**Temporal variation outweighs effects of biosolids applications in shaping arbuscular mycorrhizal fungi communities on plants grown in pasture and arable soils**

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**Abstract**

Landspreading of biosolids in agroecosystems is a common practice worldwide. Evidence suggests biosolids may be detrimental to arbuscular mycorrhizal fungi (AMF); however, previous studies focused on arable fields and often unrealistically high biosolids application levels. We investigated the effects of biosolids on AMF communities in agroecosystems, in the context of the natural seasonal dynamics of AMF community composition and diversity. A pasture and arable field under commercial farming management were annually amended with two different types of biosolids, and applied at levels meeting current European Union regulations. AMF root colonisation and community composition were measured in *Lolium perenne* roots from a pasture and *Trifolium repens* roots growing in arable soil across the seasons of two years. AMF community compositions were assessed by terminal-restriction fragment length polymorphism analyses. Biosolids had no significant effect on AMF root colonisation or community composition in either agroecosystem. Soil chemical analyses indicated several changes in the top 0–5 cm layer of the pasture soil, consisting of small increases in heavy metal concentrations in biosolids relative to control plots. Temporal AMF dynamics were detected in soils from both agroecosystem, and indicate that the effects of seasonality outweigh that of biosolids application.

**1. Introduction**

Arbuscular mycorrhizal fungi (AMF) of the phylum Glomeromycota (Schüßler et al., 2001) form symbiotic associations with the roots of most land plants, thereby mediating plant nutrient dynamics and AMF fungal carbon allocation (Smith and Read, 2008). Arbuscular mycorrhizal fungi have been shown to influence several ecosystem processes, including nutrient cycling, plant productivity and diversity, and soil aggregation (van der Heijden et al., 1998, 2006; Klironomos et al., 2000; Maherali and Klironomos, 2007; Leifheit et al., 2013).

The compositions of AMF communities associated with plant roots have been studied in various ecosystems around the world (Öpik et al., 2006, 2013). Different habitats are known to host different AMF communities (Helgason et al., 1998; Öpik et al., 2003, 2006) and AMF community compositions have been related to various soil physical and chemical characteristics (Lekberg et al., 2007; Hazard et al., 2013). Furthermore, communities of AMF are affected by agricultural management practices, with agriculture intensification reducing AMF richness (Oehl et al., 2003; Hijiri et al., 2006; Gosling et al., 2010).

The practice of applying biosolids (treated municipal sewage sludge) as a soil amendment to agriculture lands has been used for decades (Fytili and Zabaniotou, 2008). Biosolids have been shown to improve soil physical conditions, supply nutrients, enhance microbial activity and benefit plant productivity (Garcia et al., 1994; Pascual et al., 1999; Sullivan et al., 2006a, Cogger et al., 2013). While many studies have investigated the effects of biosolids on soil bacterial and fungal communities (e.g. Sullivan et al., 2006b; Anderson et al., 2008; Ippolito et al., 2009; Mattana et al., 2014), few studies have specifically investigated such effects on AMF communities.

Available evidence suggests that biosolids could alter AMF community composition, despite the nature of impacts varying between studies. AMF colonization of plant roots, spore densities in soils and species richness of spores or on roots has been found to increase, decrease or show no effect to biosolids application (Arnold and Kapustka, 1987; Weissenhorn et al., 1995; del Val et al., 1999a, b; Jacquot et al., 2000; Jacquot-Plumey et al., 2001; Barbarick et al., 2004; Toljander et al., 2008). Contradictory results between AMF biosolids studies may be attributed to several factors, including differences between biosolids, application levels, agricultural management and study methodologies. Thus, further AMF biosolids studies are required which investigate effects on AMF community compositions and that evade the factors causing contradictory results.

