Shifts in microbial communities do not explain the response of grassland ecosystem function to plant functional composition and rainfall change.

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ABSTRACT

Ecosystem functioning in grasslands is regulated by a range of biotic and abiotic factors, and the role of microbial communities in regulating ecosystem function has been the subject of much recent scrutiny. However, there are still knowledge gaps regarding the impacts of rainfall and vegetation change upon microbial communities and the implications of these changes for ecosystem functioning. We investigated this issue using data from an experimental mesotrophic grassland study in south-east England, which had been subjected to four years of rainfall and plant functional composition manipulations. Soil respiration, nitrogen and phosphorus stocks were measured, and the abundance and community structure of soil microbes were characterised using quantitative PCR and multiplex-TRFLP analysis, respectively. Bacterial community structure was strongly related to the plant functional

composition treatments, but not the rainfall treatment. However, there was a strong effect of both rainfall change and plant functional group upon bacterial abundance. There was also a weak interactive effect of the two treatments upon fungal community structure, although fungal abundance was not affected by either treatment. Next, we used a statistical approach to assess whether treatment effects on ecosystem function were regulated by the microbial community. Our results revealed that ecosystem function was influenced by the experimental treatments, but was not related to associated changes to the microbial community. Overall, these results indicate that changes in fungal and bacterial community structure and abundance play a relatively minor role in determining ecosystem function responses to precipitation and plant functional composition change, and that direct effects on soil physical and chemical properties and upon plant and microbial physiology may play a more important role.

1. INTRODUCTION

In the coming century climate change will affect grasslands by altering precipitation and therefore water availability (Kardol *et al.* 2010; IPCC 2014). Changing rainfall patterns are likely to alter microbial community structure, which can have implications for many ecosystem functions, including those relating to nutrient cycling and carbon (C) sequestration (Morecroft *et al.* 2004; Gilgen *et al.* 2010). Changes to rainfall patterns will also be accompanied by changes to the diversity and functional composition of plant communities, which can be driven by a number of global change drivers including nitrogen (N) deposition and land use intensification (Manning 2012, Southon *et al.*, 2013, Allan et al 2015). It is therefore important to consider the effect of both rainfall manipulations and plant functional

identity in a systematic manner in order to understand global change impacts upon ecosystem function.

Many studies have demonstrated a link between plant functional traits and ecosystem functioning. For example, grassland ecosystem C fluxes can be explained by plant trait measurements including specific leaf area (SLA) and leaf N content (Everwand *et al.* 2014). Such traits may have direct links to function but can also serve as proxies for the traits that determine plant effects on the soil environment, e.g. those that explain the chemistry of root exudates and rhizodeposition rates (Marschner *et al.* 2001; Bais *et al.* 2006; Badri *et al.* 2009; Berg & Smalla 2009), factors which are known to alter microbial communities (De Vries et al 2012a). Accordingly, experimental manipulation of these traits can offer valuable insights into how vegetation properties regulate ecosystem functioning (Fry *et al.* 2014a).

In addition to being influenced by the plant community, microbial communities are also affected by a range of abiotic and biotic factors. These include soil moisture, which is likely to be affected by climate change in the future (Seneviratne *et al.* 2010). As a result of these relationships both climate and biodiversity change have the potential to alter the structure and abundance of microbial assemblages, with potentially far-reaching effects on soil C and nutrient cycling (Schimel & Bennett 2004; Ryan & Law 2005; Bardgett *et al.* 2008; Chapin *et al.* 2009; Bardgett et al 2013). The mechanisms underlying these changes are diverse. For example, soil microbes are likely to respond rapidly and dramatically to changes in soil moisture because their low motility and small size leave them vulnerable to localised water deficits (Manzoni *et al.* 2012), an effect that would reduce microbial activity and soil organic matter turnover. However, drying-rewetting cycles have also been shown to stimulate microbial activity, and destabilise C held in soil aggregates, resulting in rapid, short-term shifts in rates of organic matter turnover and consequently CO_2 efflux and nutrient release

(Birch 1958; Fierer & Schimel 2003; Kaisermann *et al.* 2015). These changes could alter the overall C balance of a system, as well as its capacity to support diverse plant communities.

There is a hypothetical role for microbial structure as an important mediator of plant compositional effects on soil function, but this has rarely been tested empirically (Quétier *et al.* 2007; Kardol *et al.* 2010; Butterfield & Suding 2013). For example, alteration of plant communities may cause changes in community-level plant traits such as average leaf nitrogen content. These could drive shifts in microbial abundance and community structure, which could in turn affect ecosystem function (Bardgett *et al.* 2014, Thakur *et al.* 2015). One mechanism through which plant traits affect microbes and subsequently impact function could be the selection for specific microbial communities in response to plant species specific rhizodeposits. This can be either inhibitory, e.g. through release of damaging reactive oxygen species, or stimulatory, e.g. via provision of valuable carbon-based substrates (see Hartmann *et al.* 2009 for a comprehensive review). Another example of this cascade of effects occurs when considering microbes that are adapted to colonise roots of one or a few plant species, such as *Rhizobium* spp. on legumes or many species of mycorrhizal fungi. These microbes can improve plant performance by improved access to otherwise inaccessible nutrients, resulting in increased photosynthetic capacity and carbon sequestration (De Deyn *et al.* 2008).

