

## **Grazing effects on microbial community composition, growth and nutrient cycling in salt marsh and sand dune grasslands**

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1    **Grazing effects on microbial community composition, growth and nutrient cycling in**  
2    **salt marsh and sand dune grasslands**

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## 19    **Abstract**

20    The effect of grazing by large herbivores on the microbial community, and the  
21    ecosystem functions they provide, is relatively unknown in grassland systems. In this  
22    study, the impact of grazing upon the size, composition and activity of the soil microbial  
23    community was measured in field experiments in two coastal ecosystems; one salt  
24    marsh and one sand dune grassland. Bacterial, fungal and total microbial biomass were  
25    not systematically affected by grazing across ecosystems, although within ecosystem  
26    differences could be detected. Fungal-to-bacterial ratio did not differ with grazing for  
27    either habitat. Redundancy analysis showed that soil moisture, bulk density and root  
28    biomass significantly explained the composition of phospholipid fatty acid (PLFA)  
29    markers, dominated by the distinction between the two grassland habitats, but where  
30    the grazing effect could also be resolved. PLFA markers for gram-positive bacteria were  
31    more proportionally abundant in un-grazed, and markers for gram-negative bacteria in  
32    grazed grasslands. Bacterial growth rate (leucine incorporation) was highest in un-  
33    grazed salt marsh, but did not vary with grazing intensity in the sand dune grassland. We  
34    conclude that grazing consistently affects the composition of the soil microbial  
35    community in semi-natural grasslands, but that its influence is small (7% of the total  
36    variation in PLFA composition), compared to differences between grassland types (89%).  
37    The relatively small effect of grazing translated to small effects on measurements of soil  
38    microbial functions, including N and C mineralization. This study is an early step toward

39 assessing consequences of land-use change for global nutrient cycles driven by the  
40 microbial community.

41 **Keywords** Livestock grazing . Decomposer ecology . Bacterial growth rate . PLFAs .  
42 Nutrient cycling

## 43 **Introduction**

44 Many types of semi-natural grassland, including coastal grasslands, have been  
45 traditionally managed by low intensity cattle or sheep grazing. However, in the light of  
46 removal of European Union (EU) subsidies for marginal grazing land (Strijker 2005;  
47 Taylor 2006) it is not known how grazing abandonment will affect these habitats. The  
48 effects of large herbivore removal are relatively well studied for plant, invertebrate and  
49 bird communities (Morris 2000; Pykälä 2003; Vickery et al. 2001). However, effects upon  
50 the soil microbial community, and therefore soil ecosystem functions such as plant  
51 nutrient availability and organic matter decomposition, are less well known (Smith et al.  
52 2003).

53 Cessation of livestock grazing leads to the gradual development of a plant  
54 community dominated by tall grasses or shrubs with an increased plant litter layer  
55 (Bakker et al. 1993; Janišová et al. 2011) and has variable effects on root biomass,  
56 turnover and exudation (Piñeiro et al. 2010). Soil microbial activity and abundance are  
57 directly related to litter and rhizodeposition (Beare et al. 1991; Grayston et al. 2001;  
58 Jones et al. 2004; Mawdsley and Bardgett 1997). Grazing intensity also affects abiotic

factors. Short grazed vegetation leads to greater and more variable soil temperatures than un-grazed grassland (Curry 1994). Large herbivore grazers, such as cattle, compact the soil surface via treading and change soil structure and aeration leading to waterlogging (Lambert 2000), with effects upon microbial community composition (Clegg 2006). Grazing animals also return nutrients to the soil (Bakker et al. 1993) that greatly influence microbial activity. For instance, cattle faeces are a source of soil C and can increase microbial biomass and respiration (Hatch et al. 2000; Lovell and Jarvis 1996) and livestock urine is a source of N linked to increases in respiration, nitrous oxide emissions and microbial biomass (Ritz et al. 2004). Nutrient cycling due to grazing can be expected to have differential effects in high and in low fertility systems (Holdo et al. 2007), and while sand dune grasslands typically are of low fertility, salt marsh grasslands tend to be of higher fertility.

Salt marshes differ from other terrestrial ecosystems due to regular cycles of inundation by tides that transiently saturate the soil with water, and thereby limit oxygen availability. In these ecosystems, the overriding influence of soil moisture (Waksman and Gerrettsen, 1931) is particularly emphasized. While microbial activity increases with higher water availability in dry conditions (Bapiri et al. 2010; Iovieno and Bååth 2008), the relationship changes at high water availabilities, and waterlogged soils exhibit reduced soil respiration (Luo and Zhou 2006).

It has proven difficult to generalize the impact of land-use on soil microbial communities. It has been shown that factors including tillage (Six et al. 2006; Van

Groeninger et al. 2010), N fertilization (de Vries et al. 2006; Rousk et al. 2011a) and grazing intensity (Bardgett et al. 2001; Klumpp et al. 2009; Lopez-Sangil et al. 2011) can affect the size and composition of the soil microbial community. However, the precise changes within the microbial community between different ecosystems have not been systematically addressed. To date insights have been mostly limited to individual case-studies (Strickland and Rousk 2010), and efforts to synthesize these into a general understanding of grazing have yet to be attempted. The direct influence of the micro-scale conditions on composition, activity and biomass of the microflora are important. That is, it will only be possible to generalize effects of land-use to the extent that they expose microbial communities to reproducible selective pressures such as pH changes (Rousk et al. 2010a), or organic matter quality (Rousk and Bååth 2007).