Published AMF biosolids studies thus far have focused on arable systems, and often been conducted in experimental arable fields or used experimentally constructed soils, and often used unrealistically high biosolids application levels. There is currently a dearth of information on the impact of biosolids application on AMF communities in grassland systems, with only a grassland study by Barbarick et al. (2004) that investigated AMF root colonisation of blue grama(*Bouteloua gracilis*) six years after a single application of biosolids. Understanding the impact of biosolids in grassland systems represents a significant knowledge gap, as the AMF communities associated with perennial plants in grasslands are more diverse to those in arable fields (Oehl et al., 2003, 2010; Öpik et al., 2006). Also, studies are required to investigate effects of biosolids under commercial farming systems using realistic application rates and different sewage products. Further, studies are needed which take into consideration the seasonal dynamics of AMF (Dumbrell et al*.*, 2011).

Here, we investigated the short-term effects of biosolids on AMF in a grassland and arable field under typical commercial farming management in which biosolids were applied at levels meeting current European Union regulations (DoELG, 1998). Two contrasting biosolids types, differing in dry matter, nutrient and heavy metal concentrations, were compared. The specific aims of the experiment were (1) to compare the effects of two contrasting biosolids on AMF communities colonising *Lolium perenne* from a pasture and *Trifolium repens* grown in arable soils and (2) to determine the impacts of biosolids, relative to natural seasonal fluctuations, on AMF community dynamics.

**2. Materials and Methods**

*2.1* Description of field sites and experimental design

The pasture field was located near Tinahely, County Wicklow, Ireland (52°49′30″N, 6°26′12″W) on a sandy loam soil (10.4% clay content at 0–20 cm depth; pH 5.2) with a plant community consisting almost entirely of *Lolium perenne.* The site was grazed by sheep and cattle. The arable field was located near Aughrim, County Wicklow, Ireland (52°52′34″N, 6°16′20″W) on a sandy loam soil (8.9% clay content at 0–20 cm depth; pH 6.2) and has been used for the production of spring barley since the early 1980’s following conventional farming practices. Coinciding with the months sampled in this study, seasonal climate conditions for the sites are provided in Table 1.

At the field sites, 15 plots (each 20 m × 15 m), arranged in five blocks, were established in a complete randomised block design. Each plot was separated by 7 m, and blocks by 10 m, in order to create a buffer zone between treatments. Plots were subjected to one of three treatments: Biocake, Biofert or control (no biosolids). The biosolids were supplied by the Ringsend wastewater treatment plant in Dublin, Ireland, where sewage sludge is treated by a thermal drying process resulting in ‘Class A’ pasteurised biosolids with 26% (Biocake) and 95% (Biofert) dry matter (DM) and differing in nutrient and heavy metal concentrations (Table 2). Using commercial machinery (large scale tractor-drawn applicator), biosolids were spread onto plots in March 2007 and 2008, and prior to ploughing and sowing of spring barley in the arable site. Biosolids were applied at the maximum level of 5 Mg DM ha-1 following European Union regulations (DoELG, 1998). Previous to this study, the field sites had never been treated with biosolids.

During the course of this experiment, farming practices continued as normal. At the arable field site mineral fertilizer was applied: 118 kg ha-1 of N (calcium-ammonium-nitrate); fungicides were applied: 0.25 l ha-1 of Bumper (Propiconazole), 0.5 l ha-1 of Amistar (Azoxystrobin) and 0.75 l ha-1 of Bravo (Chlorothalonil); herbicides were applied: 30 g ha-1 of Metsulfuron-methyl and 2.3 l ha-1 of CMPP [((chloro(methyl)phenoxy)) propionic acid]; and insecticide was applied: 165 ml ha-1 of Esfenvalerate.

*2.2* Collection of *Lolium perenne* from the pasture site

Whole plant samples of *Lolium perenne* were randomly collected from each plot in March 2007, June 2007, October 2007, January 2008, March 2008 and October 2008. For this, five soil monoliths of 15 cm × 15 cm area by 30 cm deep were excavated from each plot using a spade, stored at 4°C and processed within two weeks from collection. Soil was thoroughly washed from the plant roots with tap water. For each plot, one individual *L. perenne* plant was randomly selected from each of the five soil monolith samples and roots were bulked, rinsed with deionised water, blotted dry, homogenised and equally split into two sub-samples. One sub-sample was stored in 70% ethanol at room temperature for AMF root colonisation analyses, and the other flash-frozen with liquid N and stored at –80°C for molecular procedures.

