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Soil Biology and Biochemistry

DOI:
10.1016/j.soilbio.2017.10.024

Published: 01/02/2018

Peer reviewed version

Microbial competition for nitrogen and carbon is as intense in the subsoil as in the topsoil

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ABSTRACT

Most studies on plant nutrition tend to focus on the topsoil (plough layer) and frequently neglect subsoil processes. However, cereal roots can potentially acquire nutrients including organic and inorganic nitrogen (N) from deep in the soil profile. Greater knowledge on the interaction of plants and microbes in subsoil environments is required to evaluate whether deep rooting traits in cereals will achieve greater nutrient use efficiency and greater soil carbon (C) storage in cropping systems. This study aimed to evaluate the relationship between root distribution, organic and inorganic N availability and potential N supply at the critical growth period during the wheat cropping cycle in a sand textured Eutric Cambisol. Our results provide evidence of significant microbial capacity in the subsoil. The rate of plant residue turnover and the mineralization of organic C and N substrates (glucose, amino acids, peptides, protein) declined slightly with increasing soil depth; however, these rates were not correlated with basal soil respiration, microbial biomass or community structure. This suggests that the microbial population in subsoil is more C limited but that its activity can be readily stimulated upon C substrate addition. A significant potential for organic and inorganic N turnover was also demonstrated at depth with a similar abundance of ammonifiers and ammonia oxidizing bacteria (AOB) and archaea (AOA) throughout the soil profile. Again, N mineralization in subsoils appears to be substrate limited. Root density declined rapidly down the soil profile with few roots present past 50 cm; suggesting that this is the major factor limiting C recharge of soil organic matter and microbial activity in subsoils. Greater root proliferation at depth could allow greater capture of water and the recapture of N lost by leaching; however, our results suggest that plant-microbial competition for C and N is as intense in the subsoil as in the topsoil. We conclude that while deeper rooting may improve nutrient and water use efficiency it may not lead to much greater C sequestration in subsoils, at least in the short term.

Keywords: Ammonium; Dissolved organic nitrogen; Nitrate; Nitrification; Rhizosphere

1. Introduction

In high input agricultural systems, nitrogen (N) availability is largely controlled by fertilizer events and the subsequent transformation and redistribution of N within the soil (Van Egmond et al., 2002). Typically, however, only 50% of the N applied to the crop in temperate climates is taken up by the plant indicating low rates of N use efficiency (Lassaletta et al., 2014). In many countries, however, there is a move to reduce the reliance on mineral fertilizers and to use added and intrinsic soil N reserves more efficiently (Chen et al., 2016). Ultimately, this aims to reduce economic costs as well as simultaneously lowering losses via leaching (NO_3^-), denitrification (N_2/N_2O) and volatilization (NH_3). Increases in N efficiency can potentially be achieved using a range of plant-based strategies (e.g. changes in root architecture combined with deeper rooting, release of nitrification inhibitors, use of N_2-fixers; Liu et al., 2013) as well as changes in agronomic practice (e.g. improvements in fertilizer timing, formulation, placement; Hoyle and Murphy, 2011; Sartain and Obrezai, 2010). Under some of these scenarios it is likely that plants will have to take up and utilise a wider range of organic and inorganic N forms (e.g. amino acids, peptides and polyamines). We hypothesize that this will increase the competition between plant roots and soil microbial community associated with both the mineralization of N contained in soil organic matter (SOM) (via the direct release of root proteases or stimulation of SOM priming) and the capture of any N released in both the topsoil and subsoil (Bardgett et al., 2003; Farrell et al., 2013; Kaiser et al., 2015).

As soils frequently become progressively drier during the growing season, there is a decreased root capture of water and nutrients from the topsoil, leading to the growth of a few roots to depths often in excess of 1 m (DuPont et al., 2014). This suggests that the subsoil may play a more significant role in N supply later in the season, especially under reduced fertilizer input regimes. This may also promote carbon (C) sequestration in subsoils although the evidence to support this remains controversial (Agostini et al., 2015; Menichetti et al., 2015). Plant and microbial N cycling in deeper soil horizons, however, have received much less attention than in surface soils. If we are to capitalize on the deep rooting phenomenon of most cereals and the potential to manipulate root architecture (breeding, genetic modification; Fang et al., 2017), it is important that we understand water and nutrient availability in deeper soil layers as well as the microbial processes that control them (e.g. SOM dynamics; Zhang et al., 2014).

Agronomic estimates of N supply to plants are typically predicted from the amount of inorganic N released during the laboratory incubation of soils collected within the plough layer (0-30 cm). These mineralization rates are unlikely to be representative of deeper soil layers and ways of integrating potential N supply from subsoil is therefore needed. The amount and turnover of N in subsoil will largely depend on its exchange capacity, structure, organic material availability and microbial activity. It is well established that significant microbial activity may occur at depth (Doran, 1987; Soudi et al., 1990), albeit at much lower levels and with a different community structure than occurs in topsoil (Federle et al., 1986). When considering microbial processes at depth a key component with respect to N cycling is the abundance of ammonia-oxidising archaea (AOA) and bacteria (AOB) that are responsible for the rate limiting step in nitrification and thus potential N loss. Dominance of AOA relative to AOB in the *amoA* (ammonia monooxygenase) soil gene pool has been reported in many ecosystems globally. Substrate availability and pH have been identified as the major drivers of niche specialization between AOA and AOB, with AOA being reported to be more competitive in acidic, organic matter depleted soil conditions at depth than AOB (He et al., 2012; Zhang et al., 2012; Banning et al., 2015). However, variation in soil factors such as water and oxygen availability are also important factors which differ in subsoil and which may play a role in regulating population abundances to depth. The quantity and quality of organic inputs to subsoil may also be different to the soil surface due to lower rates of root and microbial turnover and the lack of leaf litter and crop residue inputs. Subsoil soil organic matter has also been suggested to be older and more recalcitrant than in the topsoil (Schrumpf et al., 2013; Torres-Sallan et al., 2017). While this may favour C sequestration, it may conversely limit N supply to the plant.