In this study we investigated the impact of grazing intensity on the active soil decomposer community of temperate upper saltmarsh and fixed sand dune grasslands. By including two independent grazing systems predicted to be of contrasting fertility, we aimed to assess and relate the influence of grazing on the composition, size and activity of the soil microbial community to the specific differences inherent between ecosystems. Microbial biomass and community composition were measured using phospholipid fatty acids (PLFAs) and bacterial growth rate by leucine incorporation. We hypothesized the principal source of variation in the microbial composition would occur between the two ecosystems, but that we would also resolve a secondary, systematic effect of grazing intensity.

## Methods

### Salt marsh

The study area, Crossens Marsh (53° 41' 15" N, 2° 57' 4" W), is a salt marsh located on the southern edge of the Ribble estuary in North-West England and is part of the Sefton Coast Special Protection Area managed by Natural England, the statutory conservation body. The marsh was historically un-grazed but was split into two management types over 40 years ago, un-grazed and cattle grazed by a boundary fence. The grazed marsh is characterized predominantly by *Festuca rubra* and the un-grazed marsh by *Elytrigia repens* (Rodwell 2000). The grazed part of the marsh covers 517 ha and is uniformly grazed by around 100 bullocks from late May to early October, approximately 0.2 cattle (*Bos taurus*) ha<sup>-1</sup>, and provides a consistent short sward height (< 8 cm) for overwintering pink-footed geese (*Anser brachyrhynchus*) to feed. Small herbivores such as field voles are also present, particularly on the un-grazed marsh. All experimental units were selected within the high marsh zone where numerous creeks are present but tidal inundations are relatively rare, limited to around eight events a year on high equinox tides. A paired experimental design was used with six experimental units of approximately 10 m x 10 m set up on each side of a 600 m long section of the fence line, 100-150 m apart, in a 'mirror image' formation, giving six grazed (G1-G6) and six un-grazed (U1-U6) units (Fig. 1a) (Ford et al. 2012). Each experimental unit was located between 20 m and 50 m from the fence line to ensure an adequate buffer zone and checked for standard elevation within  $\pm 10$  cm.

122 Sand dune grassland

123 Newborough Warren is a calcareous coastal sand dune grassland, located in NW Wales  
124 (53° 8' 59" N, 4° 21' 1" W), noted for its high plant biodiversity and designated as a  
125 National Nature Reserve, Site of Special Scientific Interest and Special Area of  
126 Conservation under the EC Habitats and Species Directive 1992 (Plassmann et al. 2010).  
127 The 389 ha site is managed by Countryside Council for Wales (CCW) and grazed by  
128 Welsh mountain ponies (*Equus ferus caballus*; 0.2 ha<sup>-1</sup>), cattle, Belted Galloways and  
129 Dexters (*Bos taurus*; 0.05 ha<sup>-1</sup>), and rabbits (*Oryctolagus cuniculus*; 45 ha<sup>-1</sup>) (Plassmann  
130 et al. 2009), designed to promote plant diversity. Grazed vegetation is characteristic of  
131 *Carex arenaria* - *Festuca ovina* - *Agrostis capillaris* dune grassland and *Festuca rubra* -  
132 *Galium verum* fixed dune grassland (Rodwell 2000). In 2003, three replicate  
133 experimental blocks were established, each containing three 10 m x 10 m experimental  
134 units: one fully grazed unit (unfenced), one rabbit grazed unit (fenced with 10 cm x 10  
135 cm mesh to exclude large grazers) and one un-grazed unit (fenced with 10 cm x 10 cm  
136 mesh and an additional 2.7 cm x 3.7 cm mesh buried 20 cm underground to prevent  
137 rabbit access) (Fig. 1b; Plassmann et al. 2009). Small mammals and invertebrate  
138 herbivores were assumed to be present within all experimental units. Fully grazed units  
139 are denoted as pony and rabbit grazed (PR1 - PR3), rabbit grazed (R1 - R3) and un-  
140 grazed (U1 - U3).

141 Soil and vegetation analyses



In autumn 2010, four soil cores (5 cm depth, 5 cm diameter) per experimental unit were taken, vegetation, roots and stones were removed and the remaining soil was sieved to  $\leq 2$  mm and stored for 1 week at 5°C before further analyses. For soil respiration, 10 g sub-samples, one from each soil core sample, were weighed into 50 ml polypropylene centrifugation vials and soil respiration rate at 22 °C measured continuously on a multichannel IR respirometer (PP-systems Ltd, Hitchin, UK). The reported soil respiration rate was the 4 hour average measurement taken after reaching a stable rate. Gravimetric soil moisture was estimated by determining the weight loss of samples dried initially at 105°C for 72 hours. Subsequently, organic matter (OM) content was estimated by loss-on-ignition from soil sub samples (375 °C for 16 hours; Ball 1964). Soil pH (5 g soil: 12.5 ml water dilution factor) was determined using a Corning pH meter 220. Samples to determine bulk density were collected during September 2009 using three intact soil cores of 3.8 cm diameter and 15 cm depth from each experimental unit. Cores were dried at 105 °C for 72 hours and the dry mass divided by the volume of the core to calculate bulk density. Soil cores for total soil C and N were air dried, thoroughly homogenised and dried at 105 °C for 3 hours prior to analysis. Samples were analysed on an Elementar Vario-EL elemental analyser (Elementar Analysensysteme GmbH, Hanau, Germany), using oxidative combustion to detect C and N. The C/N ratio was also calculated using a mass ratio.