Here we investigated the impacts of changes to rainfall and plant functional identity on microbial community structure and ecosystem functioning in a lowland grassland and assessed whether changes to function were mediated by changes in microbial abundance and/or community structure. We addressed this question within the framework of a long-running field experiment in south-east England (the DIRECT experiment), which manipulated plant functional composition and rainfall patterns over four years (Fry *et al.* 2013; 2014a). We hypothesised that 1) shifts in rainfall pattern and plant functional identity

result in changes in the microbial community structure; 2) fungal groups will be more strongly affected by plant functional group identity than rainfall as they are closely linked to plant composition via specialised biotic interactions (e.g. pathogens and symbionts; Klabi *et al.* 2014) and decomposer species litter preferences, while bacterial groups are more susceptible to rainfall change due to the osmotic stress it causes (Evans & Wallenstein 2014; Fuchslueger *et al.* 2014); and 3) some of the changes to ecosystem function caused by rainfall and plant functional composition can be attributed to concurrent changes in microbial community properties.

2. METHODS

2.1. STUDY SITE

This study took place in the fourth year of a climate change and functional composition experiment on a mesotrophic grassland in Silwood Park, Berkshire, UK, (lat. 51.406371, long. -0.648648). The soil was an acidic loamy sand (mean pH 5.5), which was regularly ploughed in the years preceding the study (Fry *et al.* 2013, 2014b, for more information see Appendix S1). The dominant species included *Holcus mollis, Holcus lanatus, Agrostis capillaris, Cirsium arvense* and *Rumex obtusifolius*, (community classification: EUNIS code E2, (European Nature Information System, http://eunis.eea.europa.eu)). There was a shallow incline east to west across the site, which led to drier average soil conditions in the west than the east and a slight shift towards a more legume dominated community in the west. To account for these gradients the plots were organised in a randomised, blocked design. Mean

annual rainfall at the site is 833 mm yr^{-1}, with cool wet winters (January average 4.8˚C) and warm dry summers (July average 17.2˚C). The site was ploughed in October 2007 and the experiment began in June 2008.

2.2 EXPERIMENTAL DESIGN

The experiment consisted of two treatments: a rainfall manipulation altering precipitation inputs in line with 2100 projections (IPCC 2007; Fry *et al*. 2013), and a plant functional composition treatment based on a bespoke trait-based species classification (Fry *et al.* 2014a). The full experimental design has been described previously (Fry et al., 2013; 2014b) but is outlined in brief as follows. Rainfall regime was manipulated to reduce net summer rainfall (JJA) by 30 % compared to ambient inputs, and to vary the intensity of rainfall events, in line with end-of-century model predictions for the region (simplified regime in Figure S1 in Supporting Information; Murphy *et al.* 2009). In winter (DJF), a projected 15 % increase in rainfall volume was simulated. This combination of reduced summer and increased winter (end-of-century) rainfall regime is referred to as the "2100" treatment throughout the paper. The summer phase of the treatment was implemented using rainout shelters built over 2.4 m x 2.4 m plots, which remained in place from June-August. We applied the rainfall treatment in the following manner: when less than 20 mm of rain fell in a 24 hour period, we reapplied 50 % of the volume to the 2100 plots and discarded the remainder. When more than 20 mm fell, we reapplied 100 % of the volume. This created a pattern of small rainfall pulses and large inputs, with a net decrease of ~30 % volume averaged across the summer. The accompanying winter regime involved a 15 % rainfall addition to the 2100 plots, after each rainfall event, with no pattern alteration.

The second treatment, functional composition, was created by manipulating the plant community that established from the existing seedbank and surrounding vegetation at the site, following ploughing in 2007. Our bespoke plant species classification grouped species according to functional effects traits known to impact C, N and water cycling (Reich 2014, Everwand *et al.* 2014). This resulted in groups classed as “perennials”, “caespitose grasses”, and “annuals”, (see Fry *et al.* 2014a and Everwand *et al.* 2014 for trait measurement methods). Perennial species have a high average specific plant area (total leaf area/aboveground biomass, and similar to specific leaf area, hereafter SPA), with N-rich leaves. Earlier work showed that the presence of perennials resulted in higher ecosystem level respiration and decomposition rates than the other groups under ambient conditions, although these diminished in the 2100 treatment in the first three years of the experiment (Fry *et al.* 2013). Caespitose grasses exhibit high above- and below-ground biomass and comparatively low tissue N content. Annual species are distinctive by their short life span, low SPA and high tissue N. To date, annuals have been largely unaffected by rainfall manipulation (Fry *et al.* 2013). A full combinatorial design led to seven factor levels: individual groups (n=3); pairs of groups (n=2); and one level of all three groups (n=1). We created the functional composition gradient in the plots by weeding selectively, initially in October 2008 then each May from 2009-2011. A dominant species, *Holcus mollis* (perennials) covered much of the plots at the beginning of the study. In order to prevent confounding the effects of perennial group removal with those of major soil disturbance and microclimate disruption, this species was retained in all plots. As *H. mollis* does not have a trait combination that is particularly representative of the perennial group (e.g. its SPA is 131 % higher and leaf N is 46 % lower than the mean of the group), selective removal of all other perennial species, which account for 34% of plant cover in control conditions, is still expected to have significant effects upon

187 the trait distribution of the remaining community. Two levels of rainfall combined factorially
188 with seven levels of composition, and four replicate blocks, resulted in 56 experimental plots.

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190 2.3 ECOSYSTEM FUNCTION MEASUREMENTS

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192 Rainfall was monitored throughout the experiment daily using a Weatherlink Vantage Pro
193 weather station (Davis Instruments, CA, USA) to guide accurate reapplication of rainfall. We
194 also measured soil moisture content (SMC) to a depth of 10 cm using a ThetaProbe Soil
195 Moisture Meter HH2 with ML2x probe (Delta-T, Cambridge, UK), in four areas of each plot
196 and then averaged these across the plot; measurements were taken twice per week between
197 3rd May and 27th July 2011, with a final measurement on 31st August 2011. We also surveyed
198 the vegetation in each plot at the beginning of June when the rain shelters were established,
199 and in late July before widespread senescence began. A 1 m x 1 m quadrat was placed in the
200 centre of each plot and the percentage cover of all individual vascular plant species was
201 recorded using visual estimation. This area was defined as the 'experimental zone', and all
202 function measurements were taken within it. Cover estimates were summed to give total
203 percentage cover of biomass.