Root length density (RLD) has been used as a proxy to predict water and nutrient uptake by plants (Taylor and Klepper, 1975; Herkelrath et al., 1977). This relationship can work well when there is adequate soil moisture available; however, it lacks precision when surface soils become prone to drying. The root systems of mature wheat plants typically extend deeper than 120 cm by the end of the growing season. However, the time at which maximal crop N demand and subsoil exploitation coincide is earlier in the season (i.e. Growth Stages GS31-71, stem elongation to the start of flowering; AHDB, 2015). Further, even though roots may extend deeper into the subsoil, their density may be extremely low (Li et al., 2017). This study therefore aimed to evaluate the relationship between root distribution, organic and inorganic N availability and potential N supply at this critical period during a wheat growing cycle. We hypothesized that the subsoil microbial population would be very low due to the lack of supply of available C and N from plant roots and associated mycorrhizas. Further, this nutrient limitation would lead to a more fungal and Gram+ dominated community and that this would be slow to respond to C substrate addition leading to a greater potential to retain C in subsoils. We also hypothesized that slow rates of organic N addition would lead to low populations of AOA and AOB and little potential to generate NO_3^-, thus also favouring N retention in subsoils.

2. Materials and methods

2.1. Site characteristics

Soil was collected from a replicated wheat field trial site located in Abergwyngregyn, North Wales (53°14'29"N, 4°01'15"W) and is classified as a sand textured Eutric Cambisol. The soil pH is 6.3 and does not vary significantly with depth (0-60 cm; $P > 0.05$). The bulk density in the topsoil (0-30 cm) is 1.48 ± 0.12 g cm^{-3} and in the subsoil (30-60 cm) 1.63 ± 0.10 g cm^{-3}. The climate at the site is classed as temperate-oceanic with a mean annual soil temperature of 11°C at 10 cm depth and a mean annual rainfall of 1250 mm yr^{-1}. The field trial consisted of six replicated plots (12.5 × 3 m) which were ploughed (0-30 cm) and planted with spring wheat (*Triticum aestivum* L. cv. Granary) in May 2013. Fertilizer was added after crop emergence (60 kg N ha^{-1} as ammonium nitrate, 80 kg K ha^{-1}, 28 kg P ha^{-1}) and dicot herbicides applied following standard agronomic practice.

Soil water content, crop height and biomass were determined weekly by destructive sampling throughout the growing season. Briefly, in six replicate plots, all the crop biomass was removed within a sub-plot (0.5 m × 0.5 m), the samples placed in paper bags and the harvested biomass dried at 80°C for 7 d to determine dry weight. At the same time, crop height was recorded at 5 points (1 m apart) within each of the six plots. Soil water content was determined weekly by destructive sampling throughout the growing season. Briefly, topsoil (0-30 cm) and subsoil (30-60 cm) samples were taken from six replicate plots, sieved to pass 2 mm and a subsample used to determine moisture content by drying at 105°C overnight.

Duplicate soil samples were collected from 4 of the 6 plots in July, 2013, when the plants had reached late stem extension (Feekes growth stage 9, Zadoks growth stage 39; Large, 1954; Zadoks et al., 1974) corresponding to the period of maximum plant N demand (AHDB, 2015). To estimate root density, intact soil cores were taken to a depth of 80 cm using a Cobra-TT percussion hammer corer (Eijkelkamp Agrisearch Equipment, 6987 EM Giesbeek, The Netherlands). After removal from the soil, the intact cores were split into 10 cm sections, the samples transferred to CO_2 permeable polythene bags and placed at 4°C to await root recovery and soil analysis. As there were very few roots in the 60-80 cm layer, soils were only analyzed to 60 cm for the microbial N cycling and N pool size estimates. For root analysis, one of the duplicate cores was maintained intact, however, for the remaining soil analyses, the second soil core was sieved to pass 2 mm, removing any vegetation, stones and earthworms and experiments started within 48 h of field collection.

2.2. Quantification of root length density and soil respiration

Roots were washed from the soil cores by a combination of mechanical shaking and flotation using a 1 mm mesh to capture roots. The roots were then placed on 20 × 20 cm clear plastic plates and root length determined with WinRhizo® (Regent Instruments Inc., Ville de Québec, Canada).

Basal respiration was determined on field-moist soil (50 cm^3) in the laboratory at 20°C over 24 h using an SR1 automated multichannel soil respirometer (PP Systems Ltd, Hitchin, UK). Visible roots were removed prior to analysis. The mean respiration rate was determined for the last 6 h of the measurement period when the CO_2 efflux rates had quasi-stabilized.

