Ectomycorrhizal fungi and N_2O production

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Abstract
Denitrification is an important biogeochemical soil process largely responsible for the production of the harmful greenhouse gas, nitrous oxide (N_2O). Several soil factors control the final N_2O:N_2 product ratio, and the availability and quality of labile carbon (C) are often regarded as the most important. Low N_2O emissions compared to agricultural sources are characteristic of boreal and temperate forest soils, which are generally a result of acidic conditions and low nitrate availability. However, the role of the dominant microbial community, the ectomycorrhizal (EcM) fungi, in such forest soils during denitrification, has been overlooked. As EcM fungi are key in transferring host C throughout the soil system, we hypothesise that EcM fungal C is important in stimulating denitrification. Results from soil incubation and bacterial culture experiments using ^{15}N-nitrate indicate that addition of C sources, derived from EcM fungi, to the soil microbial community or a bacterial culture under controlled anaerobic conditions, leads to greater production of ^{15}N-(N_2O and N_2). Therefore taking account of the presence of EcM fungi during denitrification could further our understanding of the processes involved in forest soil N_2O emissions.

Key Words
Nitrogen cycle, mycorrhizal fungi, *Paxillus involutus*, denitrifying bacteria, greenhouse gases.

Introduction
Denitrification is an important soil process responsible for significant emissions of nitrous oxide (N_2O). N_2O emissions are closely related to anthropogenic activities, with increasing concentrations in the atmosphere a direct consequence of enhanced nitrogen (N) fertiliser use (Houghton *et al.* 2001). Therefore, agricultural soils are the dominant sources of N_2O, and the factors relevant to controlling source-sink relationships have been well-studied in these systems. In comparison to agricultural soils, N_2O emissions from boreal and temperate forest soils, linked to denitrification and nitrification processes, are relatively low (Kesik *et al.* 2005). As forest systems are distinct to agricultural soils, different factors have been examined to try and improve our understanding of highly variable forest N_2O emissions, such as forest type/tree species (Priha *et al.* 1999; Maljanen *et al.* 2003), distance from trees and tree roots (Butterbach-bahl *et al.* 2002; von Arnold *et al.* 2005), and management practices (Ineson *et al.* 1991; Zerva and Mencuccini 2005). However, N_2O emissions from forest biomes have largely been investigated without consideration of the dominant soil microbes unique to these forest soils, the ectomycorrhizal (EcM) fungi.

EcM fungi form symbiotic mutualistic associations with the roots of trees, such as pine, birch and spruce. EcM fungi develop an extensive mycelial network, and in return for nutrient and moisture acquisition for their hosts, the fungi receive up to 30% of the host's photoassimilate, which is used for further growth and reproduction (Smith and Read 2008). These fungi play key roles in forest soil biogeochemical cycling, mainly through their mineralization of mineral and organic matter, uptake of essential nutrients, and exudation of labile carbon (C) and other organic compounds (Chalot and Brun 1998; Jones *et al.* 2004). The EcM fungi also have positive and negative effects on other soil microbes (Olsson *et al.* 1996; Nurmiaho-Lassila *et al.* 1997), therefore it is likely that denitrifying bacteria are also affected. The product ratio of N_2O:N_2 by denitrifying bacteria is controlled by access to labile C (Burford and Bremner 1985; Firestone 1982; Henry *et al.* 2008), and there is the potential for bacteria in close proximity to EcM mycelia to utilise C derived from EcM fungi. EcM fungal-derived C could be in various forms, such as that released by actively foraging fungal mycelia, by modification of root exudation (quantity and quality) following EcM fungal colonisation of root tips, or from C released during decay of senescent fungal mycelia. Several studies have identified the main components of EcM fungal exudates, and key differences have been found between

mycorrhizal and non-mycorrhizal roots, especially with regards to organic acids (e.g. Ahonen-Jonnarth *et al.* 2001; van Hees *et al.* 2003, 2005; Fransson *et al.* 2007). Therefore, there is the potential that C from EcM fungi affects denitrification and subsequent N_2O:N_2 ratios. Here we report on two experiments that tested the hypothesis that EcM fungal C (from synthetic sources and natural exudates) would stimulate denitrification.

