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Identifying biomarkers for shifts in microbial community structure in Irish Grasslands: the influence of context-specific drivers

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Abstract

Purpose This study investigated the comparative influence of regional/climatic difference in agronomic potential ('Region'), natural drainage ('Drainage') and grassland management intensity ('Manage') on soil physicochemical variables, microbial community structure and soil potential extracellular enzymatic activity across 37 Irish grassland sites.

Methods Soil samples were collected in a structured manner from representative sites, and soil physicochemical parameters were measured. Soil microbial community structure was determined by phospholipid fatty acid analysis and different potential soil extracellular enzymatic activities were measured through both fluorometric and colorimetric assays. Doubly-nested PERMANOVA and ANOVA models were performed in R to evaluate the impact of the examined factors on multivariate and univariate variables, respectively.

Results A doubly-nested PERMANOVA model revealed that each of the three factors exerted a significant influence on soil microbial community structure ($p < 0.05$). The factor 'Drainage' did have a stronger influence on the abundance of the fatty acid biomarker for the arbuscular mycorrizal fungi (effect size = 20.54%) than 'Region' had (effect size = 10.05%), with 'Manage' having no significant effect. In contrast to soil microbial community structure, individual soil potential extracellular enzymatic activities mostly either did not significantly respond to any factor, or significantly responded to 'Region' only ($p < 0.05$, i.e., β-N-acetyl-glucosaminidase, arylsulfatase and acid phosphatase).

Conclusion The study highlights the value of considering localized environmental drivers in studies relating to soil parameters at the national level, to support effective, context-specific management strategies for soil biodiversity conservation and national monitoring schemes of soil biology.

Keywords Phospholipid fatty acids · Enzymatic activity · Grassland management intensity · Drainage · Arbuscular mycorrhizal fungi

1 Introduction

Soil health depends on the complex interactions between soil physical, biological and chemical attributes (Creamer et al. 2010). National and international soil health policies require defining a baseline or normal range of soil conditions, which vary from site to site. This helps assess the current state of soils and informs future policy decisions (Stone et al. 2016). The European Union introduced the Soil Strategy for 2030 to address the degradation of soil health across Europe and beyond (Commission 2021). The goal is to achieve healthy soils throughout the continent by 2050. This initiative was followed by the EU Soil Biodiversity Strategy 2030, and in 2023, a proposal for a European Soil Health Monitoring Directive was introduced (European Commission 2023; Panagos et al. 2024). This directive is unique in providing broad protection for soil health, acknowledging the essential ecosystem services that healthy soils offer, highlighting the importance of preserving these benefits for future generations. To implement these policies effectively, the monitoring of soil is essential to ensure soil protection (Turbé et al.

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2010). Research recently has focused on criteria to measure soil health and ecosystem services at different scales to evaluate the potential risks of soil degradation across Europe (Bonilla-Bedoya et al. 2023; Bourdin et al. 2014; Bünemann et al. 2018; Creamer et al. 2015). In these proposed schemes, assessing soil biology is often challenging, especially on a large scale, as national projects require significant labour and resources to gather enough data for accurate evaluations, making the process costly and time consuming (Stone et al. 2016).

One often overlooked aspect is that national surveys of soil biological quality must account for country-specific differences and regional drivers, such as climate, inherent soil properties, and long-term land management practices. Since soil microorganisms respond rapidly to these factors, they serve as sensitive indicators of environmental changes and management shifts. Consequently, the quantitative analysis of microbial community structure, abundance, and activity has become a widely used approach for assessing soil health in relation to these key drivers (García-Orenes et al. 2013).

A commonly used method to analyse structural microbial components and estimate soil microbial biomass is the phospholipid fatty acid analysis (PLFA) (Francisco et al. 2016; Frostegård et al. 2011). PLFAs are components of microbial cell membranes, which are believed to be rapidly degraded after cell death (Kujur and Patel 2014). These membrane components can be extracted from the soil, separated based on polarity by solid phase extraction, and broken down into fatty acid methyl esters (FAMEs). PLFA profiles can be used to quantify biomarkers for specific groups of living microorganisms in the soil, such as Gram positive and Gram negative bacteria, fungi, and actinobacteria. These profiles differentiate microorganisms within the entire microbial community by analysing the unique lengths and structures of their signature fatty acids (Vestal and White 1989). This can include the quantitative and qualitative assessments of the total microbial biomass in a soil sample (Piotrowska-Seget and Mrozik 2003). This method has been recognised as sensitive and reproducible especially when trying to capture differences across larger scales of observations and potentially can be employed in national monitoring schemes of soil biology (Francisco et al. 2016).

