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Biology and Fertility of Soils

DOI:
10.1007/s00374-012-0721-2

Published: 01/01/2013

Peer reviewed version

Cyswllt i'r cyhoeddiad / Link to publication

Dyfyniad o'r fersiwn a gyhoeddwyd / Citation for published version (APA):
Ford, H., Rousk, J., Garbutt, A., Jones, L., & Jones, D. L. (2013). Grazing effects on microbial community composition, growth and nutrient cycling in salt marsh and sand dune grasslands. *Biology and Fertility of Soils*, *49*(1), 89-98. https://doi.org/10.1007/s00374-012-0721-2

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10. Apr. 2024

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

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Research paper for *Biology and Fertility of Soils*

Abstract

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

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

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

Introduction

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

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

59 factors. Short grazed vegetation leads to greater and more variable soil temperatures
60 than un-grazed grassland (Curry 1994). Large herbivore grazers, such as cattle, compact
61 the soil surface via treading and change soil structure and aeration leading to water-
62 logging (Lambert 2000), with effects upon microbial community composition (Clegg
63 2006). Grazing animals also return nutrients to the soil (Bakker et al. 1993) that greatly
64 influence microbial activity. For instance, cattle faeces are a source of soil C and can
65 increase microbial biomass and respiration (Hatch et al. 2000; Lovell and Jarvis 1996)
66 and livestock urine is a source of N linked to increases in respiration, nitrous oxide
67 emissions and microbial biomass (Ritz et al. 2004). Nutrient cycling due to grazing can be
68 expected to have differential effects in high and in low fertility systems (Holdo et al.
69 2007), and while sand dune grasslands typically are of low fertility, salt marsh grasslands
70 tend to be of higher fertility.

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

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

Groeningen 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^{o} 41’ 15” N, 2^{o} 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^{-1}, 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 ±10 cm.

Sand dune grassland

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

Soil and vegetation analyses

142 In autumn 2010, four soil cores (5 cm depth, 5 cm diameter) per experimental unit were
143 taken, vegetation, roots and stones were removed and the remaining soil was sieved to
144 ≤ 2 mm and stored for 1 week at 5°C before further analyses. For soil respiration, 10 g
145 sub-samples, one from each soil core sample, were weighed into 50 ml polypropylene
146 centrifugation vials and soil respiration rate at 22 °C measured continuously on a
147 multichannel IR respirometer (PP-systems Ltd, Hitchin, UK). The reported soil respiration
148 rate was the 4 hour average measurement taken after reaching a stable rate.
149 Gravimetric soil moisture was estimated by determining the weight loss of samples
150 dried initially at 105^{o}C for 72 hours. Subsequently, organic matter (OM) content was
151 estimated by loss-on-ignition from soil sub samples (375 ^{o}C for 16 hours; Ball 1964). Soil
152 pH (5 g soil: 12.5 ml water dilution factor) was determined using a Corning pH meter
153 220. Samples to determine bulk density were collected during September 2009 using
154 three intact soil cores of 3.8 cm diameter and 15 cm depth from each experimental unit.
155 Cores were dried at 105 ^{o}C for 72 hours and the dry mass divided by the volume of the
156 core to calculate bulk density. Soil cores for total soil C and N were air dried, thoroughly
157 homogenised and dried at 105 ^{o}C for 3 hours prior to analysis. Samples were analysed
158 on an Elementar Vario-EL elemental analyser (Elementar Analysensysteme GmbH,
159 Hanau, Germany), using oxidative combustion to detect C and N. The C/N ratio was also
160 calculated using a mass ratio.

161 The potential for nutrient cycling by microbes was assessed using a measure of
162 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 μg N g^{-1} 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 –

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 turnover (Lukac and Godbold 2010).