2.3. Soil solution extraction and soil chemical analysis

Soil N availability was estimated according to Jones and Willett (2006). Briefly, 5 g of field-moist soil was extracted with 25 ml of 0.5 M K_2SO_4 on a reciprocating shaker (Edmund Bühler GmbH, SM-30, Germany; 200 rev min^{-1}) for 60 min. After shaking, samples were centrifuged (10 min; 1699 *g*) and the supernatant recovered and stored at -20°C to await analysis.

Soil solution samples were analyzed for dissolved organic C and total dissolved N (TDN) using a Multi N/C 21005 (Analytik-Jena AG, Jena, Germany). Total amino acid-N was determined fluorometrically using the *o*-phthaldialdehyde-β-mercaptoethanol procedure of Jones et al. (2002). Nitrate and NH_4^+ were analyzed colorimetrically using the methods of Miranda et al. (2001) and Mulvaney (1996) respectively. Dissolved organic N (DON) was calculated by subtraction of inorganic N (NO_3^- and NH_4^+) from TDN.

Total C and N of soils were determined on ground soil using a Truspec CN analyzer (Leco Corp., St Joseph, MI, USA). Soil pH and electrical conductivity (EC) were determined in soil:distilled water extracts (1:5 v/v) with standard electrodes, while moisture content was determined by oven drying (105°C, 24 h). The gravimetric moisture contents were corrected for stone-corrected bulk density to allow expression of water content on a volumetric basis.

2.4. Net N mineralization and nitrification

Net N mineralization was determined by anaerobic incubation according to Waring and Bremner (1964) and Kresoivć et al. (2005). Briefly, 10 g of field-moist soil was placed in 50 cm^3 polypropylene tubes and anaerobic conditions imposed by filling the tubes with distilled water and then sealing the tubes. Soil samples were then incubated for 7 d in the dark at 40ºC. Subsequently, solid KCl was added to achieve a final concentration of 1 M KCl and the samples extracted by shaking for 60 min (200 rev min^{-1}). The extracts were then centrifuged (1699 *g*, 10 min) and NH_4^+ determined as described previously. Net ammonification was calculated as the amount of NH_4^+ present after 7 d minus that present at the start of the incubation.

Net nitrification was determined according to Hart et al. (1994). Briefly, 5 g of field-moist soil from each soil layer was placed in a 50 cm^3 polypropylene tube. The tubes were then loosely sealed and the samples incubated in the dark at 20°C. After 30 d, the soil was subsequently extracted with 0.5 M K_2SO_4 and NO_3^- and NH_4^+ determined as described above. Net ammonification and nitrification was calculated as the amount of NO_3^- and NH_4^+ present after 30 d minus that present at the start of the incubation.

2.5. Amino acid, peptide, protein and glucose turnover

To estimate rates of DON turnover, the mineralization of amino acids, oligopeptides and protein were determined. For comparison, the turnover of glucose was also used as a general reporter of soil microbial activity (Coody et al., 1986). Briefly, field-moist soil (5 g) was placed in 50 cm^3 polypropylene containers and 0.5 ml of either ^{14}C-labelled glucose (25 mM, 1.85 kBq ml^{-1}), amino acids (10 mM, 1.55 kBq ml^{-1}), peptides (25 mM, 1 kBq ml^{-1}) or protein (13.2 mg l^{-1}, 51 kBq ml^{-1}) added to the soil surface (Farrell et al., 2011). After the addition of each ^{14}C-substrate to the soil, a $^{14}CO_2$ trap containing 1 ml of 1 M NaOH was placed above the soil and the tubes sealed. With the exception of protein, the tubes were then incubated at 20°C for 30 min after which the NaOH traps were removed to determine the amount of substrate mineralized. In the case of protein, the procedure was identical except that the incubation period was 24 h. The ^{14}C content of the NaOH traps was determined with Wallac 1404 liquid scintillation counter (Wallac EG&G, Milton Keynes, UK) after mixing with Scintisafe3 scintillation cocktail (Fisher Scientific, Loughborough, UK). The amino acids consisted of an equimolar mix of 20 different L-amino acids (L-glycine, L-isoleucine, L-arginine, Lglutamine, L-phenylalanine, L-histidine, L-asparagine, L-valine, L-threonine, L-leucine, L-alanine, L-methionine, L-cysteine, L-lysine, L-tryptophan, L-serine, L-proline, L-glutamate, L-aspartate acid, L-ornithine) while the L-peptides consisted of a mixture of equimolar L-dialanine and L-trialanine. The mixed soluble plant protein was purified from ^{14}C-labelled tobacco leaves (American Radiolabeled Chemicals Inc., St Louis, MO, USA).

To determine the rate of arginine mineralization, 0.5 ml of a ^{14}C-labelled L-arginine solution (25 mM; 2.17 kBq ml^{-1}; Amersham Biosciences UK Ltd, Chalfont St Giles, Bucks, UK) was added to 5 g of field-moist soil and the rate of $^{14}CO_2$ evolution measured over a 48 h as described in Kemmitt et al. (2006). After 48 h, the net amount of NH_4^+ and NO_3^- produced from the added arginine was determined by extracting the soil with 25 ml 0.5 M K_2SO_4 and subsequent analysis as described previously.