The potential for nutrient cycling by microbes was assessed using a measure of mineralisable N (Rowe et al. 2011). Three N mineralisation cores, 3.8 cm diameter and

15 cm depth, were taken from each experimental unit, during September 2009. Soil cores were taken using plastic corers, capped at both ends to minimise soil disruption, and stored intact at 4 °C. Accumulated inorganic N was flushed from the cores by spraying with a solution of similar ionic concentration to UK rain over 7 days until 150 ml of leachate had been collected. Cores were incubated at 10 °C for 28 days, homogenised and a sub-sample extracted using 1 M KCl for the analysis of ammonium and nitrate content (Rowe et al. 2011). Nitrogen mineralization rate was calculated over these 28 days assuming that all previous inorganic N had been removed during the 7 day flushing period. Mineralisable N was expressed as  $\mu\text{g N g}^{-1} \text{ org. mt (organic matter) day}^{-1}$  for plant and microbial available N. Above-ground live vegetation (shoot) and plant litter were collected from five (two in sand dunes) 25 cm x 50 cm zones, cut to ground-level, in July 2009. One root core of 5 cm diameter and 10 cm depth was also taken per quadrat and washed to remove all soil. Above-ground vegetation, litter and roots were all dried at 80 °C for 24 hours and weighed to determine above-ground shoot biomass, litter biomass and below-ground root biomass, respectively. Root turnover was measured during September 2010 via four nylon 1 mm root turnover mesh strips (Normesh, UK), 2.5 cm wide x 15 cm long, placed in vertical cuts made in the soil with 2.5 cm overlap at the bottom and 2.5 cm emerging from the soil, 50 cm apart, across a 2 m transect in each unit. After 28 days the mesh strips were removed along with a slightly wider and deeper intact soil core. Cores were pushed out and divided in two along the mesh line, the number of fine roots penetrating each mesh depth zone (0 –

184 2.5 cm; 2.5 – 5 cm; 5 – 7.5 cm; 7.5 – 10 cm) were counted by eye as a proxy for fine root  
185 turnover (Lukac and Godbold 2010).

#### 186 PLFAs

187 The PLFA composition from a 1 g fresh soil sub-sample was determined according to  
188 Frostegård et al. (1993) with modifications (Nilsson et al. 2007). An internal standard  
189 (methyl nonadecanoate fatty acid 19:0) was added before the methylation step. To  
190 obtain indications of bacterial and fungal biomass specific PLFA markers were summed  
191 (Frostegård and Bååth 1996; Table 3). PLFAs were also grouped according to Gram-  
192 negative and Gram-positive bacteria (O’Leary and Wilkinson 1988; Wilkinson 1988;  
193 Table 3).

#### 194 Bacterial growth rate and turnover times

195 Bacterial growth was estimated by measuring the incorporation of leucine (Leu) into  
196 bacteria (Kirchman et al. 1985) extracted from 1 g soil sub-samples using the  
197 homogenization / centrifugation technique (Bååth 1994), with modifications (Bååth et al.  
198 2001; Rousk and Bååth 2011). We added 2 µl [<sup>3</sup>H]Leu (37 MBq ml<sup>-1</sup>, 5.74 TBq mmol<sup>-1</sup>,  
199 Perkin Elmer) that were combined with non-labelled Leu, resulting in a final  
200 concentration of 275 nM Leu in the bacterial suspensions. The samples were then  
201 incubated for 2 h at 22 °C in the dark. Bacterial growth was estimated from the amount  
202 of Leu incorporated into extracted bacteria per hour and gram of soil. The Leu method is  
203 a well-established method to estimate bacterial biomass production rate in both aquatic

(Kirchman 2001) and terrestrial (Rousk and Bååth 2011) environments, and PLFA concentrations can be used to estimate bacterial biomass (Frostegård and Bååth 1996; Strickland and Rousk 2010); thus a rough index for bacterial turnover time was calculated by dividing the bacterial biomass (nmol PLFAs g<sup>-1</sup>) by bacterial growth rate (nmol Leu incorporation g<sup>-1</sup> h<sup>-1</sup>).

## Statistical analysis

Salt marsh and sand dune grassland data were analysed separately using linear mixed effects models (lme) in R (R Development Core Team 2011). Differences between pairs of grazing treatments (Salt marsh: G and U; Sand dune: PR and R, R and U, or PR and U) for all variables were analysed. For the sand dune grassland the grazing treatment was nested within block (Fig. 1b), for the salt marsh grazing was also nested within block with 'blocks' defined as positions along the fence line (Fig. 1a) (example R code: lme (pH ~ grazing, random = ~1|block/grazing)). This approach was used to enable the raw data to be analysed accounting for replication at the level of the experimental unit or block (Salt marsh n = 6; Sand dune n = 3; Crawley 2007). To ensure normal distribution of data, lme models were run iteratively for raw, logged and square root transformed data (for percentage variables arcsine square root transformed was also run) for each soil or microbial variable. Each model was compared and the most normal or 'best' for each variable was presented in the results section, chosen on the basis of lowest Akaike information criterion (AIC) number and quantile probability plot (qqnorm) with most normal distribution (straightest line) following a visual assessment (Crawley 2007). For

overall grazing effect, results were analysed using Analysis of Variance (ANOVA) of the lme model. For the sand dune data, in addition to the overall grazing effect, we reported differences between treatment pairs directly from the lme analysis.