Soil extracellular enzyme activities are also of interest to soil scientists because they provide information on the ability of soils to perform biogeochemical reactions, they can be used as an index to detect impacts of anthropogenic management, climate and soil conditions, and they are generally simple, rapid, accurate, and inexpensive (Bondi et al. 2016; Sinsabaugh et al. 2008). Alterations in the production and activity of soil enzymes can significantly impact ecosystem function (Zuccarini et al. 2023). Consequently, the way that soil ecosystems react to changes is heavily influenced by how their soil enzymes respond (Zuccarini et al. 2023; Delgado-Baquerizo et al. 2013) and differs among the different agro-ecosystems.

In an Irish context, permanent grasslands are generally good in terms of soil biological conditions, associated with high biomass production potential (Bondi et al. 2021; Graça et al. 2021). Even if Irish grasslands have optimal growing conditions, variations do exist as a consequence of grass growth and utilization being largely controlled by soil drainage capacity, climate and management applied (Tuohy et al. 2015). Several studies have reported the effect of agricultural management practices on microbial community structure by means of lab or field scale experiments in Ireland. Grassland sward management has been shown to greatly influence soil microbial community structure within the soil profile (Richter et al. 2018; Ikoyi et al. 2024; Ryan et al. 2023). The effect of N fertiliser applications and its influence on soil bacteria and fungi communities have been explored by Duff et al. (2022); Lovell et al. 1995); while (Gebremichael et al. 2022) did show a significant effect of differing soil P levels on soil microbial respiration. (Graça et al. 2021) provided an initial understanding of the co-occurrence of factors that influence soil biology focusing on the effect of the combination of P legacy and natural drainage characteristics on the diversity and abundance of the soil microbial community. However, there are very few studies that combine observations on soil intrinsic characteristics, like climatic region, natural drainage and grassland management pressure at the national scale.

This is associated with a lack of comprehensive data on soil microbial abundance and extracellular enzymatic activity in Irish soils, making it difficult to effectively monitor changes in microbial dynamics under various environmental conditions. Consequently, the absence of such data limits the establishment of quantitative baselines for assessing soil health and its response to key drivers. An understanding of context-specific drivers influencing such variables at the national level is crucial to address this gap. Such information is essential for informing national policies and guidelines on national monitoring schemes of soil biology.

The current work aimed to provide information on microbial community structure, abundance and extracellular enzymatic activity of Irish grassland soils in the context of a national scale campaign on soil health by means of widely used indicators such as PLFAs and enzymatic activities. In particular, this study aimed to identify biomarkers that can detect changes in these variables, considering context-specific drivers that influence soil health in Irish grasslands. These included: i. different climatic regions, ii. contrasting natural drainage conditions, and iii. contrasting agricultural grassland management intensities.

The study specifically hypothesised that:

1. Drainage class would be a stronger driver of soil microbial community structure than both climatic region and grassland agricultural management intensity.
2. Drainage class would be a stronger driver of soil microbial extracellular enzymatic activities, than both climatic region and grassland agricultural management intensity with activities being lower in poorly-drained soils than well-drained soils.

2 Materials and methods

2.1 Context and site selection

The SQUARE project launched a soil sampling campaign with the intention of providing information on soil biological status at a national scale. The study focused on Irish grasslands typically grazed by livestock or mown for silage, or both. The sites were selected based on the following characteristics: i). climatic region and ii). natural soil drainage and iii). agricultural grassland management intensity, herein referred to as 'Region', 'Drainage' and 'Manage' respectively. 37 sites were selected on operational grassland farms. From each of these 37 farms, one field, representative of the main agricultural management practice, was chosen from each farm as a reference for management data collection and sampling.

i. Distribution across climatic regions

The sites were distributed across two climatic regions based on both annual rainfall level and annual sunshine hours. Data was collected from the Met Éireann database (the Irish National Meteorological Service) available online (Met Éireann 2024). This allowed the definition of:

1. West agricultural region (West), situated largely in the Western region of Ireland, where climatic conditions were characterised by lower number of sunshine hours and higher average annual rainfall, and was therefore considered suboptimal for agricultural production (O'Donnell et al. 2021) (Table S1).
2. East agricultural region (East), situated largely in the Southern and Eastern part of the country, with a significantly higher number of sunshine hours and lower rainfall, where climatic conditions were considered optimal for agricultural production (Table S1).

ii. Drainage characteristics

All the sites were classified following the Irish soil classification system (Creamer et al. 2014) into natural drainage classes. They were either poorly or well drained sites based on diagnostic features, which designates soils as poorly drained where there was evidence of saturation occurring within 40 cm of the surface horizon (mottling and/or argic/spodic horizon causing stagnation), or as well drained where no evidence of waterlogging occurred (Bondi et al. 2021). As the natural drainage class is derived from the occurring soil diagnostic feature, drainage in this study is strictly linked to the soil type and consequently summarises the intrinsic characteristics of the soil.

iii. Management pressure

The sites were also classified into management classes on the basis of an aggregated soil trafficking intensity index for compaction ($STIC^{i}$), as developed by Bondi et al. 2021. Lower index values (<0, Low) are associated with lower trafficking pressure of grazing and/or machinery, while high (>0, High) index values refer to higher trafficking pressure.