*2.3* Arable soil bioassay with *Trifolium repens*

An arable soil bioassay approach was used to bait for AMF as crop plants were not always present for sampling throughout the year. From the arable site, soil samples were collected from each of the plots in February, July and October 2007, and in January and October 2008. A total of five soil samples were collected within each plot using a standard, 20 cm depth x 5 cm diameter Edelman soil auger (Eijkelkamp Agrisearch Equipment BV, Giesbeek, NL). Soil samples were taken at 2 m intervals along a transect centred in the middle of the plot. For each plot, soil samples were bulked, homogenised and stored at 4°C until used.

 A subsample of this soil was used for a bioassay with *Trifolium repens* L. (Fabaceae)to bait for AMF. Pots (8 cm × 8 cm × 8 cm) were filled with a 1:1 mix of bulked soil and autoclaved sand (three replicate pots were prepared per plot per sampling date). Fifteen negative control pots contained autoclaved field soil and sand (1:1 mix). Seeds of *T. repens* were surface-sterilised (2.5% sodium hypochlorite for 15 min), rinsed three times in sterile water, and 10 seeds were sown into each pot. Pots were arranged randomly in a growth chamber and the seedlings were grown under environmentally-controlled conditions [8 h dark/16 h light (120 µmol photons m-2 s-1) cycle, and a constant temperature of 20°C] and plants were routinely watered as necessary. After three months, all plants were removed from the soil and roots were thoroughly washed free of soil using tap water. Roots grown in soils from the same plot were bulked, and further processed as described for the *L. perenne* root samples.

*2.4* Soil chemical analyses

Soil chemical characteristics were determined from samples collected in October 2008. For the pasture site, a total of five soil samples were collected within each plot using a standard 20 cm depth × 5 cm diameter Edelman soil auger (Eijkelkamp Agrisearch Equipment BV, Giesbeek, NL). Samples were taken such that one sample was collected close to the centre of the plot and the others 2 m from the centre in each of the four cardinal directions. As the biosolids were applied to the surface and not mechanically incorporated in the grassland system, the soil samples were split into two depths, 0–5 cm and 5–20 cm layers, bulked separately and homogenised. A subsample was passed through a 2 mm sieve, air-dried and stored at room temperature. A subsample of the bulked mixed arable soil that was collected in October 2008 was processed as above and used for soil chemical analyses.

Available P (Av. P), total P, KCl-extractable NO3-N and the heavy metals Cd, Cu, Pb, Ni and Zn were determined. For this, soil samples were sent to City Analysts Ltd., Dublin, Ireland for available and total P using nitric acid digestion and colorimetric analyses. NO3-N and heavy metal measurements were conducted by ALcontrol Laboratories, Dublin, Ireland. NO3-N was extracted in 1M KCl and concentrations were determined on a chemical analyzer and heavy metals were measured using inductively coupled plasma - mass spectrometry (ICP-MS).

*2.5* Percent root colonisation by AMF

Root samples stored in 70% ethanol were cut into 1 cm fragments and cleared in 10% (w/v) KOH at 90°C for 9 min. They were then cooled in an ice bath, rinsed in water and immersed in 1% (v/v) HCl for 30 s to acidify roots prior to staining in a solution of 1% (v/v) Shaeffer black ink (Sheaffer Pen, Ft. Madison, Iowa, USA) in 1% (v/v) HCl for 5 min at 90°C (adapted from Vierheilig et al., 1998). Stained roots were rinsed in water and destained in lactic glycerol (14:1:1 v/v/v lactic acid: glycerol: water) overnight. Percent root colonisation by arbuscules (AC), vesicles (VC) and total AMF colonisation (TC; including hyphae, arbuscules and vesicles) were quantified by compound microscopy (200× magnification) using the magnified intersect method (McGonigle et al., 1990); AC, VC and TC were recorded at 100 intersections using 25 randomly selected 1 cm root fragments.

