deficiency in hydroaeroponic culture. *Symbiosis* **53:** 123-129.

Tanaka Y, Yano K. **2005.** Nitrogen delivery to maize via mycorrhizal hyphae depends on the form of N supplied. *Plant Cell and Environment* **28:** 1247-1254.

Taylor BR, Parkinson D, Parsons WFJ. **1989.** Nitrogen and lignin content as predictors of litter decay-rates: a microcosm test. *Ecology* **70:** 97-104.

Thies JA, Merrill SB, Corley EL. **2002.** Red food coloring stain: new, safer procedures for staining nematodes in roots and egg masses on root surfaces. *Journal of Nematology* **34:** 179-181.

Thompson JP. **1996.** Correction of dual phosphorus and zinc deficiencies of linseed (*Linum usitatissimum* L) with cultures of vesicula-arbuscular mycorrhizal fungi. *Soil Biology and Biochemistry* **28:** 941-951.

Thomson AJ, Giannopoulos G, Pretty J, Baggs EM, Richardson DJ. **2012.** Biological sources and sinks of nitrous oxide and strategies to mitigate emissions. *Philosophical Transactions of the Royal Society B: Biological Sciences* **367:** 1157-1168.

Thomson BD, Clarkson DT, Brain P. **1990.** Kinetics of phosphorus uptake by the germ-tubes of the vesicular-arbuscular mycorrhizal fungus, *Gigaspora margarita*. *New Phytologist* **116:** 647-653.

Thornton B, Bausenwein U. **2000.** Seasonal protease activity in storage tissue of the deciduous grass *Molinia caerulea*. *New Phytologist* **146:** 75-81.

Tinker PB, Nye PH. **2000.** *Solute movement in the rhizosphere*. New York, USA: Oxford University Press.

Tobar R, Azcón R, Barea JM. **1994.** Improved nitrogen uptake and transport from ^{15}N-labelled nitrate by external hyphae of arbuscular mycorrhiza under water-stressed conditions. *New Phytologist* **126:** 119-122.

Toljander JF, Lindahl BD, Paul LR, Elfstrand M, Finlay RD. **2007.** Influence of arbuscular mycorrhizal mycelial exudates on soil bacterial growth and community structure. *FEMS Microbiology Ecology* **61:** 295-304.

Toma Y, Hatano R. **2007.** Effect of crop residue C:N ratio on N_2O emissions from Gray Lowland soil in Mikasa, Hokkaido, Japan. *Soil Science and Plant Nutrition* **53:** 198-205.

Toro M, Azcón R, Barea JM. **1998.** The use of isotopic dilution techniques to evaluate the interactive effects of *Rhizobium* genotype, mycorrhizal fungi, phosphate-solubilizing rhizobacteria and rock phosphate on nitrogen and phosphorus acquisition by *Medicago* sativa. *New Phytologist* **138:** 265-273.

Treseder KK. **2004.** A meta-analysis of mycorrhizal responses to nitrogen, phosphorus, and atmospheric CO_2 in field studies. *New Phytologist* **164:** 347-355.

Treseder KK, Allen MF. **2000.** Mycorrhizal fungi have a potential role in soil carbon storage under elevated CO_2 and nitrogen deposition. *New Phytologist* **147:** 189-200.

Treseder KK, Allen MF. **2002.** Direct nitrogen and phosphorus limitation of arbuscular mycorrhizal fungi: a model and field test. *New Phytologist* **155:** 507-515.

Treseder KK, Cross A. **2006.** Global distributions of arbuscular mycorrhizal fungi. *Ecosystems* **9:** 305-316.

Trouve C, Chazal PM, Gueroux B, Sauvaitre N. **1998.** Denitrification by new strains of *Thiobacillus denitrificans* under non-standard physicochemical conditions. Effect of temperature, pH, and sulphur source. *Environmental Technology* **19:** 601-610.