On the basis of these factors, the 37 sites were clustered into eight scenarios based on region, drainage class and agricultural grassland management intensity. The West region included 17 sites, and the East region included 20. In the West, 10 sites were well-drained (5 under high management intensity, 5 under low management intensity), and 7 were poorly drained (4 high management intensity, 3 low management intensity). In the East, 9 sites were well-drained (4 high management intensity, 5 low management intensity), and 11 were poorly drained (5 high management intensity, 6 low management intensity).

2.2 Soil sampling

A total of 37 fields were sampled in the Spring of 2016 (Table S1 and Figure S1). A sampling area (30×30 m) was chosen within each representative field. In each sampling area, a composite sample of nine individual subsamples was collected using a 5 cm diameter auger, following a 'W'shaped sampling design to a depth of 10 cm (Wall and Plunkett 2021). Large stones and plant material such as grass and roots were removed. The samples were then sieved through a 2 mm mesh on-site and stored at 4°C during transport. In the lab the samples were split into three portions: a field-moist subsample for soil enzymatic and microbial biomass analysis, a subsample which was further preserved at −20°C for PLFA analyses and the remaining subsample, which was dried at 40°C and used for the physicochemical analyses.

2.3 Soil analysis

2.3.1 Soil physicochemical analysis

The following physicochemical analyses were performed on the dried subsamples as previously reported (Bondi et al. 2021; Graça et al. 2021). Soil pH was determined using a 1:2.5 soil-to-water ratio (Byrne 1979) using a pH-meter. Organic matter (OM) was measured as the loss-on-ignition at 500°C (Storer 1984). Soil Organic Carbon (SOC),

total carbon (% total C) and total nitrogen (% total N) were determined by dry combustion using a CN LECO FP 2000 analyser. Mehlich-3 extractable aluminium (M3-Al), iron (M3-Fe), copper (M3-Cu), calcium (M3-Ca), magnesium (M3-Mg), manganese (M3-Mn), zinc (M3-Zn), sulphur (M3-S), potassium (M3-K) and phosphorus (M3-P) were measured by Varian VISTA Inductively coupled plasma-optical emission spectroscopy (ICP-OES). Olsen P was determined in a 1:4 soil to $NaHCO_3$ solution (0.5 mol L^{-1}, pH 8.5; (Olsen et al. 1954)). In the field moist subsamples, soil dry matter (DM) percentage after saturation and natural drainage for 24 h was determined by weight difference post-oven drying at 105 °C. Soil microbial biomass C and N were determined via chloroform fumigation as described in (Voroney et al. 2007).

2.3.2 Extracellular enzyme essays

The field moist soil subsamples were also used to asses a suit of enzymatic activities responsible for C, N and P cycles. 5 g of fresh soil were added to 50 mL of sterile distilled water (dH_2O), and enzyme extraction was carried out by shaking the mixture on a Gyratory shaker (New Brunswick Scientific) at 150 rpm for 10 min (Fox et al. 2017). Following this, the sample was centrifuged at 750 rpm for 10 min at 4 °C. Then, 200 µL of the resulting supernatant was transferred into individual wells of black microtiter plates (VWR, Radnor, PA). Five extracellular-enzymes activities were measured in all samples by using fluorogenic methylumbelliferyl (MUB)-linked artificial substrates (Sinsabaugh et al. 2008; Marx et al. 2001; Vepsäläinen et al. 2001). α– and β-glucosidase (involved in soil carbon cycling) were assessed using 4-MUB-β-D-glucoside substrate and 4-MUB-α-D-glucopyranoside substrate. Acid phosphatase (involved in soil phosphorus turnover) was assessed using 4-MUB-phosphate substrate. N-acetyl-β-D-glucosaminidase (involved in soil nitrogen cycling) was assessed using 4-MUB-N-acetyl-D-glucosaminide. Urease activity was determined following a colorimetric method as reported previously (Nannipieri et al. 1980). The stoichiometric ratios of enzymatic activities were calculated to assess microbial nutrient demand toward C, N, and P acquisition. Specifically, the activities of β-glucosidase and α-glucosidase were summed to represent total C-acquiring enzyme activity, while N-acquiring activity was represented by N-acetyl-β-D-glucosaminidase, and P-acquiring activity by acid phosphatase. From these values, C/N, C/P, and N/P ratios were calculated by dividing the respective enzyme activities, following established stoichiometric approaches (Sinsabaugh et al. 2008).