PLFAs

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

Bacterial growth rate and turnover times

Bacterial growth was estimated by measuring the incorporation of leucine (Leu) into bacteria (Kirchman et al. 1985) extracted from 1 g soil sub-samples using the homogenization / centrifugation technique (Bååth 1994), with modifications (Bååth et al. 2001; Rousk and Bååth 2011). We added 2 µl [^{3}H]Leu (37 MBq ml^{-1}, 5.74 TBq mmol^{-1}, Perkin Elmer) that were combined with non-labelled Leu, resulting in a final concentration of 275 nM Leu in the bacterial suspensions. The samples were then incubated for 2 h at 22 °C in the dark. Bacterial growth was estimated from the amount of Leu incorporated into extracted bacteria per hour and gram of soil. The Leu method is 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^{-1}) by bacterial growth rate (nmol Leu incorporation g^{-1} h^{-1}).

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

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

256 PLFAs

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

266 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

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

293 **Discussion**

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

309 It was previously found that Gram-negative PLFA markers are relatively
310 abundant in the rhizosphere (Söderberg et al. 2004) due to the presence of labile C
311 resources (Bird et al. 2011; Steer and Harris 2000). Gram-negative bacteria are normally
312 characterized by high reproductive rates and elevated activity under conditions of
313 ample nutrient supply, traits that would be an advantage in the rhizosphere (Jones et al.
314 2004; Barret et al. 2011). Moreover, the slow uptake of labile C resources into Gram-
315 positive markers compared to Gram-negative ones, further strengthens this connection
316 (Bird et al. 2011; Olsson and Johnson 2005; Treonis et al. 2004). The link between
317 grazing and rhizosphere stimulation is well known (e.g. Wardle et al. 2004) and
318 therefore, that grazing induces a shift toward Gram-negative bacteria is consistent with
319 these previous findings of soil microbial community responses.

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

328 It must be noted here, though, that while these estimates of microbial process
329 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.

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

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

365 The effect of soil pH on microbial PLFA composition has been well studied, and
366 where markers have been identified to pinpoint the direct effect of pH on the microbial
367 community PLFA composition (Rousk et al. 2010b), it was found that the relative
368 abundance of 16:1w5 and 18:1w7 should increase concomitantly with decreases in
369 cy19:0, toward higher pH. Later work verified the application of these markers to
370 identify the trajectory of change of the microbial PLFA composition due to soil pH in
371 different types of ecosystems, extending their use to agricultural, grassland and forest
372 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

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

402 **Acknowledgements**

403 We thank Inma Robinson for assistance with the respiration analyses. This study was
404 funded as part of a NERC - Centre for Ecology and Hydrology project - NECO3610. JR was
405 supported by grants from the Swedish Research Council (Vetenskapsrådet; Project No.
406 623-2009-7343 and 621-2011-5719). Thanks to Countryside Council for Wales and
407 Natural England for access to field sites.

408

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580

581 **Figure legends**

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

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

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

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

601

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

	Grazed	Un-grazed	Model SE	
Soil				
Organic matter content (%)	15.60	12.05	(1.16)	*
Basal respiration rate (μg C g^{-1} org. mt h^{-1})	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 (g cm^{-3})	0.81	0.72	(0.03)	*
C/N mass ratio	14.5	12.9	(0.55)	*
N mineralisation rate				
NO_3^- (μg N g^{-1} org. mt day^{-1})	0.54	3.75	(1.29)	***
NH_4^+ (μg N g^{-1} org. mt day^{-1})	1.19	0.34	(0.29)	**
Vegetation				
Root turnover (no. fine roots $month^{-1}$)	53.67	36.28	(3.98)	**
Root biomass (g dry wt m^{-2})	3370	960	(290)	***
Litter biomass (g dry wt m^{-2})	10	340	(70)	*
Shoot biomass (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	
Soil					
Organic matter content (%)	9.65	10.83	8.27	(0.96)	*ns*
Basal respiration rate (μg C g^{-1} org. mt h^{-1})	17.92 a	16.13 a	9.08 b	(2.31)	*
pH	6.21	6.16	6.01	(0.21)	*ns*
Gravimetric soil moisture content (%)	35.73	41.25	31.79	(3.73)	*ns*
Bulk density (g cm^{-3})	1.01	1.02	0.93	(0.04)	*ns*
C/N mass ratio	12.0	11.5	11.3	(0.31)	*ns*
N mineralisation rate					
NO_3^- (μg N g^{-1} org. mt day^{-1})	0.85 a	1.89	3.59 b	(0.91)	*
NH_4^+ (μg N g^{-1} org. mt day^{-1})	2.28	2.85	1.44	(1.00)	*ns*
Vegetation					
Root turnover (no. fine roots $month^{-1}$)	43.36 a	54.83 b	49.17 a	(3.84)	*
Root biomass (g dry wt m^{-2})	1240 a	1220 a	710 b	(210)	*
Litter biomass (g dry wt m^{-2})	120 a	220 b	280 b	(40)	*
Shoot biomass (g dry wt m^{-2})	830	800	590	(200)	*ns*