The relationship between salt marsh and sand dune grassland PLFA composition (mol-% of the 30 most abundant PLFAs; standardized to unit variance) and environmental variables (soil parameters from Table 1 and 2) from grazed and un-grazed experimental units was analyzed using redundancy analysis (RDA). RDA scaling was focused on inter-‘species’ (PLFAs) correlations and centered by species. The significance of environmental variables was tested using automatic forward selection (Monte Carlo test, 500 permutations). All multivariate analysis was carried out in Canoco v.4.5 (Ter Braak and Šmilauer, 2003). The RDA plots show a visual interpretation of the relationship between environmental variables and the distribution of PLFA markers for both salt marsh and sand dune grassland. Grazing treatment of each unit was included in the final RDA plots but was not used to influence the analysis. To determine significant effects on the PLFA composition of the microbial community, the RDA axes scores for axis 1 and 2, respectively, were subjected to subsequent 2-way ANOVAs to test for the influence of site (salt marsh vs sand dune grassland) and grazing (grazed vs un-grazed) on the distribution of samples along the two axes. For the sand dune grassland fully grazed (PR) and rabbit grazed (R) were grouped as grazed.

## **Results**

### **Soil and vegetation characteristics**

Organic matter content, bulk density, C/N ratio, net ammonification rate, root turnover and root biomass were all significantly greater on the grazed compared to un-grazed salt marsh grassland (Table 1). Net nitrification rate, soil pH, litter and shoot biomass were all significantly greater on the un-grazed compared to grazed salt marsh. Salt marsh soil basal respiration rate did not differ with grazing treatment. For the sand dune grassland, the majority of soil and vegetation variables did not differ significantly with grazing intensity (Table 2). Soil basal respiration rate and root biomass were greater in the fully and rabbit grazed than the un-grazed sand dune grassland. Litter biomass was greater in the rabbit and un-grazed than the fully grazed sand dune grassland. Net nitrification rate was greatest in the un-grazed sand dune soil.

#### PLFAs

Total PLFA, bacterial and fungal PLFA concentrations were all about 50% higher in grazed than un-grazed salt marsh but did not differ with grazing treatment in sand dune soils (Fig. 2). The relative abundances of both bacterial and fungal PLFA markers did not differ significantly with grazing treatment for either salt marsh or sand dune grassland; consequently the fungal-to-bacterial ratio did not differ between treatments (Table 4; Table 5). Gram-negative bacterial PLFAs (as defined in Table 3) were proportionally more abundant in the grazed than the un-grazed salt-marsh. Gram-positive bacterial PLFAs were proportionally more abundant in the un-grazed than the fully grazed sand dune grassland.

#### PLFAs and environmental variables

The RDA bi-plot (Fig. 3) shows the relationship between environmental variables and the distribution of PLFA markers for both salt marsh and sand dune grassland. Axis 1, and axes 1 and 2 combined, explained 89% and 96% of the variation in relative abundance of PLFA markers respectively. Monte Carlo permutation tests showed that three environmental variables explained a significant proportion of the variation; gravimetric soil moisture (F-ratio = 48.86,  $P < 0.01$ ), bulk density (F-ratio = 4.95,  $P < 0.01$ ) and root biomass (F-ratio = 4.37,  $P < 0.01$ ). All other environmental variables either correlated with these three or did not describe a significant proportion of PLFA marker occurrence. The RDA plot showed a significant distinction between the salt marsh and sand dune grassland sites along axis 1 (ANOVA site,  $P < 0.001$ ; Fig. 3a). The effect of 'grazing' and 'site: grazing' interaction were not significant for axis 1. Grazing affected the PLFA composition along axis 2 as seen by a significant division between un-grazed and grazed treatments (ANOVA grazing,  $P < 0.001$ ; Fig. 3a). The effect of 'site' and 'site: grazing' interaction were not significant for axis 2. Grazed treatments in both sites were positively associated with axis 2, while un-grazed treatments were negatively associated with axis 2. PLFA markers associated with Gram-positive bacteria (Table 3), including PLFAs i17:0 and i15:0 were relatively more abundant in soils with lower grazing pressures, while markers associated with Gram-negative bacteria, including 16:1w7t, 16:1w7c and 18:1w7, were relatively more abundant in systems with higher grazing pressures.

Bacterial growth rate and turnover times

Bacterial growth rate was greater in un-grazed than grazed salt marsh (Fig. 4), but was not significantly different between the sand dune grassland grazing treatments. The index for bacterial turnover time was also significantly lower in un-grazed compared to the grazed salt marsh ( $P<0.01$ ), but did not differ significantly with grazing treatment for the sand dune grassland.

## **Discussion**

In the present study, we aimed to assess and relate the influence of grazing on the soil microbial community. We wanted to identify how grazing could systematically affect the soil microbial community structure and quantitatively relate these differences to known differences between different ecosystems. As anticipated, most of the variation, 89%, of the microbial PLFA composition was related to site differences, clearly separating the salt marsh and sand dune communities. Soil moisture content was much greater in the salt marsh than the sand dune grassland, explaining its influence on the first axis of the RDA analysis. For axis 2, ~7% of the total variation in PLFA composition was clearly related to grazing intensity for both sites. The environmental factors related to this separation both between sites and as a consequence of grazing, included soil moisture, bulk density and root biomass. Using the PLFA markers composition to indicate how grazing intensity affected the microbial community composition we find that markers associated with Gram-positive bacteria were relatively more abundant in soils with lower grazing pressures, while markers associated with Gram-negative bacteria were relatively more abundant in soils with higher grazing pressures.



It was previously found that Gram-negative PLFA markers are relatively abundant in the rhizosphere (Söderberg et al. 2004) due to the presence of labile C resources (Bird et al. 2011; Steer and Harris 2000). Gram-negative bacteria are normally characterized by high reproductive rates and elevated activity under conditions of ample nutrient supply, traits that would be an advantage in the rhizosphere (Jones et al. 2004; Barret et al. 2011). Moreover, the slow uptake of labile C resources into Gram-positive markers compared to Gram-negative ones, further strengthens this connection (Bird et al. 2011; Olsson and Johnson 2005; Treonis et al. 2004). The link between grazing and rhizosphere stimulation is well known (e.g. Wardle et al. 2004) and therefore, that grazing induces a shift toward Gram-negative bacteria is consistent with these previous findings of soil microbial community responses.