204 Ecosystem respiration (R_{eco} mg CO_2 m^2 s^{-1}) was measured in August 2011 when the shelters
205 were removed, using a transparent plastic cuvette (300 cm^2 area, 9000 cm^3 volume) with an
206 opaque sleeve attached to a Ciras-1 infra-red gas analyser (IRGA, PP Systems, Hitchin, UK).
207 This was fitted over a collar embedded in the soil to make a gas-tight seal. The collars
208 protruded 10 cm above the soil surface. Each measurement was 120 seconds long.
209 Concurrent soil moisture and soil temperature (Checktemp Electronic Thermometer, Hanna,

Bedfordshire, UK) measurements were taken (three replicates of each) to serve as covariates (for further details of this method see Everwand *et al.* 2014).

Extractable soil nutrient levels were determined in July 2011 by taking composite samples (four sub-samples per plot) to 10 cm depth, homogenising and sieving (< 2 mm) to remove root material and stones. Extractable N and P were determined following the method of Allen (1989). In brief, for extractable N, 20 g fresh soil was mixed with 75 ml 1M KCl and placed on an orbital shaker for 60 minutes at 150 rpm before being filtered. For extractable P 10 g soil was shaken with 150 ml Truog's solution for 30 minutes before being filtered. The samples were stored for no longer than 24 hours at 5 ˚C, before being analysed colorimetrically using a Skalar SAN^{++} auto-analyser (Skalar, York, UK). Final values were corrected for soil moisture content.

We derived mineralisation rates *in situ* by inserting 5 cm wide PVC tubes 10 cm into the soil in May 2011, at the same time as soils were sampled for extractable nutrients (above). Tubes were covered to prevent leaching and incubated in plots for a period of two months. In July, we removed them and measured the extractable N as above. Mineralisation rate was then calculated by subtracting the original (May) concentration from the incubated (July) concentration and multiplying by the bulk density, to give values as g N m^{-2}. Extractions were performed on the day of soil sampling.

2.4 SOIL MICROBIAL COMMUNITY STRUCTURE

Subsamples of the sieved soil collected for nutrient analysis (see above) were taken from all 56 plots on the 31st August 2011, the day summer rainfall treatments ended and six weeks

after the second vegetation survey. Samples were frozen fresh at -20 °C, then the total DNA was extracted from 0.5 g soil using a Powersoil® DNA isolation kit (MoBio Laboratories Inc. CA, USA). The DNA was quantified and quality checked using a Nanodrop 2000 Spectrophotometer (Thermo Fisher Scientific, CA, USA). The extracted DNA was then amplified in a multiplex Polymerase Chain Reaction (PCR) procedure, with primers designed for bacterial and fungal groups in the same reaction. We amplified bacterial (16S *rRNA*) and fungal (ITS) gene sequences using the method described by Singh *et al.* (2006). Briefly, each reaction consisted of a total 50 µl reaction mixture containing 5 µl of template DNA added to a master mix with 5 µl 10x NH_4 reaction buffer, 2 µl of 50 mM $MgCl_2$, 200 µl of each 20 mM dNTP, 0.5 µl of Biotaq DNA polymerase (all reagents Bioline, MA, USA, final concentration 2.5 U) and 1 µl of 20 mg/ml Bovine Serum Albumin (BSA, Promega, WI, USA). 0.5 µl of bacterial primers (63F-VIC labelled and 1087R; final concentration 200 nM; Hauben et al., 1997; Marchesi et al., 1998) and 1 µl of fungal primers (ITS 1F-FAM labelled and ITS 4, final concentration 400nM; Gardes and Bruns, 1993; White *et al*., 1990; all primers sourced from Invitro Technology, VIC, AU). All PCRs were carried out on a Bio-Rad DNA Engine Dyad Cycler (Bio-Rad, Gladesville, NSW, AU) with the following cycle: denaturing at 95 ˚C for 15 minutes, 30 cycles of denaturing at 94 ˚C for 30 seconds, annealing at 55 ˚C for 30 seconds, elongation at 72 ˚C for 60 seconds and a final elongation period of 72 ˚C for 10 minutes. Amplification was verified visually using electrophoresis on a 1.5 % agarose gel in 1 % Tris/Borate/EDTA with an ethidium bromide (EtBr) stain.

Amplified PCR products were purified using a ChargeSwitch® PCR Clean-Up Kit (Invitrogen, VIC, AU). Cleaned PCR products (approximately 1000 ng) were digested in a 20 µl reaction mixture consisting of 2 µl of 10 x buffer, 0.2 µl of 20 mg ml^{-1} BSA, 0.5 µl *HHaI* restriction enzyme (10 U final concentration; all reagents New England Biolabs, MS, USA), made up to volume with nuclease-free water. Terminal restriction fragments (TRFs) were

resolved on an ABI3500 Genetic Analyser (Invitro Technology) following mixing of 1 µl of digested product with 10 µl of hi-dye formamide and 0.3 µl LIZ-600 size standard (Invitro Technology). Fragments were analysed using Genemapper (version 4.1, (Invitro Technology). We excluded TRFs smaller than 50 and larger than 600 base pair lengths, since these were outside the range of the size standard. TRFs were binned using T-REX (trex.biohpc.org, Culman *et al.* 2009). We examined the peaks visually then filtered them by height. Peaks lower than the lower standard deviation of the mean were excluded. Peaks were then aligned with a clustering threshold of 0.95. We further cleaned the data by excluding TRFs that only appeared in 1 % of samples. This resulted in a lower TRF number than is reported in some other studies (Kennedy *et al.* 2005; Brodie *et al.* 2008). However, reducing the number of TRFs to focus on the dominant types helps to eliminate artefactual results (Singh *et al.* 2006), and allowed us to use multivariate statistics.