Tu C, Booker FL, Watson DM, Chen X, Rufty TW, Shi W, Hu SJ. **2006.** Mycorrhizal mediation of plant N acquisition and residue decomposition: Impact of mineral N inputs. *Global Change Biology* **12:** 793-803.

van der Heijden MGA, Streitwolf-Engel R, Riedl R, Siegrist S, Neudecker A, Ineichen K, Boller T, Wiemken A, Sanders IR. **2006.** The mycorrhizal contribution to plant productivity, plant nutrition and soil structure in experimental grassland. *New Phytologist* **172:** 739-752.

van Hees PAW, Jones DL, Finlay R, Godbold DL, Lundström US. **2005.** The carbon we do not see – the impact of low molecular weight compounds on carbon dynamics and respiration in forest soils: a review. *Soil Biology and Biochemistry* **37:** 1-13.

Vandenkoornhuyse P, Mahé S, Ineson P, Staddon P, Ostle N, Cliquet JB, Francez AJ, Fitter AH, Young PW. 2007. Active root-inhabiting microbes identified by rapid incorporation of plant-derived carbon into RNA. *Proceedings of the National Academy of Sciences of the United States of America.* **104:** 16970-16975.

Vargas R, Baldocchi DD, Querejeta JI, Curtis PS, Hasselquist NJ, Janssens IA, Allen MF, Montagnani L. **2010.** Ecosystem CO_2 fluxes of arbuscular and ectomycorrhizal dominated vegetation types are differentially influenced by precipitation and temperature. *New Phytologist* **185:** 226-236.

Venterea RT. **2007.** Nitrite□driven nitrous oxide production under aerobic soil conditions: kinetics and biochemical controls. *Global Change Biology* **13:** 1798-1809.

Verbruggen E, Veresoglou SD, Anderson IC, Caruso T, Hammer EC, Kohler J, Rillig MC. **2013.** Arbuscular mycorrhizal fungi □□short-term liability but long□term benefits for soil carbon storage? *New Phytologist* **197:** 366-368.

Veresoglou SD. **2012.** Arbuscular mycorrhiza prevents suppression of actual nitrification rates in the (myco-)rhizosphere of *Plantago lanceolata*. *Pedosphere* **22:** 225-229.

Veresoglou SD, Sen R, Mamolos AP, Veresoglou DS. **2011a.** Plant species identity and arbuscular mycorrhizal status modulate potential nitrification rates in nitrogen-limited grassland soils. *Journal of Ecology* **99:** 1339-1349.

Veresoglou SD, Shaw LJ, Hooker JE, Sen R. **2012a.** Arbuscular mycorrhizal modulation of diazotrophic and denitrifying microbial communities in the (mycor)rhizosphere of *Plantago lanceolata*. *Soil Biology and Biochemistry* **53:** 78-81.

Veresoglou SD, Shaw LJ, Sen R. **2011b.** *Glomus intraradices* and *Gigaspora margarita* arbuscular mycorrhizal associations differentially affect nitrogen and potassium nutrition of *Plantago lanceolata* in a low fertility dune soil. *Plant and Soil* **340:** 481-490.

Veresoglou SD, Chen B, Rillig MC. **2012b.** Arbuscular mycorrhiza and soil nitrogen cycling. *Soil Biology and Biochemistry* **46:** 53-62.

Verhagen FJM, Hageman PEJ, Woldendorp JW, Laanbroek HJ. **1994.** Competition for ammonium between nitrifying bacteria and plant roots in soil in pots; effects of grazing by flagellates and fertilization. *Soil Biology and Biochemistry* **26:** 89-96.

Verhagen FJM, Laanbroek HJ, Woldendrop JW. **1995.** Competition for ammonium between plant roots and nitrifying and heterotrophic bacteria and the effects of protozoan grazing. *Plant and Soil* **170:** 241-250.

Verhamme DT, Prosser JI, Nicol GW. **2011.** Ammonia concentration determines differential growth of ammonia-oxidising archaea and bacteria in soil microcosms. *The ISME journal* **5:** 1067-1071.

Vierheilig H, Schweiger P, Brundrett M. **2005.** An overview of methods for the detection and observation of arbuscular mycorrhizal fungi in roots. *Physiologia Plantarum* **125:** 393-404.