The shift toward higher relative abundances of Gram-negative PLFA markers, often associated with a prolific and fast growing bacterial community, in grazed soil was completely consistent with other measurements. Nitrogen mineralization did not show consistent patterns with grazing, nor did measurements of plant productivity and turnover. Bacterial growth was lower in grazed salt marsh soils, and not clearly affected in the sand dune grassland. However, there was a clear tendency for overall microbial activity, as indicated by basal respiration, to be stimulated by grazing, with a non-significant trend in salt marsh soils and a significant increase in the sand dune system.

It must be noted here, though, that while these estimates of microbial process rates are a snapshot of the active microbial community, the PLFA concentrations (i.e.

biomass concentrations) are an aggregate measure that integrates the recent history of activity, and where high-activity events will disproportionately dominate. This means that at the end of the growth season (we sampled in November), the PLFA concentrations may be more indicative of the conditions of high growth and PLFA productivity during the summer. This due to accumulated PLFA concentrations during bursts of microbial growth being relatively slow to turn over (Rousk and Bååth 2007; 2011). The bacterial growth rates, conversely, represent the microbial activity after the growth season is over, and senescence rhizodeposition rates become minimal to prepare for winter conditions (Jones et al. 2004). Consequently, a microbial community with high reliance of plant root exudation as primary C resource (Gram-negative bacteria) is likely to decrease their growth rates in response to the down-regulated plant-C supply. This should also be reflected in longer bacterial turnover times. The C supply for Gram-positive bacteria, conversely, dominated by the soil organic matter rather than labile plant root exudates, is not likely to be drastically affected by the down-regulation of plants, also translating to smaller reductions in turnover time. While speculative, this could explain the lack of difference (sand dunes), and even tendency for higher bacterial growth in un-grazed soils (salt marsh). The basal respiration rates, measured under laboratory conditions, should reflect the current C availability in the soil, and are not likely to factor in quickly depleted plant-C components (Bengtson and Bengtsson 2007). However, this measure of gross microbial activity and C availability indicated that a microbial community that were mineralizing more C were present in grazed, compared to un-grazed sand-dunes, matching the PLFA results in that system.

Finally, it should be noted that only about 7% of the variation in PLFA composition was related to grazing intensity, despite the aggregate nature of this measurement (i.e. biomass concentration-related). This is likely to mean that any direct effects on rates due to grazing should have been expected to be small, and easily obscured by other (e.g. inter-ecosystem related) factors, including OM concentration, nutrient conditions, pH and soil moisture conditions.

We did not find consistent patterns regarding the relative importance of fungi and bacteria with grazing in this work. Previous studies have found support for and against grazing effects on the balance of fungal and bacterial decomposers (Strickland and Rousk 2010), and a conclusive pattern appears elusive, or context dependent. While it is possible that there are effects on the balance of fungal-to-bacterial balance within the decomposer community, these effects are too small to be discernible when grazing accounts for <10% of the overall PLFA variation.

The effect of soil pH on microbial PLFA composition has been well studied, and where markers have been identified to pinpoint the direct effect of pH on the microbial community PLFA composition (Rousk et al. 2010b), it was found that the relative abundance of 16:1w5 and 18:1w7 should increase concomitantly with decreases in cy19:0, toward higher pH. Later work verified the application of these markers to identify the trajectory of change of the microbial PLFA composition due to soil pH in different types of ecosystems, extending their use to agricultural, grassland and forest soils (Rousk et al. 2011a). When applying this framework to the present dataset, it

suggests that soil pH is unrelated to any systematic variation in PLFA markers across the soil samples, and that the effect is consistent for both ecosystems. When considering the pH ranges of soils included in this study (pH 6.0-6.2 or 7.2-8.1 in sand dune and saltmarsh grassland, respectively), this outcome seems reasonable in the light of minimal differences in both soil bacterial growth and PLFA composition in this range (pH 6-8; Rousk et al. 2011b).

## **Conclusions**

We were able to demonstrate systematic effects of large herbivore grazing on the structure of the soil microbial community, with consistent patterns across different semi-natural ecosystems. Moreover, we were able to relate these effect-sizes to the expected large differences in microbial community composition between different ecosystems: while 89% of the microbial PLFA variation was due to ecosystem differences, 7% of the variation was directly related to the grazing intensity by large herbivores. These results suggest that previous assessments of microbial communities focused on investigating the effects of grazing may in fact have been confounded by system-associated differences in land management, such as fertilisation regime, rather than grazing *per se*. Higher grazing intensity correlated with dominance of PLFA markers abundant in Gram-negative bacteria, often associated with the use of labile C resources, while lower grazing intensity or removal of grazing correlated with a dominance of Gram-positive bacteria. We found no consistent effect of grazing on the fungal-to-bacterial balance. While effects were small next to differences between ecosystems, the

consistent shift in the soil microbial community composition coincided with a shift toward higher basal respiration in systems with higher grazing pressure, highlighting the importance of the soil microbial community for basic ecosystem services such as decomposition. While we are approaching a general understanding of global warming effects due to the direct temperature effects on microbial decomposition of organic matter (Conant et al. 2011; Kirschbaum 2004), we are further from a general understanding of land-use effects on the global C cycle. This study is an early step in this direction.

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## Figure legends

**Fig. 1** Experimental design at **a** Crossens Marsh salt marsh (all units are 10 m x 10 m square at 20 - 30 m, 30 - 40 m or 40 - 50 m from the fence line) and **b** Newborough Warren sand dune grassland. Diagrams are not to scale.