2.5 BACTERIAL AND FUNGAL ABUNDANCE

We quantified soil bacterial and fungal DNA separately using quantitative PCR (qPCR) using GoTaq® qPCR Master Mix (Promega, NSW AU). All qPCRs were performed using a Rotor-Gene 6000 cycler (Corbett Life Science, NSW, AU). For bacteria cycling conditions were as follows; 95 ˚C for two minutes, then 40 cycles of denaturation at 95 ˚C for 15 seconds, annealing at 53 ˚C for 30 seconds, elongation at 60 ˚C for 60 seconds, then a final elongation period of 72 ˚C for 10 minutes. At the end of this period, the amplification specificity was determined using a dissociation curve which ramped up the temperature from 60-98 ˚C in 30 second increments. Amplification efficiency was >99 % in all cases. For bacteria, we created a calibration curve using cloned 16S rRNA gene PCR products using the TOPO TA cloning

kit (Life Technologies, VIC, AU). The gene copy number (hereafter termed abundance) was determined following spectrophotometric quantification using the equation from Godornes *et al.* (2007). Details of the Master Mix and standards can be found in Appendix S2.

The fungal reaction began at 95 ˚C for two minutes, the 40 cycles of denaturation at 95 ˚C for 15 seconds, annealing at 48 ˚C for 20 seconds, 72 ˚C for 45 seconds and 83 ˚C for 15 seconds, with a final elongation period of 72 ˚C for 10 minutes. The dissociation curve was set to run from 58 – 95 ˚C in 30 second increments. Amplification efficiency was again 99 %. Gene copy number is referred to as copies g $soil^{-1}$.

2.6 STATISTICAL ANALYSIS

All analyses were performed using R2.15.0 (R Core Development Team 2012). We analysed the effects of the rainfall treatment on soil moisture measurements for individual time points using a one-way analysis of variance (ANOVA) with block as an error term and rainfall regime as the main effect. We then looked for treatment effects on arcsine transformed plant percentage cover data from the July vegetation survey using multivariate ANOVA (MANOVA) with Pillai's Trace (Bray & Maxwell 1982). In this analysis the percentage cover values of all species were used in the MANOVA. Pillai's Trace was used because the cover data was zero-inflated and this metric is less sensitive to violations of assumptions (such as unbalanced factor levels) than other metrics (Scheiner 2001). The functional composition treatment was included as a categorical explanatory variable, with each group converted to a binary presence or absence factor, while the rainfall change treatment was represented as a two level factor. Block was represented as an error term with four levels. All

two-way interactions between the rainfall and functional composition variables were included. This analysis was followed by an inspection of treatment effects on individual species, using two-way ANOVA with a Bonferroni correction (Holm 1979).

Treatment effects on microbial community structure were evaluated in August 2011 using MANOVA, as described above for plant species cover, with concatenated TRFs as the response variable. Bacterial and fungal community data were analysed separately. Again, we tested for the effects of rainfall change treatment and plant functional group presence or absence, with block as an error term. Significant dissimilarities between treatments were then represented visually by using linear discriminant analysis (LDA; Bray & Maxwell 1982) in the MASS package of R. Individual bacterial and fungal TRFs were analysed with post-hoc two-way ANOVAs with Bonferroni correction to detect whether particular TRFs were associated with specific plant functional groups or were sensitive to the rainfall change treatment. The m-TRFLP data were subsequently analysed using non-metric multidimensional scaling (NMDS; vegan package of R) in order to create NMDS axes for use in subsequent statistical models. Dissimilarities were calculated using the Bray-Curtis index. Closer points on the NMDS axes indicate more similar communities. Eigenvectors produced by LDA are unsuitable for this type of modelling because the treatments are demarcated *a priori* (Legendre & Legendre 2012). We then evaluated abundance of the bacterial and fungal groups using ANOVAs, with rainfall treatment and binary variables of functional group presence or absence as explanatory variables and block as an error term.

To test whether the rainfall change and functional composition treatments affected ecosystem functioning, we constructed two-way ANOVAs to describe the ecosystem function data, with the treatments as factors and all first order interactions included, and block as an error term. The response variables evaluated were ecosystem respiration (R_{eco}), soil extractable

ammonium (NH_4), nitrate (NO_3), phosphate (PO_4), and N mineralisation rate, which were log transformed where they did not meet assumptions of ANOVA.

We followed these analyses with ANCOVAs, with the same model structure as the previous ANOVAs but with the addition of any microbial parameters (community structure or abundance) that had also been changed by the treatments as covariates. The microbial parameters included in this section of the analyses were bacterial abundance and the structure of the bacterial and fungal communities, as represented by the first two NMDS axes for each (see results). The covariates were added in order of the degree of significance of treatment effects upon them (most significant first), and models were simplified using likelihood ratio F tests (LRTs) to remove non-significant covariates. Treatment effects were not removed. This was done to assess whether treatment effects on ecosystem function were regulated by the microbial community. If treatment effects are weakened by the addition of the microbial parameters, this indicates that part of the treatment effect is regulated by the microbial community, as variance is shared. Alternatively, if treatment effects are strengthened due to the addition of new significant terms, which shrink error variance (increasing sum of squares) and increase F ratios, or are unaltered, this implies that variation in ecosystem functioning caused by the treatments is unrelated to change in microbial communities, which explain independent variation in ecosystem function.