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
PLFA biomarkers			
Bacteria	Multiple groups	i15:0, a15:0, 15:0, i16:0, 16:1ω9, 16:1ω7c, 10Me16:0, cy17:0, a17:0, 18:1ω7, 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	18:2ω6,9	Frostegård and Bååth (1996)
Fungal / bacterial ratio	Multiple groups	Fungi / Bacteria	Frostegård and Bååth (1996)

621 **Table 4** Relative proportions of PLFA markers for grazed and un-grazed saltmarsh soil (n = 6)
622

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

623 Treatment means and model standard error from linear mixed effects model (ANOVA) output
624 Significant differences between grazing treatments *(P<0.05), **(P<0.01), non significant results *ns*
625

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

	PR	R	U	Model SE	
Bacteria (%)	52.2	51.2	53.2	(1.19)	*ns*
Fungi (%)	5.5	6.0	4.7	(0.01)	*ns*
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)	*ns*
Fungal/bacterial ratio	0.11	0.12	0.09	(0.02)	*ns*

628 Treatment means and model standard error from linear mixed effects model (ANOVA) output
629 Significant differences between grazing treatments *(P<0.05), non significant results *ns*
630

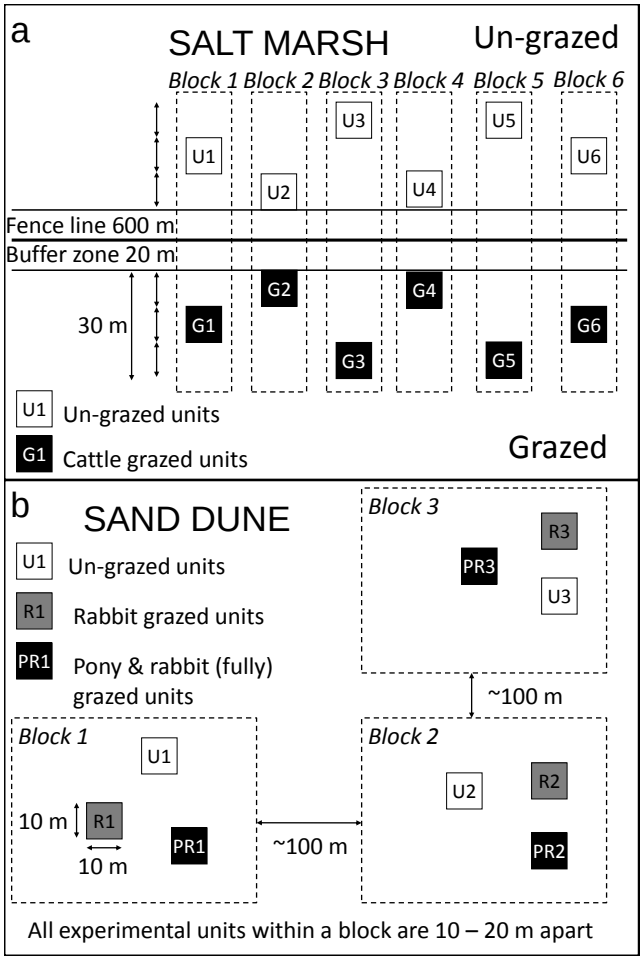
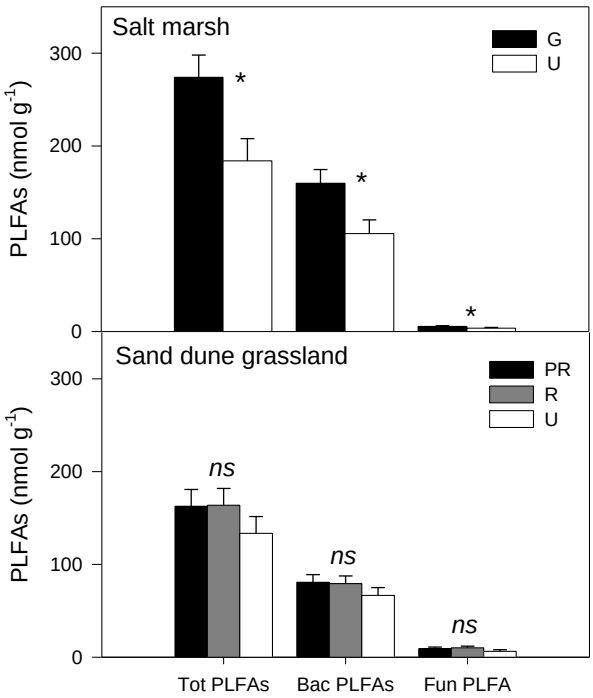