**Fig. 2** Total, bacterial and fungal PLFA concentrations for salt marsh (G = grazed; U = un-grazed) and sand dune grassland (PR = fully grazed; R = rabbit grazed; U = un-grazed). Treatment means and model standard error from linear mixed effects model (ANOVA) output. Significant differences between grazing treatments indicated by  $^*(P<0.05)$ , non significant results by *ns*.

**Fig. 3** RDA plots showing relationship between salt marsh and sand dune grassland experimental units and environmental variables (**a**), and distribution of PLFA markers (**b**). Significant environmental variables (Canoco v.4.5; Monte Carlo test, 500 permutations) have larger, bold font. Axis one explained 89%, axis two 7%, of the variation in relative abundance of PLFA markers. PLFA markers indicative of Gram-positive bacteria and Gram-negative bacteria as in O'Leary and Wilkinson (1988) and Wilkinson (1988) respectively. PCA scores along axis 2 show a significant grazing effect (ANOVA:  $P<0.001$ ).

**Fig. 4** Bacterial growth rate for salt marsh (G = grazed; U = un-grazed) and sand dune grassland (PR = fully grazed; R = rabbit grazed; U = un-grazed). Treatment means, error bars as standard deviation of the mean. Significant differences between grazing treatments indicated by  $^*(P<0.05)$ , non significant results by *ns*.



**Table 1** Soil and vegetation characteristics of the salt marsh in grazed and un-grazed experimental units (n = 6)

	Grazed	Un-grazed	Model SE	
<i>Soil</i>				
Organic matter content (%)	15.60	12.05	(1.16)	*
Basal respiration rate ( $\mu\text{g C g}^{-1}$ org. mt h <sup>-1</sup> )	23.92	23.25	(2.75)	ns
pH	7.15	8.07	(0.12)	***
Gravimetric soil moisture content (%)	126	111	(10.6)	ns
Bulk density ( $\text{g cm}^{-3}$ )	0.81	0.72	(0.03)	*
C/N mass ratio	14.5	12.9	(0.55)	*
<i>N mineralisation rate</i>				
NO <sub>3</sub> <sup>-</sup> ( $\mu\text{g N g}^{-1}$ org. mt day <sup>-1</sup> )	0.54	3.75	(1.29)	***
NH <sub>4</sub> <sup>+</sup> ( $\mu\text{g N g}^{-1}$ org. mt day <sup>-1</sup> )	1.19	0.34	(0.29)	**
<i>Vegetation</i>				
Root turnover (no. fine roots month <sup>-1</sup> )	53.67	36.28	(3.98)	**
Root biomass ( $\text{g dry wt m}^{-2}$ )	3370	960	(290)	***
Litter biomass ( $\text{g dry wt m}^{-2}$ )	10	340	(70)	*
Shoot biomass ( $\text{g dry wt m}^{-2}$ )	320	690	(70)	*

Treatment means and model standard error from linear mixed effects model (ANOVA) output

org. mt = organic matter

Significant differences between grazing treatments \*( $P < 0.05$ ), \*\*( $P < 0.01$ ) \*\*\*( $P < 0.001$ )

Non significant results ns

609 **Table 2** Soil and vegetation characteristics of the coastal grassland for three grazing treatments (PR = fully  
610 grazed, R = rabbit grazed, U = un-grazed; n = 3)

	PR	R	U	Model SE	
<i>Soil</i>					
Organic matter content (%)	9.65	10.83	8.27	(0.96)	<i>ns</i>
Basal respiration rate ( $\mu\text{g C g}^{-1}$ org. mt h <sup>-1</sup> )	17.92 a	16.13 a	9.08 b	(2.31)	*
pH	6.21	6.16	6.01	(0.21)	<i>ns</i>
Gravimetric soil moisture content (%)	35.73	41.25	31.79	(3.73)	<i>ns</i>
Bulk density ( $\text{g cm}^{-3}$ )	1.01	1.02	0.93	(0.04)	<i>ns</i>
C/N mass ratio	12.0	11.5	11.3	(0.31)	<i>ns</i>
<i>N mineralisation rate</i>					
NO <sub>3</sub> <sup>-</sup> ( $\mu\text{g N g}^{-1}$ org. mt day <sup>-1</sup> )	0.85 a	1.89	3.59 b	(0.91)	*
NH <sub>4</sub> <sup>+</sup> ( $\mu\text{g N g}^{-1}$ org. mt day <sup>-1</sup> )	2.28	2.85	1.44	(1.00)	<i>ns</i>
<i>Vegetation</i>					
Root turnover (no. fine roots month <sup>-1</sup> )	43.36 a	54.83 b	49.17 a	(3.84)	*
Root biomass ( $\text{g dry wt m}^{-2}$ )	1240 a	1220 a	710 b	(210)	*
Litter biomass ( $\text{g dry wt m}^{-2}$ )	120 a	220 b	280 b	(40)	*
Shoot biomass ( $\text{g dry wt m}^{-2}$ )	830	800	590	(200)	<i>ns</i>

611 Treatment means and model standard error from linear mixed effects model (ANOVA) output

612 org. mt = organic matter

613 Significant differences between grazing treatments (a is different from b) \*( $P < 0.05$ ), \*\*( $P < 0.01$ )

614 Non significant results *ns*

615

**Table 3** PLFA markers used for taxonomic groups. Note that gram-positive and gram-negative bacteria are subsets of total bacteria.