*2.6* T-RFLP analysis

Frozen root samples were homogenised in liquid N (using a mortar and pestle) and total DNA was extracted from 100 mg of homogenate using the DNeasy Plant Mini Kit (QIAGEN, Hilden, Germany). An 800bp region of the 18S rDNA gene was amplified using the AMF-specific primers AML1 and AML2 (Lee et al., 2008). AML1 and AML2 were 5’ end-labelled with the fluorescent dyes HEX and FAM, respectively. Polymerase chain reaction (PCR) was conducted in a total volume of 50 µl which contained 44 µl of Megamix (Microzone, Haywards Heath, UK), 25 pmol of each primer, and 4 µl of template DNA. Amplification was performed using a G-Storm GS1 Thermal Cycler (Gene Technologies Ltd., Essex, UK) and a programme consisting of: 3 min at 94°C, 40 cycles of 1 min at 94°C, 1 min at 57°C, 1 min at 72°C and a final extension for 5 min at 72°C. PCR products were purified using the QIAquick PCR Purification Kit (QIAGEN, Hilden, Germany).

For terminal-restriction fragment length polymorphism (T-RFLP) analysis, purified PCR products were digested with the restriction enzymes *Hin*fІ (New England BioLabs, Ipswich, MA, USA) and *Hsp*92ІІ (Promega, Madison, WI, USA) in two separate reactions. These enzymes have previously been shown to yield discriminatory terminal-restriction fragments (T-RFs) from DNA fragments amplified using the primers NS31 and AM1 (Vandenkoornhuyse et al., 2003; Johnson et al., 2003); such discrimination was validated *in silico* by restriction analysis of 100 AML1/AML2-amplified AMF sequences downloaded from the European Molecular Biology Laboratory (EMBL) Nucleotide Sequence Database (http://www.ebi.ac.uk/embl/). The digestion reaction, purification of digested products and T-RF analysis were conducted as described in more detail in Hazard et al. (2013). The resulting T-RF profiles were manually analysed using the program GeneMarker (SoftGenetics, State College, PA, USA). Only T-RFs with peak heights above 50 fluorescent units and between 75–500 bp in size were considered for further analyses.

A representative clone library was constructed from the pasture and arable samples from October 2007 in order to identify AMF (via NCBI BLAST search of clone sequences and phylogenetic analyses) and their associated T-RFs (via virtual restriction enzyme digests of sequences) and to confirm the T-RFLP data through identification of clone sequences to check for non-specific amplification (see Supplemental Materials and Methods). AMF within the Glomeraceae, Claroideoglomeraceae, Acaulosporaceae, Ambisporaceae and Diversisporaceae were identified (Fig. S1 and S2). Unique T-RFs were associated with some of the identified AMF (see Table S1 and S2), however further cloning and sequencing would have been necessary for development of a complete AMF T-RF database. Five of 129 clone sequences were not of AMF origin, but had affinity to the same plant sequence in the EMBL database (accession EF024034). The contaminant plant sequences yielded T-RF products less than 75 bp in size and thus did not contribute to the AMF T-RF profiles (Table S2).

*2.7* Statistical analyses

Statistical analyses from the data derived from the pasture and arable site were conducted separately, as the sampling approaches used were different between these sites. The significance of the effect of treatment (Biocake, Biofert and control) and sampling time on differences between AMF root colonisation (TC, AC and VC) and mean number of T-RFs was determined using multivariate analysis of variance (MANOVA) and analysis of variance (ANOVA), respectively, incorporating a nested design [treatment(sampling time)]. A nested approach was used instead of repeated measures, as different individual plants from the plots and pots were sampled across time. Blocks were not included in the final model as they had no significant effect on the observations (*P* > 0.09). When the main model was significant (*P* < 0.05), pair-wise differences were determined using Tukey’s honestly significant difference (HSD) test (α = 0.05). To improve normality, AMF root colonisation data (TC, AC and VC) were arcsine square root-transformed. Significant correlations between AMF root colonization variables were determined using Pearson Product-Moment Correlation analysis. Soil chemical parameters were compared using a two-way ANOVA with the treatments, and soil depth (0–5 cm versus 5–20 cm) as factors for the pasture site. Statistical tests were performed using SPSS version 15.0 (SPSS Inc., Chicago, Illinois, USA).