2.3.3 PLFA extraction and analysis methodology

Soil subsamples stored at −20ºC, then freeze-dried. Soil lipid extraction was performed as described in Bligh and Dyer (1959) and under the guidelines described in (ISO 2010). Briefly, lipids were extracted from 2 g of lyophilized soil and separated by solid-phase extraction using an SI-column and organic solvents as eluents (chloroform, acetone and methanol) to eluate neutral lipids, glycolipids and phospholipids, respectively, the latest being eluted directly to MIDI screw-capped tubes. Phospholipid eluent was evaporated to dryness under N_2 at 40ºC, saponified, methylated and extracted using the MIDI technical note (MIDI, Inc., Newark, DE, United States) according to Sasser (1990). Gas chromatography fatty acid methyl esters (FAME) were measured by Gas Chromatography (GC), identified and quantified using standards (internal FAME 19:0 and calibration mixtures) and Sherlock MIS database. Briefly, MIDI FAME samples were transferred to GC vials with a 200 µl glass tube insert, dried under N_2 at 40ºC, re-dissolved in 100 µl hexane/methyl tert-butyl ether containing 0.05 mg/ml nonadecanoic acid methyl ester (19:0 FAME) as internal standard, and analysed by gas chromatography. Details of the gas chromatography analysis have been described fully (Francisco et al. 2016). Only PLFAs that represented more than 0.5% abundance in each sample were retained (Jiménez et al. 2019). The biomarker groups were defined as described in Table 1 and

Table 1 Component phospholipid fatty acids of identified groupings

Biomarker groups	PLFA
Bacteria	i15:0, a15:0, 15:0, i16:0, 16:1ω9, i17:0, a17:0, 17:0, 17:1ω8, cy17:0, 18:1ω7, cy19:0
Fungi	16:1ω5, 18:1ω9, 18:2ω6
$Gram^+$ bacteria	i14:0, i15:0, i16:0
$Gram^-$ bacteria	cy17:0, cy19:0, 16:1ω7c, 18:1ω7
Arbuscular mycorrhizal fungi	16:1ω5
Saprothrophic fungi	18:2ω6

abundance of each individual fatty acid is reported in the supplementary material (Table S2).

2.4 Data analysis

All statistical analyses were conducted in the R statistical software program, primarily using the vegan package. The PLFA abundance matrix was first normalized by relative abundance and a Bray–Curtis dissimilarity matrix was constructed using the 'vegdist' function (Barreiro et al. 2022). Visualization of the microbial community was done through non-metric multi-dimensional scaling (NMDS), with a maximum of 100 iterations and no autotransformation. The effect of the factors 'Region', 'Drainage' and 'Manage' on soil microbial community structure was assessed via Permutational Multivariate Analysis (PERMANOVA, Monte Carlo, 9999 permutations, (Anderson 2001) using a doubly nested design (i.e. 'Drainage' nested within 'Region' and 'Manage' nested within 'Drainage'). A nested (rather than factorial) approach was chosen, as the differing climatic conditions in both regions renders the intensity of grassland management in both regions to be non-equivalent (Collins and Cummins 1996). A permutational analysis of multivariate dispersion (PERMDISP) was used to assess homogeneity of multivariate dispersions, as this is a necessary assumption of PERMANOVA. The centroid distance (in Euclidian ordination space) of the microbial community structure was calculated using the 'betadisper' function (Fox et al. 2021). A distance based linear model (DISTLM) was conducted to determine which soil physicochemical and climatic variables influenced soil microbial community structure in both the West and East regions (Verniest and Greulich 2019). The effect of 'Region', 'Drainage' and 'Manage' on univariate variables was tested using analysis of variance (ANOVA), with the doubly nested design as described above, using the 'aov' function. To validate the assumption of the model, residual diagnostics were performed namely; Tukey-Ascombe, QQ, leverage and histogram of standardized residual plots. Similarly, the effects of 'Drainage' and 'Manage' separately within the West and East regions was done using a nested ANOVA model (with 'Manage' nested within 'Drainage'), with residual diagnostics performed as above.

3 Results

3.1 Soil physicochemical variables were primarily influenced by 'Region' and 'Manage'

Overall, the factor 'Region' had the strongest influence on the measured soil physicochemical variables. There was no significant effect of the factor 'Drainage' on any physicochemical variable ($p > 0.05$, Fig. 1, Table 2). Two variables displayed a significant effect of the factors 'Region' and 'Manage' namely: soil pH and SOC (all at least $p < 0.05$, Fig. 1A and E). Soil pH was higher in the West (6.34) than in the East (5.86), with an effect size of 8.13%. In contrast, 'Manage' had a negative effect on pH (effect size = −8.60%). SOC was also higher in the West (5.06%) compared to the East (3.71%), with a stronger effect size for 'Region' (36.53%) than for 'Manage' (26.72%). However, when analysed within each region, the factor 'Manage' had no significant effect on either pH or SOC ($p < 0.05$).