3. RESULTS

3.1. EFFECTS OF RAINFALL CHANGE AND PLANT COMMUNITY MANIPULATION TREATMENTS

353 The summer of 2011 was warm and fairly wet, with small inputs of rain occurring almost
354 daily. There was only one large (>20 mm) rainfall event that warranted 100 % reapplication
355 in the 2100 treatment (Figure 1). This rainfall event occurred in early summer and was
356 exceptionally large for the region (35.5 mm), and resulted in the soil moisture in the 2100
357 rainfall treatment becoming significantly higher than that of the ambient approximately two
358 days after the rainfall event and remaining higher for about 20 days, consistent with previous
359 work at this site (Fry *et al.* 2013).

360 In July, there was an average of one fewer species in the perennial plots under 2100 rainfall
361 compared with the ambient plots (species richness: $F_{1,42}$= 5.50, p= 0.024; Table S1), but any
362 effect of functional group removal on overall vegetation cover was not significant (Table S1).
363 Instead, the strongest observed trend was a negative effect of the 2100 rainfall treatment on
364 total vegetation cover, with a 14% reduction in 2100 plots ($F_{1,42}$= 15.95, p<0.001; Table S1).
365 When cover of each species was analysed simultaneously using MANOVA, a general decline
366 of perennial species abundance was observed (Table S2; $F_{1,42}$=3.95, p=0.002). The
367 leguminous annual herb *Vicia tetrasperma* was also significantly reduced by the 2100
368 treatment (1.42% under ambient, 0.79% under 2100 plots; $F_{1,42}$= 4.91, p= 0.032). Finally,
369 there were no treatment effects on the perennial dominant *Holcus mollis* (Table S3).

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371 ### 3.2 TREATMENT EFFECTS ON SOIL MICROBIAL COMMUNITY STRUCTURE
372 ### AND ECOSYSTEM FUNCTION

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374 MANOVA revealed a strong effect of the functional composition treatment upon bacterial
375 community structure (all bacterial TRFs collected into a vector), although there was no effect

of the rainfall treatment. The effects of functional composition were manifested as interaction effects between the presence of annuals and perennials (Figure 2a; Table 1, $F_{1,40}$= 7.24, p= 0.033), and caespitose grasses and annuals (Figure 2a; Table 1, $F_{1,40}$= 26.73, p= 0.003). This means that all removal treatments affected bacterial community structure relative to the controls. In contrast to the responsiveness of bacterial communities, treatment effects on fungal community structure were far weaker and only interaction effects between the rainfall change treatment and functional group presence were evident, with no strong main effects. In this case fungal community structure was altered when perennials were removed from the sward, and when rainfall was manipulated compared to unmanipulated plots (Table 2, $F_{1,39}$=64.25, p=0.015). There were also significant interactions between the manipulations of perennials and caespitose grasses, so the fungal community was different in structure when both groups were present compared with when they were absent, or if one was present alone (Figure 2b; Table 2, $F_{1,39}$=61.92, p=0.016). This was also true of the four combinations of caespitose grass and annual plants (Figure 2b; Table 2, $F_{1,39}$=39.65, p=0.025). When the abundance of individual TRFs were considered, only three were significantly affected by the treatments, with two being associated with the presence of annual plants (Table S5).

The NMDS for bacterial and fungal community structures resulted in low stress values for each (0.096 for bacteria and 0.140 for fungi, k=2), which indicates a good fit to the data. For the bacteria, both axes were strongly positively correlated with TRF size, so bigger fragments were associated with higher values on the axes (Figure S2). We used the first two axes for each in the statistical modelling portion of the analysis.

The qPCR data showed that relative bacterial abundance (gene copies g^{-1} soil) was significantly higher in the 2100 rainfall treatment compared with ambient (Table 3; Figure 3a-c; $F_{1,41}$=12.63, p<0.001). Bacterial abundance was also increased in plots where perennials were present (Figure 3a; $F_{1,41}$=6.99, 0.012). There was also a highly significant interaction

between rainfall treatment and the presence of caespitose grasses; when this functional group was present there was little effect of 2100 rainfall on bacterial numbers, while there was a much lower bacterial number under ambient conditions relative to 2100 rainfall in its absence (48700.00 and 30373.00 respectively; Figure 3b; $F_{1,41}$=11.20, p=0.002). There were no significant effects of either treatment on fungal copy number at the end of the rainfall change period (Table 3).

Effects of treatments on ecosystem function were inconsistent, although one general pattern was that there were no significant main effects of the rainfall change treatment on any function (Table 5). Ecosystem respiration was not significantly affected by the rainfall and plant composition treatments, as either a main or interaction effect (Figures 4a and 4d). In contrast, concentrations of both ammonium and nitrate in the soil were significantly lower when caespitose grasses were present relative to when they were removed (NH_4: Figure 4b, 5.10 and 5.96 mg kg^{-1} respectively; NO_3: Figure 4c, 2.56 and 3.40 mg kg^{-1}, respectively) There was also a significant interaction between the rainfall change treatment and the presence of annual plant species in relation to nitrate. This took the form of higher NO_3 concentrations when annuals were present in ambient rainfall plots. N mineralisation rates were significantly higher when perennial species were removed from the plots (Figure 4e; 7.70 and 2.86 g N m^2 d^{-1}).