Non-metric multidimensional scaling (NMDS) was used to visualise the AMF communities across the different treatments and sampling times. Bray-Curtis resemblance matrices were generated based on presence/absence T-RF data (Bray and Curtis, 1957; Clarke, 1993). The resemblance matrices were plotted in 2-dimensions by NMDS ordination (25 restarts, 0.01 minimum stress, Kruskall fit scheme 1, stress value < 0.14) (Kruskal and Wish, 1978). Stress (goodness of fit of the plot) was calculated as described by Kruskal (1964), with a stress level of ≤ 0.1 corresponding to an ideal representation of the data cloud (Clarke, 1993). One-way permutation multivariate analysis of variance (PERMANOVA) with a nested design was performed on Bray-Curtis resemblance matrices (incorporating 999 permutations under a reduced model for *F* statistics) to determine the significance of differences in AMF communities in terms of treatments within each sampling time and between sampling times (Anderson, 2001). PERMANOVA pairwise tests were computed (incorporating 999 permutations) when the main test showed a significant effect at *P* < 0.05. Analyses were computed using PRIMER v6.1.9 (Primer-E Ltd, Plymouth, UK).

**3. Results**

*3.1* Percent root colonisation

Biosolids application had no significant effect on percent root colonisation (total colonization, TC; arbuscules, AC; vesicles, VC) by AMF (Pasture: *P* = 0.290; Arable: *P* = 0.064, MANOVA) (Fig. 1 and 2). Although not significant, mean TC and AC are often the highest in the arable control plots in comparison to the biosolids plots across the sampling times (Figure 2).

Significant differences between sampling times were found (Pasture: *P* = 0.001; Arable: *P* = 0.001, MANOVA). For *Lolium perenne* from the pasture site, considerable variation was found in both TC and AC, but not in VC, between the different sampling times (Fig. 3a). TC significantly differed between March and June 2007; and June 2007, October 2007 and January 2008 (*P* < 0.028, Tukey’s HSD). There was a significant correlation between TC and AC numbers (*r* = 0.526, *P* < 0.001, Pearson Product-Moment Correlation analysis) and the highest mean TC and AC was in June 2007 (68 and 29% root colonisation, respectively), while the lowest was in January 2008 (50 and 17%, respectively). Annual differences in TC and AC were not significant (comparing March 2007 with March 2008 and October 2007 with October 2008) (*P* > 0.856, Tukey’s HSD). Variations in VC between sampling times were not significant (*P >* 0.052, Tukey’s HSD), ranging from 12% in March 2007, to 16% in June 2007.

Compared to *L. perenne*, the *Trifolium repens* growing in the arable soil had less significant variation in AMF colonisation (Fig. 3b). TC differed significantly between February 2007 and July 2007, October 2007 and 2008 (*P* < 0.005, Tukey’s HSD). TC was highest in February 2007 (58%) and lowest in October 2007 (41%). AC was only significantly different between October 2008 and the other sampling dates (*P* < 0.005, Tukey’s HSD) and was highest in February 2007 (6%) and lowest in October 2008 (1%). There was little variation in VC across sampling dates except between October 2007 and 2008 (*P* = 0.025, Tukey’s HSD), with 2 and 4% VC respectively.

*3.2* AMF communities

 Biosolids application had no significant effect on the similarity of AMF community structure. (Pasture: *P* = 0.192; Arable: *P* = 0.522, PERMANOVA; NMDS plots not shown). Also, within each sampling time, biosolids application had no significant effect on the mean number of T-RFs (Pasture: *P* = 0.098; Arable: *P* = 0.680, ANOVA) (Fig. 4).

 The AMF community structure significantly differed between sampling times (Pasture: *P* = 0.001; Arable: *P* = 0.001, PERMANOVA). The NMDS plot of T-RF data supports a seasonal and temporal change in the *L .perenne* AMF community from the pasture, with months within the same year and corresponding months from different years showing separation and overlap (Figure 5a). However, because the average Bray-Curtis similarities within and between the sampling times were generally similar; PERMANOVA pairwise testsbetween sampling times were not significant (*P* ≥ 0.089). NMDS ordination based on the *T. repens* T-RF profiles from the arable soil also suggests a shift in the AMF community, with samples from the same sampling time grouping together, but with an extensive overlap between sampling times (Fig. 5b). The average Bray-Curtis similarities within and between the sampling times were generally high (>50%). However, the AMF community profile of *T. repens* from October 2008 formed a distinct grouping, which was significantly different from the other sampling times (*P* ≤ 0.013, pairwise PERMANOVA).