Soil Dry Matter (DM), % total C, % total N, M3-Mg and M3-Ca were all significantly influenced by the factor 'Region' (all at least $p < 0.05$, Fig. 1B, C, D, F and G), but not by the factors 'Drainage' or 'Manage' ($p > 0.05$). % total C, % total N and M3-Ca had higher mean values in the West region (5.96%, 0.53% and 4747.07 mg kg^{-1}, respectively) than in the East region (4.54%, 0.46% and 1679.15 mg kg^{-1}, respectively). DM and M3-Mg had higher mean values in the East region (70.14% and 191.96 mg kg^{-1}, respectively) compared to the West region (63.16% and 123.77 mg kg^{-1}, respectively). Finally, there was no significant effect ($p > 0.05$) of any of the examined factors on soil available P (Olsen_P), nor on the totals level of the remaining soil elements as measured by Mehlich extraction (i.e., P, K, Fe, Al, Mn, Cu, S and Zn, Table 2.

3.2 Soil microbial community structure was significantly influenced by all three factors, with 'Drainage' having a stronger effect on arbuscular mycorrhizal fungi (AMF) and saprophytic fungi (SF)

A doubly-nested PERMANOVA model was used to determine the influence of the factors 'Region', 'Drainage' and 'Manage' on microbial community structure based on phospholipid fatty acid biomarkers. A wide range of fatty acids biomarkers were identified, 47 in total (Table S2), most of which were found in all sites of the survey. Each of the three factors did have a significant effect on soil microbial community structure (all $p < 0.05$, Table 3A), and is visualized in Fig. 2. The community centroid distances between the levels within each factor were quite comparable (Table 3A). The effect of 'Drainage' was, however, compounded by the significant dispersion ($p = 0.01$) between the levels of that factor as indicated by the PERMDISP test (Table 3A).

The effect of both 'Drainage' and 'Manage' were examined within both the West and East regions. In the West region, only the factor 'Drainage' showed a significant effect ($p = 0.013$), but not the factor 'Manage' ($p = 0.703$), with no significant effect of dispersion seen ($p > 0.05$, Table 3B). Within the East region, the effect of both the factors 'Drainage' ($p = 0.032$) and 'Manage' ($p = 0.002$) were significant, however, 'Drainage' was significantly