3.3 TREATMENT EFFECTS AND MICROBIAL CORRELATIONS WITH ECOSYSTEM FUNCTION

When microbial parameters that had been significantly affected by the treatments were added to the ecosystem function ANOVA models as covariates, there was a tendency for main treatment effects to become stronger (with lower p values), most likely due to the fact that these covariates accounted for additional variance, as opposed to the same variance (Table 5). This indicates that microbial properties are related to ecosystem functioning rates but that change in microbial properties did not fully account for the functional changes caused by the treatments. When the fungal NMDS first axis was added as a covariate, significant treatment effects on R_{eco} became apparent. Furthermore, an interaction was observed between the rainfall change treatment and presence of perennial species in the plots, when fungal community structure was included in the model. R_{eco} was higher in ambient plots where perennial species were present, and the fungal community was rich in TRF lengths 379, 117 and 127, and lower in rainfall change plots with perennials present, with a fungal community rich in TRF lengths 426, 436 and 169 (Table 5; rainfall change x perennial presence: $F_{1,41}$=11.80, p=0.002, Fungal NMDS1: $F_{1,41}$=5.95, p=0.02).

Some of the variance in extractable soil NH_4 concentrations was explained by bacterial NMDS axis 1 (Table 5; Figure S2a; $F_{1,41}$=4.87, p=0.034); concentrations were higher where bacterial communities were dominated by larger fragment sizes. This strengthened the effect of caespitose grass presence on NH_4 that was reported above (F value changed from 7.30 to 8.32, p value from 0.01 to 0.006), indicating that, while bacterial community structure was not modified by the treatments, it was related to the soil extractable NH_4 concentrations (model AIC changed from 89.1 to 83.4). In contrast, extractable soil NO_3 was not significantly altered by any microbial covariate, while soil extractable P concentrations were positively associated with bacterial abundance (Table 5; $F_{1,41}$=7.76, p=0.009). There was also a weak significant interaction between caespitose grass presence and rainfall, with much

higher PO_4 concentrations in rainfall change treatment soils where caespitose grasses were absent, relative to ambient rainfall (Table 5, $F_{1,41}$=4.55, p=0.041).

3.4 MICROBIAL COMMUNITY ASSOCIATIONS WITH ECOSYSTEM FUNCTION INDEPENDENT OF TREATMENTS

Fungal gene copy number was the only microbial parameter not significantly affected by the treatments. Inclusion of this parameter in the models indicated that fungal copy number influenced the function to some extent but that its effects were independent of the functions' response to climate or vegetation change. Fungal gene copy number added explanatory power to the model describing mineralisation rate. Mineralisation rate was lower where there were high numbers of fungal gene copies in the soil (Figure 5b; $F_{1,41}$=5.49, p=0.026), and this had very little effect on the main treatment effect. Model AIC was slightly lowered from 151.38 to 146.68.

4. DISCUSSION

Four years of continuous rainfall and plant functional composition manipulation in this grassland system have yielded distinct plant and microbial communities. While our approach precludes the ability to identify specialist microbial groups, we have nevertheless shown some interesting links between rainfall manipulations, plant community and microbial communities. With regard to our first hypothesis, the abundance and structure of the bacterial community were significantly affected by all plant functional group and rainfall treatments.

In contrast, fungal community structure was more strongly related to presence or absence of annual plants, indicating an association between plant tissue lifespan and turnover on fungal communities (Aerts & Chapin 2000; Roumet *et al.* 2006), thus supporting our second hypothesis. Our third hypothesis was supported to some extent; ecosystem respiration, extractable soil NH_4 and N mineralisation were all altered by changes in plant functional composition, and were also related to variation in the microbial community. Although treatment effects on process rates may not be directly attributable to associated changes in the microbial community, variation in microbial communities between plots was significantly related to ecosystem function. This indicates that including measures of bacterial and fungal community structure could add explanatory power to studies on ecosystem stocks and fluxes of carbon and nutrients (Mikola & Setälä1998; Bardgett *et al.* 2013), but that they may not always be required to understand ecosystem function responses to vegetation and rainfall change (but see Kardol *et al.* 2010; Latz *et al.* 2012).

Our results support the hypothesis that the bacterial community is less resistant to change than the fungal community (Marschner *et al.* 2004; Allison & Martiny 2008; Bever *et al.* 2010). Bacterial cells are free-living for the most part, and are more vulnerable to osmotic changes and fluctuations in nutrient levels than fungi (Chowdhury *et al.* 2011; Manzoni *et al.* 2012). While bacteria associate with fungi, often using the hyphae as highways through the soil (Warmink *et al.* 2011), and directly feeding from hyphae and exudates (Leveau & Preston 2008), fewer direct links with plant species or genera have been described. This is an emerging area of research and our data suggest that there may be closer links than currently recognised (Berg & Smalla 2009; Latz *et al.* 2012; Haichar *et al.* 2014).

4.1 BACTERIAL RESPONSES

In contrast to the response of fungi, rainfall treatment effects on bacterial abundance support our second hypothesis: that bacteria are responsive to rainfall change, most likely because it places them under osmotic stress. Bacterial abundance was related to both the rainfall change and plant functional composition treatments, while bacterial community structure was only affected by the latter, thus suggesting that the entire community was equally susceptible to drought. Changes in bacterial abundance caused by plant composition change could be attributable to the alteration of niche opportunities created during plant compositional manipulations (Bardgett *et al.* 2005). This indicates that the bacterial community in these grasslands is closely associated with the plant community, and that manipulation of the relatively small range of plant species at our site is sufficient to alter the bacterial community, as reported in similar lowland grassland studies (Latz *et al.* 2012).

Bacterial community structure was only affected by the functional composition manipulations. Effects of the perennial plant functional group treatment were apparent on the soil bacteria community structure, despite our decision to retain *H. mollis* in every plot to reduce weeding-disturbance effects. While we feel that we are justified in retaining this species because of its uniform cover and dominance, it is possible that its retention reduced the impact of perennial group removal.

4.2 FUNGAL RESPONSES

Fungal community structure showed a fairly weak response to both rainfall change and plant functional group treatments, while fungal abundance was not significantly affected by either.