Methods

N_2O production from soil incubations with EcM fungal sources

Soil incubations were established containing sieved (< 2 mm) forest soil collected from the organic layer (0 – 10 cm depth; pH 3.5) and the underlying mineral layer (pH 3.7) of a semi-natural pine forest (northeast Scotland, UK). Mannitol and oxalic acid (known components of EcM fungal exudates) and glucose (an easily assimilated C source) were added to the soils as single doses at 3.6 g C/L. Nitrate was added as $K^{15}NO_3$ (10 at% excess ^{15}N) at 5 g N m^{-2}. The control treatment received nitrate addition only. Carbon and N additions were in solution, thus increasing the water-filled pore space of each soil type to 70%; this ensured denitrification was the dominant N_2O-producing process (Bateman and Baggs 2005). Each treatment was replicated three times. N_2O production was measured periodically over 14 days from sealed incubations and soil was sampled from additional replicate incubations.

N_2O production by a denitrifying bacterial culture with EcM fungal exudates

EcM fungal C was collected from two mycorrhizal sources: mycorrhizal exudate was collected from mycorrhizal birch seedlings (with the EcM fungus *Paxillus involutus*) growing in a sterile liquid nutrient medium, and C representing water-soluble C that would be released from decomposing fungal mycelia was collected from dried fungal cultures of *P. involutus*. Non-mycorrhizal root exudate was collected from birch seedlings (without *P. involutus*) growing in a sterile medium. These three C sources were provided as the sole C supply (10 µg C/L) to an anaerobic culture (with 25 mM $K^{15}NO_3$, 10 at% excess ^{15}N) of the denitrifying bacterium *Paraccocus denitrificans* 1222. Nutrient media (with and without glucose) were also provided as control treatments. Each treatment (C source) was replicated five times. N_2O production was measured every 4 hr over a 12 hr growth period.

Results and discussion

N_2O production from soil incubations with EcM fungal sources

Total N_2O production over 14 days was significantly higher ($P < 0.001$) in the organic soil than in the mineral soil (Figure 1). In the organic soil, addition of mannitol and oxalic acid (known components of EcM fungal exudate) had significant effects on N_2O production over 14 days compared to glucose and control treatments, whereas in the mineral soil, addition of different C sources had no effect on N_2O production (Figure 1). In the organic soil, mannitol as a C source increased N_2O production, while addition of oxalic acid reduced N_2O production. ^{15}N analyses of gas samples indicated higher enrichment of ^{15}N-N_2 from the organic soil oxalic acid treatment, suggesting that this C source stimulated greater nitrate reduction to N_2 compared to other C sources. The results suggest that when added as single C sources, mannitol and oxalic acid alter forest soil N_2O production, and that the use of oxalic acid in denitrification can lower the N_2O:N_2 product ratio. Thus oxalic acid is a higher quality C source for denitrifying bacteria compared to glucose or mannitol. EcM fungal biomass is lower in the mineral layer compared to the organic layer (Genney *et al.* 2006), and as N_2O production was not affected, this suggests that the microbial biomass is smaller and not 'conditioned' to assimilate EcM fungal C to the same degree as the microbes in the organic layer.