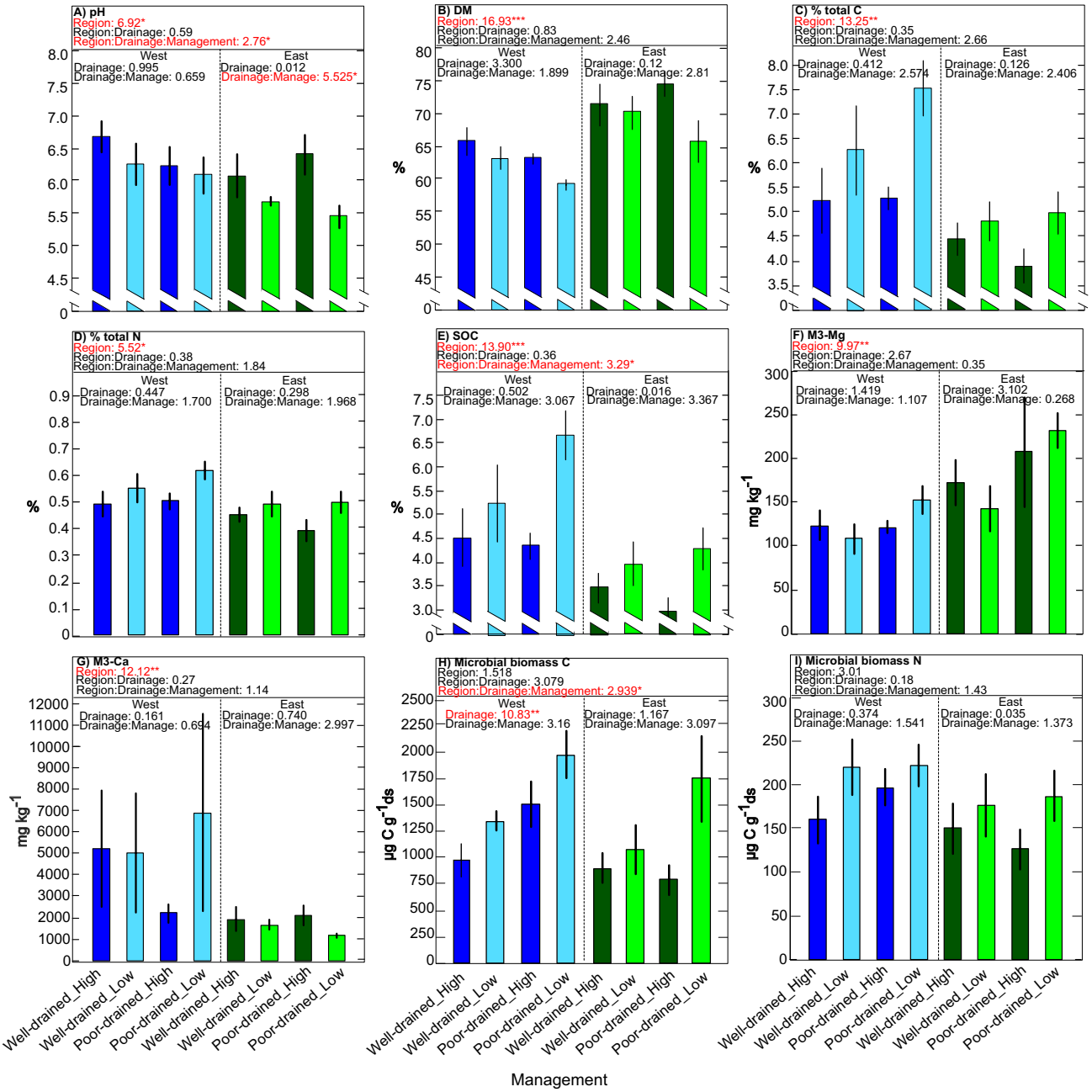

Fig. 1 Boxplot showing the effect of region (blue = West, green = East), drainage and management on **A**) pH, **B**) soil dry matter (DM), **C**) % total C, **D**) % total N, **E**) soil organic carbon (SOC), **F**) M3-Mg, **G**) M3-Ca, **H**) microbial biomass C, and **I**) microbial biomass N. Also shown as an insert panel is the F – value for each experimental variable (Region, Drainage, Manage) across the two regions, and the F value for each experimental variable within the West and East region, with significant effects highlighted in red. Significance codes: '***' $p \leq 0.001$ '**' $p \leq 0.01$ '*' $p \leq 0.05$ from an analysis of variance model. Bars represent means and error bars represent standard error

influenced by dispersion ($p = 0.009$, Table 3C). While pH was the only variable which was significantly correlated with microbial community structure in the West region ($R^2 = 0.191$, $p < 0.05$, Table S3), a number of variables were significantly correlated with microbial community structure in the East. In this region, pH had the strongest correlation ($R^2 = 0.256$, $p < 0.001$) followed by DM ($R^2 = 0.064$, $p = 0.04$), M3-Al ($R^2 = 0.130$, $p = 0.002$) and M3-Mn ($R^2 = 0.082$, $p = 0.015$). Climatic variables which were significantly correlated included both mean annual rainfall ($R^2 = 0.073$, $p = 0.021$) and direct normal irradiance ($R^2 = 0.071$, $p = 0.023$).

More specifically, if we look at the influence of these three factors on the abundance of individual phospholipid

Table 2 Mean and ± SE of the soil physicochemical variables which did not display a significant effect of any examined factor. Soil physicochemical variables included; Olsen soil available P (Olsen_P), Mehlich extractions of phosphorus (M3-P), potassium (M3-K), iron (M3-Fe), aluminium (M3-Al), manganese (M3-Mn), copper (M3-Cu), sulphur (M3-S) and Zinc (M3-Zn). Also shown is the F – value for each experimental factor ('Region', 'Drainage', 'Manage') and significance codes: '***' $p \leq 0.001$ '**' $p \leq 0.01$ '*' $p \leq 0.05$ from a nested analysis of variance model. West: West region; East: East region; Well-drained: well- drained soils; Poor-drained: poor drained soils; High: high agricultural grassland management intensity; Low: low agricultural grassland management intensity

	Units				West	West	West	West	East	East	East	East
		Region	Drainage	Manage	Well-drained	Well-drained	Poor-drained	Poor-drained	Well-drained	Well-drained	Poor-drained	Poor-drained
		F—value	F—value	F—value	High	Low	High	Low	High	Low	High	Low
log (Olsen_P)	mg kg^{-1}	0.314	0.115	1.131	4.7 (± 1.59)	8.2 (± 1.78)	8.7 (± 2.77)	5.6 (± 0.40)	25.4 (± 15.74)	7.6 (± 3.51)	6.8 (± 2.04)	7.7 (± 2.02)
log (M3-P)	mg kg^{-1}	0.08	0.40	0.40	44.1 (± 10.40)	60.3 (± 9.87)	61.8 (± 11.75)	53.4 (± 8.99)	132.5 (± 87.59)	59.4 (± 11.37)	52.0 (± 9.86)	52.1 (± 10.21)
M3-K	mg kg^{-1}	3.47	0.104	0.35	74.7 (± 19.91)	121.6 (± 22.52)	94.3 (± 25.57)	80.4 (± 16.90)	147.6 (± 53.47)	137.6 (± 42.14)	138.3 (± 36.67)	125.5 (± 19.29)
M3-Fe	mg kg^{-1}	0.055	0.018	2.519	270.5 (± 40.68)	270.8 (± 36.77)	257.5 (± 35.81)	281.4 (± 19.68)	319.1 (± 22.42)	245.7 (± 17.32)	204.9 (± 13.87)	328.9 (± 46.28)
M3-Al	mg kg^{-1}	2.05	1.61	0.45	599.5 (± 139.50)	543.8 (± 131.73)	577.2 (± 164.64)	579.7 (± 97.65)	717.8 (± 110.18)	860.7 (± 89.61)	524.3 (± 107.57)	665.2 (± 45.01)
M3-Mn	mg kg^{-1}	0.81	1.86	0.95	78.0 (± 22.32)	59.3 (± 22.17)	42.7 (± 9.96)	21.4 (± 2.66)	51.1 (± 6.62)	78.9 (± 17.48)	78.2 (± 17.65)	52.4 (± 13.80)
M3-Cu	mg kg^{-1}	1.77	0.59	0.43	8.4 (± 3.05)	5.5 (± 1.22)	6.1 (± 2.17)	6.9 (± 4.61)	7.5 (± 3.93)	4.6 (± 2.17)	4.0 (± 0.30)	3.2 (± 0.46)
M3-S	mg kg^{-1}	0.18	0.21	1.16	22.9 (± 3.12)	24.7 (± 0.49)	22.9 (± 0.70)	28.2 (± 0.40)	24.7 (± 4.62)	22.3 (± 1.68)	21.9 (± 0.68)	25.6 (± 2.14)
log (M3-Zn)	mg kg^{-1}	0.05	0.19	1.17	3.8 (± 0.78)	3.8 (± 0.62)	4.3 (± 1.49)	4.3 (± 1.74)	15.4 (± 11.68)	3.4 (± 1.15)	3.3 (± 0.79)	4.4 (± 0.52)