2.6. Mineralization of plant-derived C

The microbial turnover of complex, plant-derived C across the different soil depths was evaluated according to Glanville et al. (2012). Briefly, high molecular weight (MW) plant material was prepared by heating 2.5 g of ^{14}C-labeled *Lolium perenne* L. shoots (Hill et al., 2007) in distilled water (25 ml, 80°C) for 2 h. The extract was then centrifuged (1118 *g*, 5 min) and the soluble fraction removed. The pellet was then resuspended in distilled water and the heating and washing procedure repeated twice more until >95% of the water soluble fraction had been removed. The pellet remaining was dried overnight at 80ºC and ground to a fine powder.

The mineralization dynamics of the high MW plant material was determined by mixing 100 mg of ^{14}C-labelled plant material with 5 g of field-moist soil. The production of $^{14}CO_2$ was monitored as described above for the low MW substrates but over 40 d. To ensure that water was not limiting, the experiment was also repeated but after the simultaneous addition of distilled water (to reach field capacity) and the ^{14}C-labelled plant material.

2.7. Nucleic acid extraction and quantitative PCR (qPCR)

For each soil sample, DNA was extracted from duplicate 800 mg sub-samples using UltraClean® DNA Isolation Kit (MoBio Laboratories Inc., Carlsbad, CA, USA). Cell lysis was performed using a Mini Bead beater (BioSpec Products Inc., Bartlesville, OK) at 2500 rev min^{-1} for 2 min. Duplicate DNA extractions were combined to give a total extract volume of 100 µl.

Functional genes, archaeal and bacterial *amoA*, were quantified using a ViiA7 qPCR machine (Thermo Fisher Scientific, Scoresby, Australia). Each 20 µl qPCR reaction contained 10 µl of Power SYBR® Green PCR Master Mix (Thermo Fisher Scientific). 0.2 µl of the specific forward and reverse primer at a concentration of 10 µM, 2 µl BSA (Ambion UltraPure BSA; 5 mg ml^{-1}; Thermo Fisher Scientific), 8 ng template DNA and sterile water to 20 µl. Primers and thermal cycling conditions for both bacterial (primers amoA-1F and amoA-2R) and archaeal (primers Arch-amoAF and Arch-amoAR) *amoA* genes were as described previously (Banning et al., 2015). Melting curves were generated for each qPCR run and fluorescence data was collected at 78°C to verify product specificity. Each qPCR reaction was run in triplicate. Standard curves were generated using dilutions of linearized cloned plasmids. Template amplified with each primer pair described above, was cloned with the P-GEM T-easy system (Promega Inc., Madison, WI), plasmid DNA extracted and inserts sequenced using Big Dye Terminator chemistry (Australian Genome Research Facility, Western Australia) to confirm correct length and identity. The standard curve gene sequences were as described previously (Barton et al., 2013). Standard curves generated in each reaction were linear over four orders of magnitude (10^4 to 10^7 gene copies) with r^2 values greater than 0.99. Efficiencies for all quantification reactions were 80-100%.

2.8. Microbial community structure

Microbial community structure was measured by phospholipid fatty acid (PLFA) analysis following the method of Buyer and Sasser (2012). Briefly, samples (2 g) were freeze-dried and Bligh-Dyer extractant (4.0 ml) containing an internal standard added. Tubes were sonicated in an ultrasonic bath for 10 min at room temperature before rotating end-over-end for 2 h. After centrifuging (10 min) the liquid phase was transferred to clean 13 mm × 100 mm screw-cap test tubes and 1.0 ml each of chloroform and water added. The upper phase was removed by aspiration and discarded while the lower phase, containing the extracted lipids, was evaporated at 30°C. Lipid classes were separated by solid phase extraction (SPE) using a 96-well SPE plate containing 50 mg of silica per well (Phenomenex, Torrance, CA). Phospholipids were eluted with 0.5 ml of 5:5:1 methanol:chloroform:H_2O (Findlay, 2004) into glass vials, the solution evaporated (70°C, 30 min). Transesterification reagent (0.2 ml) was added to each vial, the vials sealed and incubated (37°C, 15 min). Acetic acid (0.075 M) and chloroform (0.4 ml each) were added. The chloroform was evaporated just to dryness and the samples dissolved in hexane. The samples were analyzed with a 6890 gas chromatograph (Agilent Technologies, Wilmington, DE) equipped with autosampler, split-splitless inlet, and flame ionization detector. Fatty acid methyl esters were separated on an Agilent Ultra 2 column, 25 m long × 0.2 mm internal diameter × 0.33 µm film thickness. Standard nomenclature was followed for fatty acids (Frostegård et al., 1993).

2.9. Statistical and data analysis

Statistical analysis of the results was carried out by ANOVA followed by Tukey HSD post hoc test and linear regression using SPSS v14 (IBM UK Ltd, Hampshire, UK) with $P < 0.05$ used as the level to define significance. Analysis of differences in qPCR abundances of bacterial and archaeal *amoA* across soil depth was performed by analysis of variance (one-way ANOVA) using GenStat (15^{th} edition; Lawes Trust, Harpenden, UK). Principal component analysis was performed in R'.

3. Results

3.1. Crop and soil characteristics

As expected, crop height showed a sigmoidal extension pattern over the growing season with full stem extension evident after 8 weeks (Fig. 1a). Crop biomass also showed a sigmoidal growth pattern, however, above-ground biomass continued to increase up until week 13 due to progressive grain filling (Fig. 1a).