Taxonomic group	PLFA group	Specific PLFA markers	Reference
<i>PLFA biomarkers</i>			
Bacteria	Multiple groups	i15:0, a15:0, 15:0, i16:0, 16:1w9, 16:1w7c, 10Me16:0, cy17:0, a17:0, 18:1w7, cy19:0	Frostegård and Bååth (1996)
Gram positive bacteria	Branched PLFAs	i15:0, a15:0, i16:0, i17:0, a17:0	O'Leary and Wilkinson (1988)
Gram negative bacteria	Cyclopropyl and mono PLFAs	cy17:0, 16:1w7c, 16:1w7t and 18:1w7	Wilkinson (1988)
Fungi	Polyunsaturated PLFAs	18:2w6,9	Frostegård and Bååth (1996)
Fungal / bacterial ratio	Multiple groups	Fungi / Bacteria	Frostegård and Bååth (1996)

**Table 4** Relative proportions of PLFA markers for grazed and un-grazed saltmarsh soil (n = 6)

	Grazed	Un-grazed	Model SE	
Bacteria (%)	60.2	59.7	(0.53)	<i>ns</i>
Fungi (%)	1.9	1.8	(0.23)	<i>ns</i>
Gram positive bacteria (%)	15.4	15.9	(0.47)	<i>ns</i>
Gram negative bacteria (%)	33.0 a	30.5 b	(0.85)	*
Fungal/bacterial ratio	0.03	0.03	(0.01)	<i>ns</i>

Treatment means and model standard error from linear mixed effects model (ANOVA) output

Significant differences between grazing treatments \*( $P < 0.05$ ), \*\*( $P < 0.01$ ), non significant results *ns*

**Table 5** Relative proportions of PLFA markers for sand dune grassland soil (PR = fully grazed, R = rabbit grazed, U = un-grazed; n = 3)

	PR	R	U	Model SE	
Bacteria (%)	52.2	51.2	53.2	(1.19)	<i>ns</i>
Fungi (%)	5.5	6.0	4.7	(0.01)	<i>ns</i>
Gram positive bacteria (%)	16.4 a	15.3 a	19.2 b	(0.84)	*
Gram negative bacteria (%)	25.6	25.9	23.0	(1.07)	<i>ns</i>
Fungal/bacterial ratio	0.11	0.12	0.09	(0.02)	<i>ns</i>

Treatment means and model standard error from linear mixed effects model (ANOVA) output

Significant differences between grazing treatments \* ( $P < 0.05$ ), non significant results *ns*

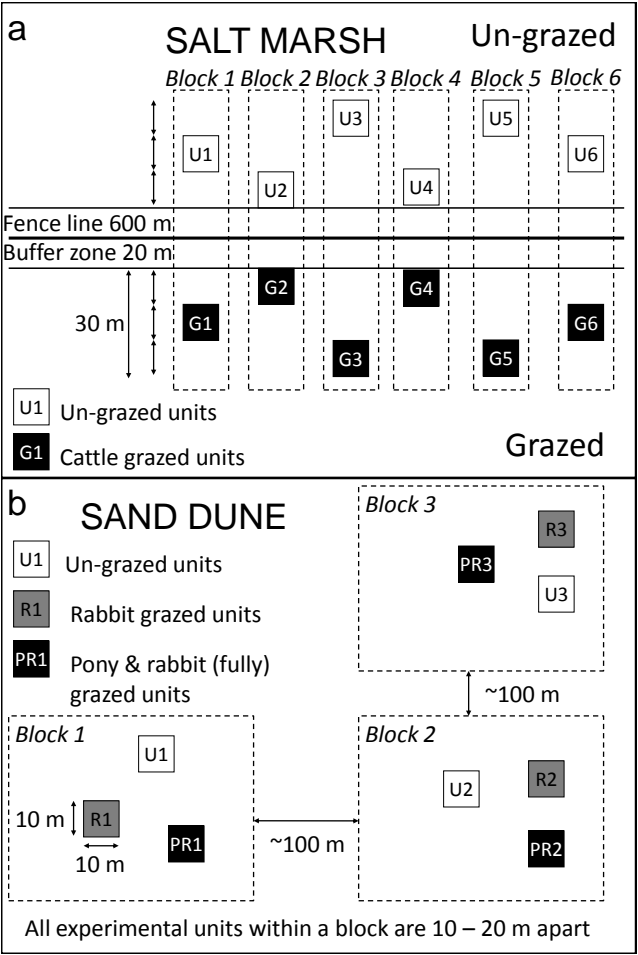
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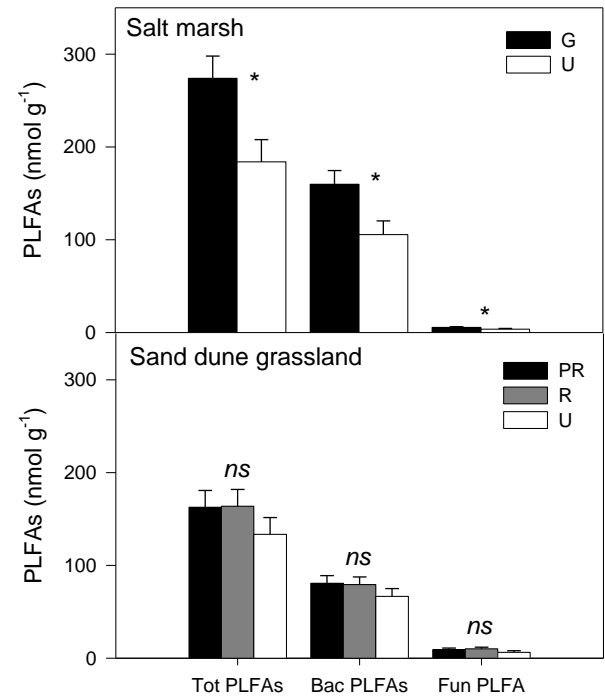
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**Fig. 1**