Table 3 The effect of region ('Region'), drainage ('Drainage') and management ('Manage') on microbial community structure as deduced from a doubly nested PERMANOVA model (A). Shown is the degrees of freedom (df), sum of squares (SS), F-statistic (F) and the mean distance (Mean distance). The effect of the factors 'Drainage' and 'Manage' within the West (B) and East (C) regions are also shown. Significance code: '***' $p \leq 0.001$ '**' $p \leq 0.01$ '*' $p \leq 0.05$

		Df	SS	F	Mean distance	PERMDISP (p)
A) All sites						
	Region	1	0.017	4.421***	0.070	0.525
	Region:Drainage	2	0.022	2.881**	0.079	0.01**
	Region:Drainage:Manage	4	0.030	1.961*	0.066	0.382
	Residual	29	0.111			
	Total	36	0.179			
B) West						
	Drainage	1	0.011	2.969*	0.073	0.353
	Drainage:Manage	2	0.006	0.770	0.056	0.606
	Residual	13	0.047			
C) East						
	Drainage	1	0.011	2.830*	0.076	0.009**
	Drainage:Manage	2	0.024	3.059**	0.074	0.160
	Residual	16	0.064			

fatty acid biomarkers, there was no effect of any of the examined factors on the sum of the community bacterial fatty acid biomarkers (PLFA_Bacteria) or the sum of the community fungal fatty acid biomarkers (PLFA_Fungi, both Table 4) nor the Bacterial:Fungal ratio (Fig. 3C, all $p > 0.05$). There was, however, a significant effect of one or more of these factors on the phospholipid fatty acid biomarkers for Gram positive and Gram negative bacteria, as well as AMF and SF (Fig. 3B and E). The abundance of the Gram-positive bacteria was significantly influenced by 'Manage' in the East region ($p = 0.031$), but no such effect was observed in the West ($p > 0.05$; Fig. 3A). In the East, the effect size was 8.20%, with higher mean values of the Gram-positive fatty acid biomarker in the high management sites (5.48 nmol g^{-1}) compared to the low management sites (5.06 nmol g^{-1}), regardless of drainage class. In contrast, the abundance of the Gram-negative bacteria was not significantly influenced by 'Manage' ($p > 0.05$), but was influenced by 'Region' ($p = 0.018$). The relative effect size of 'Region' was 5.31%, with higher mean values in the West (14.28 nmol g^{-1}) compared to the East (13.56 nmol g^{-1}; Fig. 3D). No significant effects were found for any factor on the Gram-positive to Gram-negative bacteria ratio ($p > 0.05$; Fig. 3F).

The fatty acid biomarker for arbuscular mycorrhizal fungi (AMF) was significantly influenced by both 'Region' and 'Drainage' (both $p < 0.05$; Fig. 3B), with 'Drainage' showing the stronger effect. The relative effect size of 'Region' was 10.05%, with higher AMF abundance in the West (2.11 nmol g^{-1}) than in the East (1.92 nmol g^{-1}). In comparison, 'Drainage' had a larger effect size of 20.54%. When analysed within regions, 'Drainage' significantly influenced AMF abundance in both the West ($p = 0.01$) and East ($p = 0.018$). In the West, AMF levels were higher in well-drained sites (2.28 nmol g^{-1}) than in poorly drained ones (1.87 nmol g^{-1}), with a drainage-related effect size of 22.27%. A similar pattern was observed in the East, where well-drained sites had higher AMF levels (2.09 nmol g^{-1}) than poorly drained sites (1.78 nmol g^{-1}), corresponding to an effect size of 16.89%. Saprophytic fungi (SF) were influenced only by 'Drainage' ($p = 0.03$; Fig. 3E). This effect was region-specific, however, as in the East its abundance was significantly higher in well-drained soils (1.01 nmol g^{-1}) compared to poorly drained soils (0.67 nmol g^{-1}). No significant drainage effect was observed in the West ($p > 0.05$).