Corresponding with the period of maximum crop development and low rainfall, soil water content declined dramatically between weeks 5-8 in both the topsoil and subsoil; with soil water in the subsoil being consistently lower than in the topsoil ($P < 0.05$; Fig.1b). At week 9, significant amounts of rainfall caused recharge of the soil profile with the topsoil retaining significantly more water than the subsoil ($P < 0.01$; Fig. 1b).

Root length density decreased down the soil profile, with the vast majority located in the topsoil (Fig. 2a). Less than 4% of total root length density was in the subsoil below 30 cm. Soil total and bio-available C pools also decreased with increasing depth (Table 1). Soil basal respiration was significantly greater ($P < 0.05$) in the 0-20 cm layer (Fig. 2b) with the pattern matching that of root density distribution.

3.2. Mineral N cycling

Ammonium and nitrate concentrations in the field-collected samples were significantly greater in the surface (0-10 cm) layer ($P < 0.05$) than in the deeper soil horizons (Table 1). Overall, the patterns of N mineralization in the aerobic and anaerobic incubations were similar, decreasing in an exponential pattern down the soil profile (Fig. 3). The concentration of NH_4^+ after 30 d of aerobic incubation only increased significantly in the 10-20 cm soil layer (Fig. 3). Aerobic net N mineralization within the 0-20 cm layer of the soil profile was significantly greater ($P < 0.05$) compared to the 40-60 cm layer (Fig. 3). In contrast to the aerobic incubation, the anaerobically incubated soils showed large increases in NH_4^+ concentration at all depths, with the largest increase occurring in the surface soil layer (Fig. 3).

3.3. Low molecular weight carbon substrate mineralization

Mineralization rates of low molecular weight C molecules tended to decrease slightly with depth (Fig. 4). While substrate mineralization in the topsoil (0-30 cm) was significantly greater compared to the subsoil there was still considerable mineralization occurring at 50-60 cm (Fig. 4). There was a 10,000 fold difference between protein and amino acid mineralization rates, with rates in the order amino acid > peptide > glucose > protein.

3.4. Arginine and plant residue turnover

The initial (0-6 h) arginine C mineralization rate decreased with soil depth ($P < 0.05$) (Fig. 5a). However, by 48 h the amount of arginine mineralization was statistically similar at all soil depths. While the rate of mineralization was linear in the topsoil, however, a lag phase in mineralization was observed in the subsoil horizons (data not shown). The net amount of NH_4^+ produced from the added arginine significantly increased with soil depth (Table 2). In contrast, however, the net amount of NO_3^-

decreased significantly with increasing soil depth. Overall, the ratio of C mineralization to N immobilization was greater in the topsoil than in the subsoil.

The rate of ^{14}C-labelled plant residue mineralization was much slower than those of the simple C substrates. Notably there was no significant difference in turnover rates between soil depths (P > 0.05; Fig. 5b).

3.5. AOA and AOB gene abundances

Nitrification capacity, as assessed by amoA gene abundance, was present throughout the soil profile. At every soil depth AOA gene abundance was significantly lower (P < 0.01) than AOB (Fig. 6). For AOB *amoA* gene copies ranged from 1×10^7 to 2×10^8 g^{-1} dry soil while AOA *amoA* gene copies ranged from 2 to 5×10^5 g^{-1} dry soil. There was no significant effect of depth on AOA population abundance (P > 0.05) but there was a significant effect of depth on AOB population abundance (P < 0.05) whereby AOB gene abundance was significantly lower (P < 0.05) in the subsoil below 30 cm than in topsoil (Fig. 6).

3.6. Microbial community structure

Total PLFA significantly decreased below 30 cm depth; with the amount of total PLFA relatively constant within topsoil and subsoil layers (Fig. 7a). Overall, the relative proportion of major microbial groups was quite similar at the different soil depths. The proportion of fungi and actinomycetes significantly increased with soil depth (P < 0.01 and 0.001 respectively) while the relative abundance of Gram-positive and Gram-negative bacteria both reduced (P < 0.01). The relative abundance of putative arbuscular mycorrhizal PLFAs (16:1 w5c) was similar at all depths (data not presented). Principal component analysis of the PLFA data revealed a separation of the topsoil and subsoil microbial communities (Fig. 8).