Vilcáez J, Watanabe T. **2009.** Inhibitory effect of gamma-irradiated chitosan on the growth of denitrifiers. *International Journal of Microbiology* **2009:** 418595.

Villegas J, Fortin JA. **2001.** Phosphorus solubilization and pH changes as a result of the interactions between soil bacteria and arbuscular mycorrhizal fungi on a medium containing NH_4^+ as nitrogen source. *Canadian Journal of Botany* **79:** 865-870.

Villegas-Pangga G, Blair G, Lefroy R. **2000.** Measurement of decomposition and associated nutrient release from straw (*Oryza sativa* L.) of different rice varieties using a perfusion system. *Plant and Soil* **223:** 1-11.

Vinther PF, Hansen EM, Olesen JE. **2004.** Effects of plant residues on crop performance, N mineralisation and microbial activity including field CO_2 and N_2O fluxes in unfertilised crop rotations. *Nutrient Cycling in Agroecosystems* **70:** 189-199.

Vos C, Schouteden N, van Tuinen D, Chatagnier O, Elsen A, De Waele D, Panis B, Gianinazzi-Pearson V. **2013.** Mycorrhiza-induced resistance against the root□knot nematode *Meloidogyne incognita* involves priming of defense gene responses in tomato. *Soil Biology and Biochemistry* **60:** 45-54.

Vos J, van der Putten PEL, Birch CJ. **2005.** Effect of nitrogen supply on leaf appearance, leaf growth, leaf nitrogen economy and photosynthetic capacity in maize (*Zea mays* L.). *Field Crops Research* **93:** 64-73.

Walker C, Vestberg M. **1994.** A simple and inexpensive method for producing and maintaining closed pot cultures of arbuscular mycorrhizal fungi. *Agricultural Science in Finland* **3:** 233-240.

Wallenstein MD, Myrold DD, Firestone M, Voytek M. **2006.** Environmental controls on denitrifying communities and denitrification rates: insights from molecular methods. *Ecological Applications* **16:** 2143-2152.

Wang F, Jiang R, Kertesz MA, Zhang F, Feng G. **2013.** Arbuscular mycorrhizal fungal hyphae mediating acidification can promote phytate mineralization in the hyphosphere of maize (*Zea mays* L.). *Soil Biology and Biochemistry* **65:** 69-74.

Wang X, Pan Q, Chen F, Yan X, Liao H. **2011.** Effects of co-inoculation with arbuscular mycorrhizal fungi and rhizobia on soybean growth as related to root architecture and availability of N and P. *Mycorrhiza* **21:** 173-181.

Wang ZP, Ineson P. **2003.** Methane oxidation in a temperate coniferous forest soil: effects of inorganic N. *Soil Biology and Biochemistry* **35:** 427-433.

Weiss RF. **1974.** Carbon dioxide in water and seawater: the solubility of a non-ideal gas. *Marine Chemistry* **2:** 203-215.

Weiss RF, Price BA. **1980.** Nitrous oxide solubility in water and seawater. *Marine Chemistry* **8:** 347-359.

Welc M, Ravnskov S, Kieliszewska-Rokicka B, Larsen J. **2010.** Suppression of other soil microorganisms by mycelium of arbuscular mycorrhizal fungi in root-free soil. *Soil Biology and Biochemistry* **42:** 1534-1540.

Whalen SC, Reeburgh WS, Sandbeck KA. **1990.** Rapid methane oxidation in a landfill cover soil. *Applied and Environmental Microbiology* **56:** 3405-3411.

Whitby CB, Hall G, Pickup R, Saunders JR, Ineson P, Parekh NR, McCarthy A. **2001.** ^{13}C incorporation into DNA as a means of identifying the active components of ammonia-oxidizer populations. *Letters in Applied Microbiology* **32:** 398-401.

Whiteside MD, Digman MA, Gratton E, Treseder KK. **2012.** Organic nitrogen uptake by arbuscular mycorrhizal fungi in a boreal forest. *Soil Biology and Biochemistry* **55:** 7-13.