a
SALT MARSH
Un-grazed
Block 1
Block 2
Block 3
Block 4
Block 5
Block 6
U1
U2
U3
U4
U5
U6
Fence line 600 m
Buffer zone 20 m
30 m
G1
G2
G3
G4
G5
G6
U1 Un-grazed units
G1 Cattle grazed units
Grazed
b
SAND DUNE
U1 Un-grazed units
R1 Rabbit grazed units
PR1 Pony & rabbit (fully) grazed units
Block 3
R3
PR3
U3
~100 m
Block 1
U1
10 m
R1
10 m
PR1
~100 m
Block 2
U2
R2
PR2
All experimental units within a block are 10 – 20 m apart

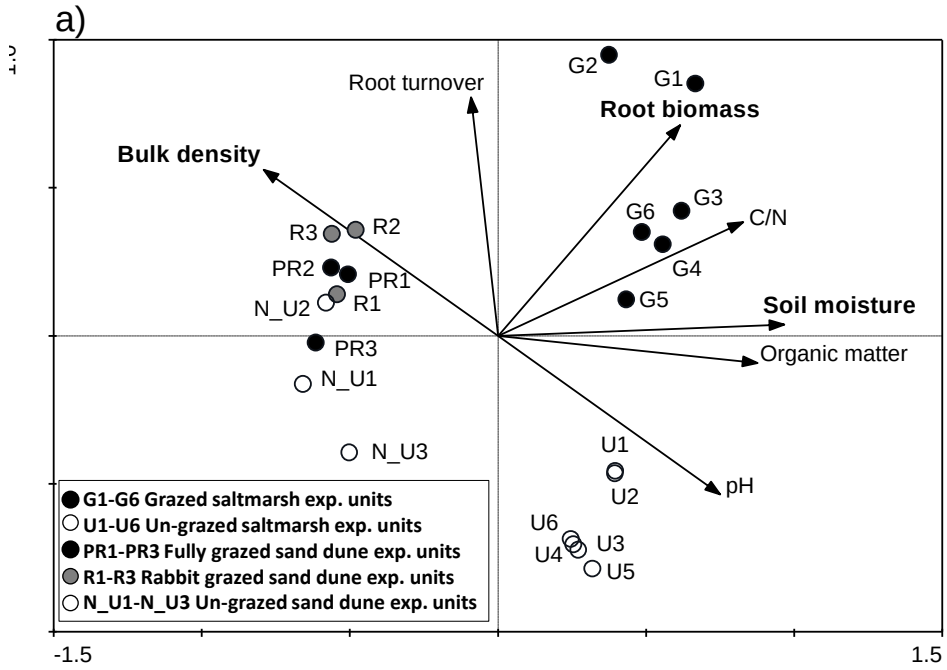
Fig. 1

636



637

638 **Fig. 2**



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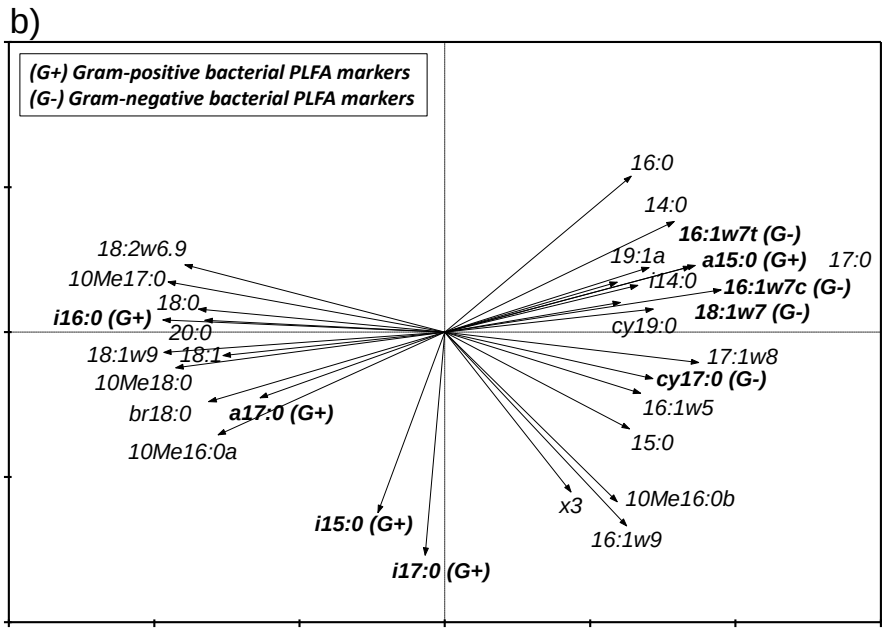
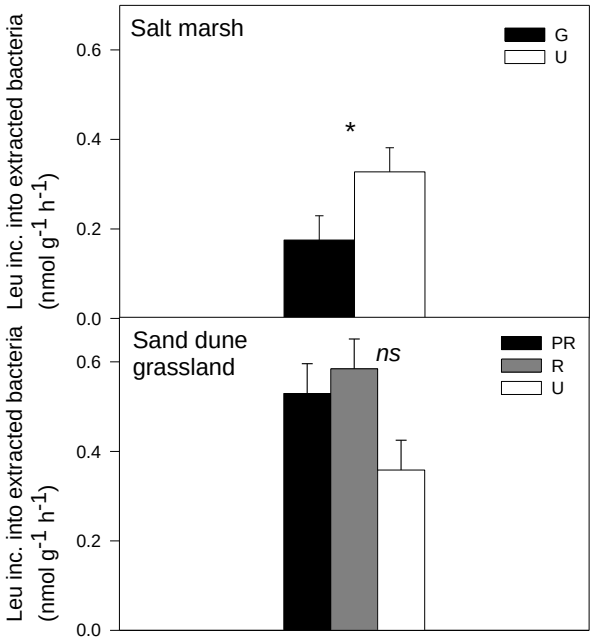


640

641 **Fig. 3**


Salt marsh
G
U
*
Leu inc. into extracted bacteria (nmol g-1 h-1)
0.0
0.2
0.4
0.6
Sand dune grassland
PR
R
U
ns
Leu inc. into extracted bacteria (nmol g-1 h-1)
0.0
0.2
0.4
0.6

642

643 **Fig. 4**