These results suggest that fungal communities in the mesotrophic grasslands of southern Britain may be relatively tolerant of future changes in rainfall. Plant functional composition manipulations only significantly affected the fungal community in weak interactions with the rainfall treatment. Therefore, our findings do not fully support the hypothesis that plant functional composition is a more important regulator of fungal communities than rainfall change. There was also very little direct effect of rainfall change on fungal community structure or fungal abundance. This is in line with current literature, which indicates that fungal mycelia are relatively resistant to, or even increased by, fluctuating water availability (Birhane *et al.* 2012; Kaisermann *et al.* 2015). Where fungal community composition was significantly altered by rainfall changes it was when perennial and caespitose grass species, but not annual plant species, were present. This is interesting because annual species often (but not always) have lower incidences of associations with mycorrhizal fungi (Akhmetzhanova *et al.* 2012; Alguacil *et al.* 2012), a phenomenon associated with the need for yearly reinfection (Saikkonen *et al.* 1998) and their tendency to colonise disturbed habitats where intact mycorrhizal mycelium may be absent. Furthermore, the rapidly changing rhizosphere under annual dominated assemblages is not conducive to hosting food webs associated with slower-growing soil fungi, which suggests that in our study fungal community structure shifted in response to the temporal stability of plants and associated soil inputs (De Vries *et al.* 2007).

The weak but complex effects of plant community manipulations on fungal community structure could indicate that there are plant species- or trait-specific characteristics that influence fungal community development. Two possible hypotheses arise from the results; more testing is required to ascertain mechanisms. The fungal community associated with perennial species may be less diverse (lower TRF number) due to slower root and shoot turnover and possibly more complex root tissue and exudate chemistry and recalcitrant litter

(Warembourg & Estelrich 2001). Another possible explanation could be due to root architecture; in our system perennial species such as *Rumex obtusifolius* and *Cirsium arvense* are tap-rooted, and deeper, less complex roots may host fewer microbial groups (Roumet *et al.* 2006).

4.3 TREATMENT EFFECTS ON FUNCTION

We found previously that plant functional composition manipulations, implemented over the preceding four years, altered rates of CO_2 exchange and nutrient cycling (Fry *et al.* 2013, 2014a). Here, as before, ecosystem respiration was strongly reduced by summer rainfall reductions in perennial plots, while plots dominated by annuals were unaffected, demonstrating consistency of treatment effects. In the study described here, we suggest that these effects are not mediated by microbes, although microbes do appear to alter ecosystem respiration independently of the plant community or rainfall regime. Temporal and seasonal measures of microbial community activity, abundance and composition, along with more detailed measures of ecosystem respiration are needed to fully understand these links.

In our system effects of vegetation and precipitation change on ecosystem function may have operated via changes to plant and microbial physiology and via impacts on the physical and chemical environment. For example, shifts in the soil physical environment as a result of altered root architecture and concomitant changes in soil structure are also likely to affect ecosystem functions by altering rates of root exudation, soil aeration and enzyme activity. Such effects may not be in line with the biomass ratio hypothesis, which assumes that the effect of plant traits on ecosystem function is proportional to their relative abundance or

biomass (Grime 1998). Extractable soil N concentrations (both NH_4 and NO_3) were partially explained by the cover of caespitose grasses, which are rare in our system (never more than 3% cover). Their disproportionately high effects on the soil may be because they are large tussock grasses that tend to collect nutrients as 'resource islands' within their rhizosphere soil and grow rapidly (Derner & Briske 2001). Soil NH_4 levels were also partly explained by the bacterial community structure, a variable that was itself strongly linked to plant functional group presence or absence. There is much evidence in the literature demonstrating links between plant compositional manipulations and microbial community responses, which in turn affect function (van der Putten *et al.* 2007; Berg & Smalla 2009), but in our study functional composition effects on microbial communities did not appear to affect function. Nonetheless, our results indicate that traits associated with plant assemblage longevity can lead to discrete and specific microbial communities, which could ultimately impact ecosystem functioning over longer timescales than those measured here.

Soil extractable P was strongly positively associated with bacterial abundance, with a weak association with rainfall and caespitose grasses in our study. Labile pools of PO_4 are primarily cycled by plants and microbes in semi-natural systems, although in low-P soils plants have many means of mobilising P from soil and bedrock (Lambers *et al.* 2006). Higher bacterial numbers were generally associated with higher P, which could indicate that the bacterial community is driving organic matter turnover and the provisioning of inorganic P. It is possible that many plant compositional impacts on function will operate via rhizosphere and rhizoplane microbial communities which we did not measure. However, the strong effects of bacterial abundance on soil P could indicate that the bacterial community is driving organic matter turnover and provisioning of nutrients in the bulk soil, as well as in direct proximity to plant roots.

4.4 INDEPENDENT MICROBIAL CORRELATIONS WITH FUNCTION

In our study measures of the microbial community explained significant amounts of variation in ecosystem function but this variance was not shared with treatment effects on function. This suggests that treatment effects on ecosystem function were not mediated by the microbial community. Instead microbial effects were independent of the treatments, but important nonetheless. Fungal abundance was significantly negatively associated with N mineralisation rate. The links between the fungal community and nitrogen cycling measurements were not interlinked with treatment effects, and while we could not speculate on the fungal to bacterial ratio here due to the limitations of qPCR, more fungal dominated soils are associated with slower process rates (De Vries *et al.* 2012a), so our data are in line with broader landscape-scale studies. Future experiments in these systems would also benefit from investigation into the responses of associated taxa, as relationships between plants, climate and soil communities are likely to shape the patterns in ecosystem function that we observe (Waldrop & Firestone 2006; Davison *et al.* 2011; Montesinos-Navarro *et al.* 2012).