Wilson GWT, Rice CW, Rillig MC, Springer A, Hartnett DC. **2009.** Soil aggregation and carbon sequestration are tightly correlated with the abundance of arbuscular mycorrhizal fungi: results from long-term field experiments. *Ecology Letters* **12:** 452-461.

Withers PJA, Edwards AC, Foy RH. **2001.** Phosphorus cycling in UK agriculture and implications for phosphorus loss from soil. *Soil Use and Management* **17:** 139-149.

Woldendorp JW, Laanbroek HJ. **1989.** Activity of nitrifiers in relation to nitrogen nutrition of plants in natural ecosystems. *Plant and Soil* **115:** 217-228.

Wrage N, Velthof GL, van Beusichem ML, Oenema O. **2001.** Role of nitrifier denitrification in the production of nitrous oxide. *Soil Biology and Biochemistry* **33:** 1723-1732.

Wright SF, Upadhyaya A. **1998.** A survey of soils for aggregate stability and glomalin, a glycoprotein produced by hyphae of arbuscular mycorrhizal fungi. *Plant and Soil* **198:** 97-107.

Wuebbles DJ, Hayhoe K. **2002.** Atmospheric methane and global change. *Earth-Science Reviews* **57:** 177-210.

Xavier LJC, Germida JJ. **2003.** Selective interactions between arbuscular mycorrhizal fungi and *Rhizobium leguminosarum* bv. *viceae* enhance pea yield and nutrition. *Biology and Fertility of Soils* **37:** 261-267.

Ye RW, Averill BA, Tiedje JM. **1994.** Denitrification - production and consumption of nitric-oxide. *Applied and Environmental Microbiology* **60:** 1053-1058.

Yoshida LC, Gamon JA, Andersen CP. **2001.** Differences in above- and below-ground responses to ozone between two populations of a perennial grass. *Plant and Soil* **233:** 203-211.

Yu KW, Wang ZP, Vermoesen A, Patrick WH, Van Cleemput O. **2001.** Nitrous oxide and methane emissions from different soil suspensions: effect of soil redox status. *Biology and Fertility of Soils* **34:** 25-30.

Yu L, Tang J, Zhang R, Wu Q, Gong M. **2013.** Effects of biochar application on soil methane emission at different soil moisture levels. *Biology and Fertility of Soils* **49:** 119-128.

Yue J, Shi Y, Zheng X, Huang G, Zhu J. **2007.** The influence of free-air CO_2 enrichment on microorganisms of a paddy soil in the rice-growing season. *Applied Soil Ecology* **35:** 154-162.

Zarea MJ, Ghalavand A, Goltapeh EM, Rejali F, Zamaniyan M. **2009.** Effects of mixed cropping, earthworms (*Pheretima* sp.), and arbuscular mycorrhizal fungi (*Glomus mosseae*) on plant yield, mycorrhizal colonization rate, soil microbial biomass, and nitrogenase activity of free-living rhizosphere bacteria. *Pedobiologia* **52:** 223-235.

Zhang W, Li F, Nie L. **2010.** Integrating multiple 'omics' analysis for microbial biology: application and methodologies. *Microbiology* **156:** 287-301.

Zhu X, Burger M, Doane TA, Horwath WR. **2013a.** Ammonia oxidation pathways and nitrifier denitrification are significant sources of N_2O and NO under low oxygen availability. *Proceedings of the National Academy of Sciences of the United States of America* **110:** 6328-6333.

Zhu X, Silva LCR, Doane TA, Horwath WR. **2013b.** Iron: the forgotten driver of nitrous oxide production in agricultural soil. *PLoS ONE* **8:** e60146.

Zhu YG, Miller RM. **2003.** Carbon cycling by arbuscular mycorrhizal fungi in soil-plant systems. *Trends in Plant Science* **8:** 407-409.

Zumft WG. **1997.** Cell biology and molecular basis of denitrification. *Microbiology and Molecular Biology Reviews* **61:** 533-616.