4. Discussion

4.1. Changes in microbial biomass, activity and community structure with depth

As expected, root abundance, microbial biomass and basal respiration all declined with soil depth (Kramer et al., 2013; Li et al., 2014; Loeppmann et al., 2016). In many cases, these changes can be attributable to excess acidity and toxic levels of Al^{3+} in the subsoil (Tang et al., 2011). In our study, however, soil pH did not vary down the profile and therefore this does not represent a confounding factor. Microbial community structure also shifted down the soil profile with the fungal-to-bacterial ratio increasing with depth, presumably due to increased C limitation and the lower N requirement of fungi rather than due to a shift in soil pH. This is in agreement with the results of Sanaullah et al. (2016) in grasslands but contrasts with the results of Kramer et al. (2013) and Stone et al. (2014) who showed either no effect or a strong decrease in fungal-to-bacterial ratio with depth. Based on the slow growth of Gram+ bacteria and there greater ability to survive C starvation (De Vries and Shade, 2013), we expected to see an increased Gram+-to-Gram- ratio with depth. Although our results do support this to some extent, the overall effect was quite small. Despite the low microbial biomass, however, we demonstrate that high rates of both soluble and insoluble C and N turnover can occur at depth. Generally, however, microbial processes have a tendency to be greatest in surface layers, especially when soil disturbance is minimised (Murphy et al., 1998). Numerous factors could contribute to lower microbial activity in deep soils. The results obtained here show a much lower abundance of roots at depth so it is likely that there is less soluble organic C or fresh particulate C being delivered to the subsoil via root exudation and root/mycorrhizal turnover (Fontaine et al., 2007). The lack of earthworm presence in our soil also prevents the bioturbation-driven delivery of C to the subsoil and limits subsoil biological hotspots in the form of deep vertical earthworm burrows (Uksa et al., 2015; Hoang et al., 2016). Microbes at depth therefore experience strong C limitation which is supported by the decrease in C-to-N ratio with depth in some soils (Rumpel and Kögel-Knabner, 2011). In addition, the increasing DOC-to-DON ratio with depth suggests that the DOC may be becoming more chemically recalcitrant (i.e. humic-like) down the soil profile. Other mechanisms which may also restrict microbial activity in subsoils include: (1) an increased bulk density which may suppress root growth; (2) greater structural aggregation which may both restrict root access and promote the physical protection of C; (3) a greater abundance of clay and oxyhydroxides which may stimulate the chemical protection of C; and (4) greater moisture contents and resulting anoxia which may supress root and microbial activity (Kinyangi et al., 2006; Rumpel and Kögel-Knabner, 2011). In the context of our well drained, sandy-textured soil we expect the influence of these factors to be relatively low.

Soluble organic N concentrations decreased with soil depth suggesting that the microbial community could also be N limited at depth. Based on the evidence presented, we ascribe these low concentrations to the low rate of DON supply from rhizodeposition and SOM turnover combined with the rapid microbial removal of labile DON from solution. As added soluble-N was readily mineralized to NH_4^+ in our subsoils we conclude that subsoil microbial activity is driven more by C limitation rather than by N limitation. This view is also supported by Jones et al. (2005) who demonstrated that the microbial use of DON compounds was largely insensitive to N fertilizer regime and more related to C availability than N availability in a range of agricultural soils. In addition, we observed a relatively high concentration of NO_3^- at depth. NO_3^- tends only to be utilized in large amounts by microorganisms under severe N deficiency due to the energetic costs associated with its assimilation (in comparison to DON and NH_4^+), again suggesting that the subsoil microbial community is not N limited (Abaas et al., 2012).

4.2. Implications for subsoil C storage

Reduced microbial activity at depth has led to the suggestion that subsoils may have the potential to lock up additional C and that this could help offset anthropogenically derived greenhouse gas emissions (Lynch and Wojciechowski, 2015; Pierret et al., 2016; Gocke et al., 2017; Torres-Sallan et al., 2017). One widely proposed mechanism to stimulate this transfer of C into subsoils is the use of crops with deep rooting traits (Lavania and Lavania, 2009; Kell, 2011) or shifts towards less intensive land management systems (Ward et al., 2016). It should be noted, however, that much controversy surrounds the stability of C in subsoils with many reports suggesting it persists for long time periods and is more stable than C in topsoils (Kramer and Gleixner, 2008; Müller et al., 2016). The evidence presented here clearly showed that while the rates of plant residue turnover were initially slower in subsoils in comparison to topsoils (0-24 h), these differences disappeared over longer incubation times (e.g. 30 d). This suggests that the subsoil microbial community quickly adapted to an increased C supply. This directly challenges the assumption that increasing the rate of C supply to subsoils will lead to greater long term C storage. It is also consistent with measurements showing that most subsoil C is of recent origin and not very stable (Hobley et al., 2017; Zhang et al., 2014). Our results also support the results from Brauer et al. (2013) and Matus et al. (2014) who suggest that C storage in subsoils is mainly driven by microbially-processed C being translocated down the soil profile as DOC and then becoming chemically protected, rather that C generated in situ within subsoils.

It should be emphasized that the discussion above mainly relates to the potential for accumulating subsoil C over a limited number of cropping cycles (i.e. 1-10 y). Over longer time scales it is conceivable that small amounts of C may become progressively stabilized in subsoils leading to substantial C increases over decadal time scales. Current evidence suggests that long-term shifts in agronomic management (>40 years) targeted at surface residue management and tillage regime can substantially increase topsoil C levels, but that they have limited capacity to alter subsoil C storage (Jarvis et al., 2017; Kinoshita et al., 2017). This provides strong evidence that C migration from top- to sub-soils is not an effective mechanism for promoting C storage in deeper soil layers. An alternative to relying on roots to deliver C to subsoils is the deep incorporation (>50 cm depth) of crop residues into soil (Alcantara et al., 2017; Cui et al., 2017). Unlike cereal roots whose C-to-N ratio ranges from 15-30, the low N content of crop residues (C-to-N ratio = 50-80) is more likely to favour C retention (and may additionally suppress N losses via leaching). It is clear, however, that more long-term field trials are required to critically address whether deeper rooting crops lead to enhanced C sequestration.