 The mean number of T-RFs significantly changed over time (Pasture: *P* = 0.001; Arable: *P* = 0.001, ANOVA) (Fig. 4). For the pasture, the mean number of T-RFs progressively increased from March 2007 through January 2008, followed by a decrease in March 2008, and an increase in October 2008 (Fig. 4a). Of the 25 T-RFs detected, nine were present across all sampling times, but with varying frequencies (Table S3). Five of the remaining 16 T-RFs occurred frequently (≥ 47% of samples) for one or two sampling times (Table S3). For the arable site, the mean number of T-RFs progressively increased from July 2007 through January 2008 (Fig. 4b). Sixteen of the 32 detected T-RFs were present at all sampling times, but with variable frequencies, and nine T-RFs were detected infrequently across sampling times (Table S4). Some T-RFs occurred seasonally; five T-RFs had high frequencies in February 2007 and January 2008 (present in 67–80% of all samples), but had low frequencies at other times (present in 0–27% of samples). Other T-RFs were different between the same months of different years; 11 T-RFs present in October 2007 (although detected at low frequencies, 6.7% of samples) were not present in October 2008, and for many T-RFs, frequencies dramatically decreased in October 2008 (Table S4).

*3.3* Effects of biosolids on soil chemical parameters

Biosolids application significantly affected some chemical properties of the pasture soil (determined at the end of the study in October 2008) in the 0–5 cm layer, but not in the 5–20 cm layer (Table 3). The Biocake application resulted in significantly higher concentrations of the heavy metals Cd, Cu, Pb and Zn within the top soil layer, compared to the control treatment (*P* < 0.05, ANOVA). The Biofert application did not significantly affect heavy metal contents in soils (Table 3). Available P was significantly different between the Biocake and Biofert treatments *(P* = 0.044, Tukey’s HSD) but overall these treatments were not significantly different from the control. When comparing across treatments, all variables were significantly different between the two soil depth (*P* < 0.001, ANOVA), except for Ni (*P* = 0.053, Tukey’s HSD), with overall greater levels of heavy metals in the top layer. No differences in the soil chemical parameters measured from the arable field were detected between the treatments (*P* < 0.190, ANOVA) (Table 3). Soil heavy metal concentrations for the pasture and arable field were all under the legal limit according to national sewage sludge regulations (DoELG, 1998).

**4. Discussion**

*4.1* Biosolids effects on AMF

The effects of Biocake and Biofert applied as organic amendment on arbuscular mycorrhizal fungi under normal, commercial farming practices was investigated under field conditions using the maximum allowed application level of biosolids (5 Mg DM ha-1 y-1). The repeated application of the biosolids did not significantly alter the AMF communities or the colonisation of roots by AMF in the pasture and arable soil. However, there was a near significant trend of lower AMF colonisation of *T. repens* in the biosolids amended arable soil across the sampling time. Previous work on the effects of biosolids on AMF showed significant changes in AMF root colonisation and spore diversity but following greater levels of biosolids application, with treatments often reapplied annually over a longer period of time (Koomen et al., 1990; del Val et al., 1999a,b; Jacquot-Plumey et al., 2001; Barbarick et al., 2004). For example, after three years of repeated application of biosolids at a level of 10 Mg ha-1 y-1, Jacquot-Plumey et al. (2001) found a decrease in root colonisation and change in the relative diversity of AMF on bioassay plants (Medicago truncatula) grown in soils obtained from an arable field. In the study conducted by Sullivan et al. (2006b), the abundance of AMF ester-linked fatty acid methyl esters (EL-FAME) biomarkers in a grassland soil decreased between control and biosolids amended plots at levels ≥2.5 Mg ha-1 after one and two years. Furthermore, Sullivan et al. (2006a) showed that application levels of ≥5 Mg ha-1 significantly increased some soil chemical properties at 0–15 cm depth, including micronutrients, macronutrients and metals. In the current study, soil chemical analyses indicated that biosolids application had minor impacts on soil chemistry, with small increases in concentrations of some heavy metals only in the top 0–5 cm layer of the pasture soil. Since soil chemical changes consisted of relatively small increases in some heavy metal concentrations between biosolids and control plots, this could be one explanation why no significant biosolids effects on AMF root colonisation and community composition were detected.