4.5 CONCLUSIONS

Ultimately in our study, microbial community composition was more strongly affected by the plant community manipulations than changes in rainfall regimes. It is, therefore, likely that future non-random extinctions of plant species in response to global change factors will have a major impact on microbial communities (De Vries *et al.* 2012b, Allan *et al.* 2015). The evidence collected here suggests however that while shifts in plant functional composition

will likely impact ecosystem functioning, these changes may not always be mediated by concomitant changes in fungal and bacterial community composition and abundance, Instead, it seems that in our system that direct effects on soil physical and chemical properties and upon plant and microbial physiology may play a more important role. Nevertheless, there is evidence from here and elsewhere that microbial community composition is important in controlling function (Van der Heijden *et al.* 2008; Kardol *et al.* 2010). Future experimental studies of climate change and/or plant community impacts on ecosystem functioning should assess the likely benefit, in terms of explanatory power, of including of bacterial and fungal parameters. The improvement in explanatory power is of particular interest due to the relatively limited plant species pool in these grasslands; while patterns of microbial activity and links to function have been made at the country- and landscape- scale (Liu *et al.* 2010; De Vries *et al.* 2012), this study shows that these relationships can be found at the experimental plot scale as well. In our study, microbial community structure was more strongly affected by plant community composition than the rainfall regime. Our results suggest that plant community changes in response to climate change and other global change drivers can result in non-random shifts in microbial community structure and abundance. This finding is particularly important as climate change and other global change drivers are is projected to cause major shifts in plant community composition, with potentially strong effects on the structure and functioning of microbial communities.

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954 SUPPORTING INFORMATION

955 Additional supporting information may be found in the online version of this article:

956

957 Figure S1: Schematic of the 2100 rainfall regime, showing a simulated version of the 2100
958 rainfall change treatment.

959

960 Appendix S1: Description of the DIRECT experimental site, with an emphasis on soil
961 parameters.

962 Appendix S2: Details of Master Mix and standards used in qPCR.

963 Table S1: Rainfall and plant composition treatment effects in July 2011.

964 Table S2: Full output of MANOVA of the effect of rainfall change and functional group
965 manipulations upon species percentage cover in July 2011.

966 Table S3: ANOVA model output showing the effect of the treatments on *Holcus mollis*
967 percentage cover in the plots.

968 Table S4: Results from ANOVAs of individual bacterial TRFs derived from MANOVA
969 output with Bonferroni correction.

970 Table S5: Results from ANOVAs of individual fungal TRFs, derived from MANOVA output
971 with Bonferroni correction.

972 Figure S2: Correlations of TRF size with NMDS axis values.

973

974 TABLES

975

976 Table 1: Full output of MANOVA of the effect of rainfall and functional group manipulations
977 upon bacterial TRF distribution in August 2011. TRFs are Terminal Restriction Fragments,
978 derived from multiplex Terminal restriction fragment length polymorphism (m-TRFLP).
979 Emboldened text denotes significance at the $p<0.05$ level.
980 Table 2: Full output of MANOVA of the effect of rainfall and functional group manipulations
981 upon fungal TRF distribution in August 2011. TRFs are Terminal Restriction Fragments,
982 derived from multiplex Terminal restriction fragment length polymorphism (m-TRFLP).
983 Emboldened text denotes significance at the $p<0.05$ level. The error d.f. is derived from the
984 total number of TRF types in the study.

985 Table 3: ANOVA of treatment effects on bacterial and fungal abundance as represented by
986 gene copy number, produced by qPCR.

987 Table 4: Treatment effects on ecosystem functions measured at the end of the summer rainfall
988 manipulation period (August 2011).

989 Table 5: Models of ecosystem functioning showing treatment effects and retained microbial
990 covariates. AICc is used for small samples. All R^2 values are adjusted R^2.

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993 FIGURES

994

995 Figure 1: Rainfall and percentage soil moisture from May 2011 to August 2011. Error bars
996 represent standard error of the mean. Significance stars are used as follows: * = $p<0.05$, ** =
997 $p<0.01$, *** = $p<0.001$.

998 Figure 2: Linear Discriminant Analysis of m-TRFLP data for a) Bacteria in August
999 (difference between groups explained: LDA1= 64.66%, LDA2= 19.52%), b) Fungi in August
1000 (difference between groups explained: LDA1= 44.69%, LDA2= 26.49%). Open circles:
1001 Perennials, open diamonds: Caespitose Grasses (CG), closed diamonds: Annuals, open
1002 triangles: Perennials and CG, closed circles: CG and Annuals, x: Perennials and Annuals,
1003 crosses: all species.

1004 Figure 3: Main treatment effects on relative bacterial copy numbers obtained from qPCR in
1005 August 2011, comparing effects of rainfall change and presence or absence of a) Perennials,
1006 $F_{1,42}$= 5.89, p= 0.019. b) Caespitose Grasses, $F_{1,42}$= 10.51, p= 0.002, c) Annuals, $F_{1,42}$=
1007 121.85, $p<0.001$. Error bars represent the standard error of the mean.

1008 Figure 4: Treatment effects upon ecosystem functions. Grey bars are the 2100 rainfall
1009 treatment, white are ambient. a) Ecosystem respiration: Rainfall change x Perennial presence:
1010 $F_{1,33}$=11.80, p=0.002. b) Soil extractable ammonia: Perennial presence: $F_{1,33}$=7.30 p=0.011,
1011 Caespitose Grass presence: $F_{1,33}$=5.95 p=0.02. c) Soil extractable nitrate: Rainfall Change x
1012 Annual presence: $F_{1,33}$=6.47 p=0.016. d) Soil extractable phosphate: Rainfall Change x
1013 Caespitose Grass presence: $F_{1,33}$=4.55 p=0.041. e) Mineralisation rate: Perennial presence:
1014 $F_{1,33}$=8.87 p=0.007.

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