4.3. Variation in soil N cycling with depth

Protein represents the major input of organic N into cropping soil systems. Therefore, the mineralization of protein, oligopeptides and amino acids is an important part of the N cycle and supplies the substrate for inorganic N production and therefore root N uptake (Jones et al., 2013). In topsoils it has been proposed that the breakdown of proteins to peptides is the main rate limiting step in the soil N cycle (Jan et al., 2009) and the evidence presented here clearly suggests that this is also the case for subsoils. However, when expressed per unit of microbial biomass, protein breakdown rate was much greater in subsoils than topsoils. Contrary to Loeppmann et al. (2016), this could either imply that the proteases have a greater substrate affinity at depth or it may relate to greater substrate availability (i.e. less substrate sorption to the solid phase).More work is therefore required to understand the factors regulating the production and behaviour of proteases in subsoils. Pinggera et al. (2015) recently demonstrated that subsoil protease activity was upregulated when abundant substrate was available but was repressed if sufficient inorganic N was present. In line with our results, this suggests that the microbial community will readily respond to substrate addition. Further, it also suggests that addition of high C:N residues (e.g. values >20) might stimulate positive priming and the mining of subsoil SOM to release N.

Arginine addition caused the rapid mineralization of amino acid-N to NH_4^+ at all soil depths, again demonstrating that ammonification was not a rate limiting step at any point in our soil profile. In addition, our soil incubation results showed that NH_4^+ only increased under anaerobic conditions, when C degradation and nitrification are oxygen limited. As rapid ammonium oxidation occurred readily under aerobic conditions it also supports the premise that N cycling in both topsoils and subsoils is limited by upstream elements in the N cycle (i.e. substrate availability for protease action).

Our results also reveal much greater nitrification potential in surface soils than at depth which may be indicative of a larger active community of nitrifiers. Therefore, more NH_4^+ would be transformed to NO_3^- in the surface soil and potentially more could be lost as N_2O or N_2. We show that the vertical distribution of both AOA and AOB were strongly correlated with each other (r^2 = 0.92, P<0.01) and also with total microbial PLFA (r^2 > 0.82, P<0.05). Further, AOB abundance closely correlated with the wider Gram- bacterial community of which it forms part (r^2 = 0.87, P<0.05). This implies that nitrification has no specialist niche in the soil profile relative to the more general aspects of soil organic C and N cycling. The results also do not support the proposal that AOB and AOA communities behave differently in different soil layers (Wang et al., 2014). In surface soils we found an increased abundance of AOB when compared to lower depths, and additionally report that AOA abundance was much lower than for AOB (ca. 200-fold) and did not vary as greatly with depth. Our results contrast with Fisher et al. (2013), Uksa et al. (2014), Wang et al. (2014) and Liu et al. (2016) who all showed that AOA abundance was much greater than AOB, particularly in subsoils. From our results, we infer that AOB are likely driving nitrification in this system due to the increase in measured nitrification in conjunction with an increase in AOB but not AOA abundance in surface soils. It is also likely that AOB are more active and can respond more quickly to additions of organic N and NH_4^+ derived from this (Di et al., 2010). This is consistent with a number of other studies (Di et al., 2009, 2010; Barton et al., 2013; Banning et al., 2015) who also suggest that AOB are likely driving topsoil nitrification.

In addition, we found that NH_4^+ concentrations in the field were significantly greater in the topsoil than at depth, which we ascribe to its higher organic matter and cation exchange capacity. This likely favours AOB over AOA with previous studies showing that AOA may only have a competitive advantage at low ammonium concentrations due to their greater substrate affinity (Martens-Habbena et al., 2009) or due to greater sensitivity to growth inhibition at high ammonium concentrations (Prosser et al., 2012). Soil pH is often described as having a significant influence on AOA and AOB abundance, although reports are not consistent. Some studies have reported AOB to be more sensitive than AOA to pH changes (Nicol et al., 2008; Yao et al., 2011). For example Yao et al. (2011) observed that AOB were more abundant in neutral and alkaline conditions than in acidic conditions, whereas there was no correlation between pH and AOA abundance. In contrast, Pereira e Silva et al. (2012) found soil pH did not influence AOB abundance but did increase AOA abundance; while Nicol et al. (2008) reported only AOA abundance and not AOB, was influenced by pH. In the current study pH did not change with depth and thus the increased abundance of AOB in the surface is not likely related to pH in this study.

It should also be noted that although there may be a significant reserve of nutrients at depth, these may be also physically or chemically protected, especially in well structured subsoils. It is therefore important for future studies to consider not only the size of the nutrient pool, but also the gross flux through this pool and its bio-accessibility

4.4. Implications of N cycle variations with depth for root uptake

Deeper rooting may promote the more efficient use of nutrients such as N and P (Lynch and Wojciechowski, 2015). However, we hypothesized that roots at the surface would be involved more in nutrient uptake than those at depth. The greater root length observed in this study corresponds with the areas of greater microbial activity, N concentrations and turnover rates. Therefore, it is likely that much more N is taken up by surface roots than those at depth. The surface soil is also likely to be the area where microbial N demand is greatest. Greater root length in topsoils would therefore allow for greater competition with microbes. The greater bulk density at depth may also suppress root growth making the access of nutrients more difficult (Salome et al., 2010).