N_2O production from direct EcM fungal C sources by a denitrifying bacterial culture

In this culture experiment, different C sources (mycorrhizal root exudate, non-mycorrhizal root exudate, mycorrhizal fungal extract, and nutrient media controls) were provided at the same C concentration to anaerobic bacterial cells. The maximum growth rates and final biomass from all treatments were similar after 12 hr growth period, however, N_2O and N_2 production was significantly different. Peak N_2O production occurred at different times for each treatment ($P < 0.001$), and bacterial cells growing in the mycorrhizal fungal extract had the highest N_2O production at 12 hr (Figure 2). There was a clear preference shown by *P. denitrificans* for the two mycorrhizal C sources, as ^{15}N-N_2O and ^{15}N-N_2 production were significantly greater in these treatments compared to the non-mycorrhizal root exudate and control treatments ($P < 0.05$). Qualitative analyses (dissolved organic carbon and specific UV absorbance) of the different C sources indicated that the mycorrhizal exudate and extract C sources contained a higher proportion of aromatic compounds, compared to the non-mycorrhizal root exudate, which are known to be produced by *P. involutus*

and other EcM fungi (Dickinson and Hutchison 1997). These novel results show the direct use and preference for mycorrhizal C sources by denitrifying bacterial cells.

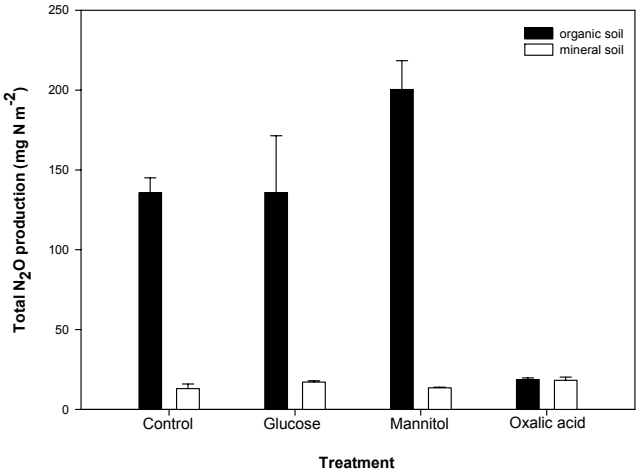

Figure 1. Total N_2O production over 14 days from forest organic and mineral soil layers after addition of glucose, mannitol and oxalic acid as single doses (3.6 g C/L).

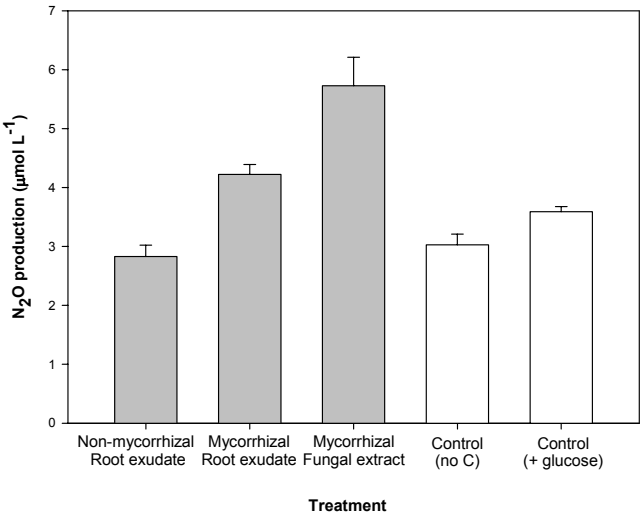

Figure 2. Final N_2O production after 12 hr growth by *P. denitrificans* 1222, under anaerobic conditions, with different C sources added at 10 µg C/L: non-mycorrhizal root exudate; mycorrhizal root exudate; mycorrhizal fungal extract; and controls (media without added C, and media with added glucose).

Conclusion

EcM fungi are the dominant microbes in acidic soils of boreal and temperate forests, and despite their important roles in biogeochemical cycling, this group of microbes has been largely ignored as a potential factor controlling N_2O production via denitrification. The key results indicate (1) that EcM fungi may have a role to play in denitrification, via provision of high quality C to denitrifying bacteria; and (2) that bacterial denitrifiers in the forest organic soil layer may be 'conditioned' to utilising mycorrhizal C compounds. Therefore, in order to understand the mechanisms and factors controlling N_2O emissions from boreal and temperate forest soils, the presence of EcM fungi and their potential contribution to denitrification and N_2O production rates, should be taken into account and investigated further.

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