While our results suggest that biosolids application to pastures and arable fields following current regulation may not cause deleterious effects to AMF communities in the short term, long-term effects could occur. Current regulations base the levels at which biosolids can be applied to land on the heavy metal concentrations of the receiving soil and of the biosolids. It is still unknown whether the current regulations will prevent long-term deleterious effects of such metals on AMF communities as heavy metals could accumulate to levels that could affect AMF diversity. For instance, del Val et al. (1999a) showed that heavy metals from biosolids deleteriously affected the external mycelial growth of some AMF fungi at high levels of biosolids after 10 years of application. Nevertheless, over time, species of AMF may develop enhanced tolerance to increased levels of heavy metals in soils (Gildon and Tinker, 1983; Weissenhorn et al., 1993; Diaz et al., 1996).

*4.2* AMF seasonal dynamics

This study corroborates previously reported seasonal differences in AMF colonisation patterns, although patterns observed here were not necessarily similar to those of published studies (Sanders and Fitter, 1992; Lugo et al., 2003). Here, total AMF root colonisation of *L. perenne* from the pasture site was greatest in June, consistent with increased plant growth rates, and was least in January when growth would be minimal. Several studies have reported similar patterns in AMF colonisation (Sigüenza et al., 1996; Mohammad et al., 1998; Lugo et al., 2003; Bohrer et al., 2004; Escudero and Mendoza, 2005).

Arbuscular colonisation also followed the same pattern as total AMF colonisation, and other studies have reported before maximum arbuscular colonisation during the growing season (Lugo et al., 2003; Garcia and Mendoza, 2008; Madyam and Jumpponen, 2008). As arbuscules are thought to be the AMF-host plant exchange site for P and possibly other nutrients (Smith and Read, 2008), high arbuscular colonisation found during the growing season could reflect the increased plant host demand for nutrients. Arbuscular colonisation has previously been linked with host metabolism; Garcia and Mendoza (2008) showed that arbuscular colonisation was greatest at the beginning of the growing season, as were concentrations of P and N in the plant-host tissue.

In our study, total AMF colonisation and arbuscular colonisation of *L. perenne* roots in the same months of different years were not significantly different (March 2007 & March 2008, October 2007 & October 2008), suggesting that the seasonal pattern in root colonisation may be similar across years. While Madyam and Jumpponen (2008) found arbuscular colonisation of grass roots to be consistently greatest in mid-summer in two consecutive years, most other studies did not find similar patterns in total AMF colonisation or hyphal colonisation across years (Sanders and Fitter, 1992; Muthukmar et al., 2002; Escudero and Mendoza, 2005; Mandyam and Jumpponen, 2008).

Using T-RFLP analyses, seasonal and temporal (between years) shifts in the AMF community compositions of *L. perenne* were found. However, development of a complete TRF database would have better aided in linking the community shift with identified AMF species. The T-RF frequency patterns observed may suggest that some dominant AMF were present in roots throughout and between years, others were only present seasonally, and rarer species fluctuated with respect to presence and frequency. For example, T-RFs associated with *Rhizophagus irregularis* were found frequently (in at least 11 of the 15 plant samples) across all sampling times (Table S3). The *Rhizophagus irregularis* species group is known for frequently colonising various plant species from diverse ecosystems worldwide (Öpik et al., 2006). In a pyrosequencing study by Dumbrell et al*.* (2011) AMF community composition of grassland plant roots had a distinct warm to cold seasonal shift with overdominance by an AMF taxon being replaced by a more even and diverse AMF community. Seasonal changes in AMF communities have been suggested to reflect a combination of plant and fungal phenology and the abiotic environment (Bever et al., 2001; Lui et al., 2009; Dumbrell et al., 2011).