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638 **Fig. 2**

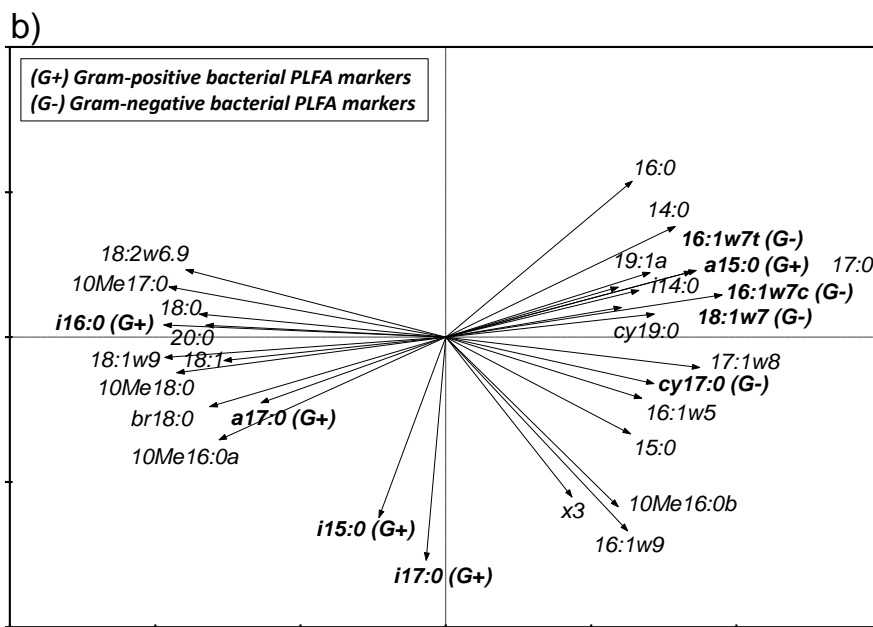
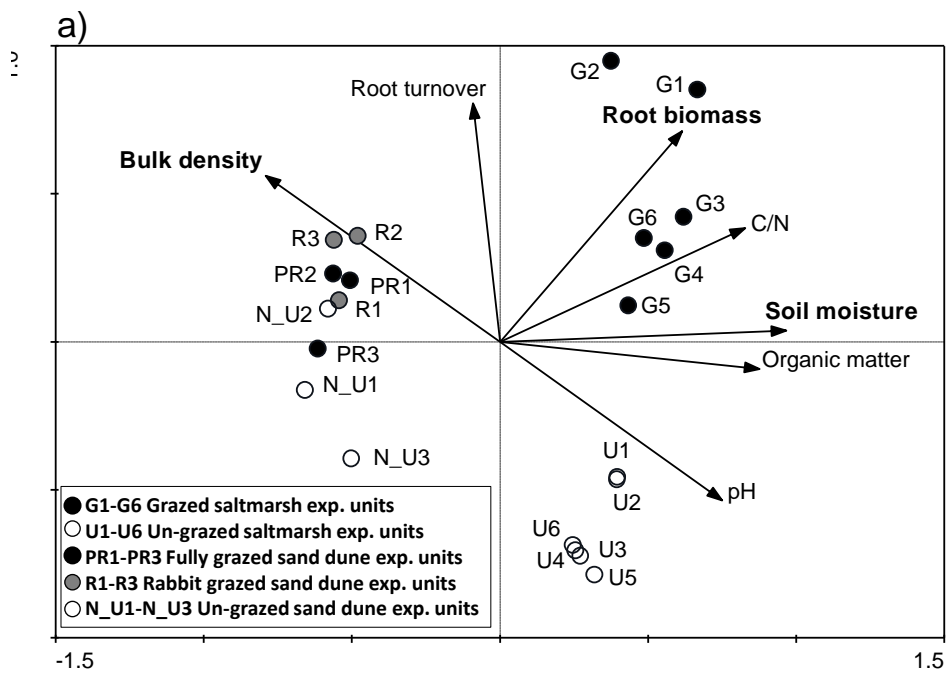
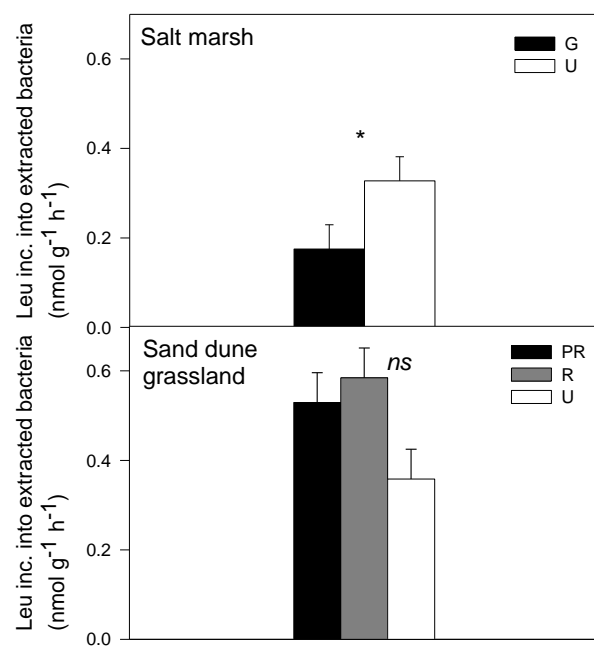


Fig. 3





**Fig. 4**