Water uptake is as important a function of plant roots as nutrient uptake. The uptake rate of water is often proportional to root length density (Hinsinger et al., 2009; Hodge et al., 2009). During drought, soil surfaces dry, limiting both water and, potentially, nutrient uptake in roots near the surface. This can lead to near-surface roots dying and greater root growth at depth (Smucker et al., 1991). In dry conditions, deeper roots could become vital for maintaining plant nutrient uptake. In addition, more roots at depth could lead to a greater input of exudates which would increase microbial activity and decrease nutrient loss (Fisk et al., 2015). It may also promote the microbial priming of subsoil SOM and the loss of stable C from soil (Fontaine et al., 2007).

Contrary to expectation, the subsoil appeared to retain less water than the topsoil. We ascribe this to its lower SOM content which is known to aid water retention and promote soil structure (Rawls et al., 2003). Further, the subsoil dried out and rewet at a similar rate to the topsoil. This does not support the hypothesis that soil moisture becomes proportionally more available in subsoil as the soil progressively dries out due to evapotranspiration losses. Our results suggest that irrespective of root length density, water is removed evenly throughout the soil profile to balance plant demand, or less likely, that plant-mediated hydraulic lift is redistributing water from deeper soil layers to the surface. This suggests that drying out of the soil profile does not induce spatial niche partitioning in N availability.

4.5. Conclusions

In terms of plant-microbial nutrient cycling, subsoils remain understudied in comparison to topsoils. In addition to providing water to plants, however, recent reviews have suggested that subsoils may represent an important store of nutrients and have the potential to sequester large amounts of C (Torres-Sallan et al., 2017). Consequently, there is a growing view that subsoils should be actively managed to optimise their functioning (e.g. by mechanical or plant-based interventions; Kell et al., 2011; Tang et al., 2011; Alcantara et al., 2016, 2017). The results presented here suggest that although the subsoil has a low and slightly different microbial community than the topsoil, in terms of C cycling, the subsoil microbial community rapidly responds to new inputs of organic C and N. This suggests that the use of deeper rooting plants may not enhance long-term C storage in subsoils, especially if they destabilize subsoil SOM through rhizosphere priming. Our results also show that, as expected, root proliferation is greatest in the region of the soil profile where nutrient cycling is greatest. At present, the routine sampling of agricultural subsoils is costly and problematic. Further, subsoils can be expected to have higher spatial heterogeneity than topsoils. Combined, this makes it difficult to make informed decisions for active subsoil management. We conclude that the potential future importance of subsoils in sustainable agriculture may have been overstated.

Acknowledgements

This research was funded by the UK Natural Environment Research Council (NE/I012303/1), the Sêr Cymru LCEE-NRN project, Climate-Smart Grass and the Australian Research Council Future Fellowship Scheme (FT110100246) awarded to DVM.

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List of Figure Captions

Fig. 1. Crop biomass and stem extension (Panel A) and soil water content in the topsoil (0-30 cm) and subsoil (30-60 cm) layers (Panel B) during the wheat cropping cycle from planting to harvest. Values represent mean ± SEM (n = 6). * and ** in Panel B indicate significant differences between depths at the $P < 0.05$ level and $P < 0.01$ level respectively. Note: Soil profiles for biochemical and molecular analysis were collected 8 weeks after planting.

Fig. 2. Density of primary (first order) and lateral (second and third order) roots (Panel A) and basal soil respiration at different depths in an agricultural wheat cropping soil (Panel B). Values are means ± SEM (n = 5). All measurements were made 8 weeks after planting. Different letters indicate significant differences between depths at the $P < 0.05$ level (Tukey's HSD).

Fig. 3. Net ammonification after incubation under anaerobic conditions for 7 days or net mineralization (ammonification and nitrification) after incubation under aerobic conditions for 30 days at different soil depths in an agricultural wheat cropping soil. Values represent means ± SEM (n = 4). Different letters indicate significant differences between depths at the $P < 0.05$ level (Tukey's HSD; lowercase for anaerobic incubation and uppercase for aerobic incubation).

Fig. 4. Mineralization of ^{14}C-labelled glucose, amino acids, oligopeptides and protein at different soil depths in an agricultural wheat cropping soil. Values represent means ± SEM (n = 4). Different letters indicate significant differences between depths at the $P < 0.05$ level (Tukey's HSD).

Fig. 5. Cumulative percentage of ^{14}C- arginine mineralization (Panel A) and cumulative percentage of ^{14}C-*Lolium perenne* shoots mineralization (Panel B) at different soil depths in an agricultural wheat cropping soil. Values represent means ± SEM (n = 4).

Fig. 6. Bacterial (AOB) and archaeal (AOA) *amoA* gene copy numbers at different soil depths in an agricultural wheat cropping soil. Values are means ± SEM (n = 4). Different letters indicate significant differences between depths at the $P < 0.05$ level (Tukey's HSD).

Fig. 7. Total microbial PLFA (Panel A) and the relative abundance of specific microbial PLFA markers (Panel B) at different soil depths in an agricultural wheat cropping soil. Values are means ± SEM (n = 4). In Panel B the fungal PLFA marker data have been multiplied x10 for scaling purposes. Different letters indicate significant differences between depths at the $P < 0.05$ level (Tukey's HSD).

Fig. 8. Principal component analysis for PLFAs (taxonomic groups based on PLFAs) as a function of soil depth. Two scales are used, the ± 3.0 scale refers to the loadings of the samples at different depths and the ± 1.0 scale refers to the loadings of the different taxonomic groups (variables). The percent of variation is included on each Principal Component (PC).