A temporal shift in the AMF community of *Trifolium repens* roots growing as bait plants in the arable soils was also detected. This result may reflect seasonal and successional sporulation dynamics; previous spore-based studies have shown differences between species in terms of season and over time (Gemma and Koske, 1988; Gemma et al., 1989; Lee and Koske, 1994; Pringle and Bever, 2002; Oehl et al., 2009). The microcosm study of Oehl et al. (2009) showed seasonal sporulation patterns for species in the genera *Acaulospora*, *Ambispora*, *Archaeospora*, *Scutellospora* and *Cetraspora*, while *Glomus* species exhibited a more successional dynamic. *Acaulospora* species have been shown to peak in sporulation from spring to early summer (Oehl et al., 2009), and have also been more frequently found as sequences on the roots of arable crops during this time in comparison to other seasons (Daniell et al., 2001; Hijri et al., 2006). Yet in the current study, *Acaulospora* species were frequently present in plots across all sampling dates, regardless of season (Table S3 and S4).

Although a temporal shift in the AMF community was detected, considerable overlap between the AMF communities between the sampling dates occurred, with the exception of the AMF community sampled in year two (October 2008). T-RF frequency differences between October 2007 and 2008 were strikingly different. Some T-RFs were present in one year and not the following year; and for many T-RFs, plot frequencies dramatically increased or decreased between years. These results could be a reflection of the disturbance undergone between years as ploughing, sowing and harvesting of spring barley would have taken place between October 2007 and 2008. Tillage has previously been shown to cause a change in the community structure of AMF spores in the soil and on crop roots (Jansa et al., 2002, 2003).

*4.3* Conclusion

In conclusion, annual application of two different types of biosolids to a pasture and arable field did not significantly affect AMF root colonisation or the community composition of AMF on plant roots in the short term, but temporal and seasonal dynamics were detected. These results suggest that the short-term use of biosolids in agroecosystems, following current regulations, may not be detrimental to AMF diversity. However, further studies are needed to investigate the long-term effects of biosolids on AMF communities in agroecosystems whilst taking into consideration the seasonal dynamics of AMF.

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**Figure Legends**

**Figure 1** Seasonal and temporal variations in the percentage of arbuscular mycorrhizal fungi (AMF) colonising *Lolium perenne* roots under different biosolids treatment regimes in **pasture**; AMF infection structures (a) total colonisation (hyphae, arbuscules and vesicles), (b) arbuscular and (c) vesicular colonisation. Bars indicate mean values (± SEM) for 5 replicate samples taken for each time point and treatment.

**Figure 2** Seasonal and temporal variations in the percentage of arbuscular mycorrhizal fungi (AMF) colonising bioassay *Trifolium repens* roots under different biosolids treatment regimes in an **arable** field; AMF infection structures (a) total colonisation (hyphae, arbuscules and vesicles), (b) arbuscular and (c) vesicular colonisation. Bars indicate mean values (± SEM) for 5 replicate samples grown in soils from each time point and treatment.

**Figure 3** Seasonal and temporal variations in the percentage of arbuscular mycorrhizal fungi (AMF) colonisation of (a) *Lolium perenne* roots from a pasture, and (b) *Trifolium repens* roots grown in arable soils. AMF infection structures: TC, total colonisation (hyphae, arbuscules and vesicles); AC, arbuscular colonisation; VC, vesicular colonisation. Bars indicate mean values (± SEM) for 15 replicate samples taken for each time point. Means with different letters are significantly different, based on Tukey’s HSD test of arcine square root-transformed data (*P* < 0.05).

**Figure 4** The mean number of T-RFs associated with (a) *Lolium perenne* from a pasture and (b) *Triflium repens* grown in arable soils subjected to different biosolids treatments and harvested at different times. Bars indicate mean values (± SEM) for 15 replicate samples taken per time point. Means with different letters are significantly different, based on Tukey’s HSD test of raw data (*P* < 0.05).

**Figure 5** Non-metric multidimensional scaling plot, based on Bray-Curtis resemblance matrices generated using the arbuscular mycorrhizal fungal community terminal-restriction fragment data for roots of (a) *Lolium perenne* collected from pasture plots and (b) *Trifolium repens* grown in arable soil collected from plots sampled at different time points.