3.3 Soil enzymatic activity was most influenced by 'Region'

The activities of three enzymes were significantly influenced by the factor 'Region', namely: β-N-acetyl-glucosaminidase (Fig. 4A, involved in the mineralization of organic N compounds), acid phosphatase (Fig. 4B, involved in the hydrolysis of organic P compounds) and arylsulfatase

(Fig. 4C, involved in sulphur mineralization). For each of the three enzymes, the activity was higher in the East region (186.62, 76.05, 236.34 µmol h^{-1} g^{-1} dry soil, respectively) with respect to the West region (95.80, 35.30, 158.47 µmol h^{-1} g^{-1} dry soil, respectively), for β-N-acetyl-glucosaminidase, acid phosphatase and arylsulfatase, respectively. The factor 'Drainage' also had a significant effect on acid phosphatase, though the relative effect size was smaller than that of 'Region' at 26.53%. There was a significant effect of 'Drainage' on the activity of this enzyme in the East region ($p = 0.043$), but not in the West ($p > 0.05$). Within the East region, a higher mean value of acid phosphatase was seen in the well-drained sites, compared to the poor-drained sites (101.48 µmol h^{-1} g^{-1} dry soil ~ 55.25 µmol h^{-1} g^{-1} dry soil, respectively). There was no significant effect of any of the examined factors on the enzymatic activities of urease, α – glucosidase and β – glucosidase (Table 4). Enzyme stoichiometric ratios (C:N, C:P, and N:P) based on the activities of carbon-, nitrogen-, and phosphorus-acquiring enzymes were also calculated. The stoichiometric ratio C:N was highly significantly affected by 'Region' ($p < 0.001$, Fig. 4D), with a higher mean value in the West (2.89) compared to the East region (1.94), suggesting a greater relative microbial demand toward carbon acquisition in the West. Similarly, there was also a significant effect of 'Region' on the C:P ratio ($p = 0.02$, Fig. 4E) again with higher values in the West region (10.01) relative to the East (5.92), indicating relatively greater enzymatic allocation toward phosphorus acquisition in the East region. In contrast, the N:P ratio did not show any significant effect of 'Region' (all $p > 0.05$, Fig. 4F), there was also no significant effect of 'Drainage' or 'Manage' detected for any calculated ratio.

4 Discussion

4.1 Comparable influence of the factors 'Region', 'Drainage' and 'Manage' on soil microbial community structure

The first hypothesis of this study postulated that soil drainage ('Drainage'), a singular aspect of grassland management, would be a stronger driver of soil microbial community structure than climatic region ('Region') and management intensity ('Manage'). When looking at soil microbial community structure, each of the three examined factors 'Region', 'Drainage' and 'Manage' did have a significant, comparable, influence. The similar mean centroid distances of each of the three factors would also indicate that the structure of the soil microbial community was influenced by all the three factors, rather than being shaped by any single factor alone. The factor 'Drainage' did have the largest mean distance of the examined factors, however, dispersion

Table 4 Mean and ± SE values for a number of soil microbial measures which did not display a significant effect to any examined factor. These included the fatty acid biomarkers for total bacteria (PLFA_Bacteria, sum of i15:0, a15:0, 15:0, i16:0, 16:1ω9, i17:0, a17:0, 17:0, 17:1ω8, cy17:0, 18:1ω7, cy19:0 PLFA) and fungi (PLFA_Fungi, sum of 16:1ω5, 18:1ω9, 18:2ω6 PLFA). Soil enzymatic values included Urease, α -glucosidase and β – glucosidase. Also shown is the F – value for each experimental variable ('Region', 'Drainage', 'Manage') and significance codes: '***' $p \leq 0.001$ '**' $p \leq 0.01$ '*' $p \leq 0.05$ from a nested analysis of variance model. West: West region; East: East region; Well-drained: well- drained soils; Poor-drained: poor drained soils; High: high agricultural grassland management intensity; Low: low agricultural grassland management intensity

	Units	Region F—value	Drainage F—value	Manage F—value	West Well-drained High	West Well-drained Low	West Poor-drained High	West Poor-drained Low	East Well-drained High	East Well-drained Low	East Poor-drained High	East Poor-drained Low
PLFA_Bacteria	nmol g^{-1}	0.009	2.579	1.298	20.53 (±0.52)	21.58 (±0.65)	21.60 (±0.49)	22.36 (±0.17)	21.10 (±0.22)	21.07 (±0.43)	21.32 (±0.15)	21.89 (±0.38)
PLFA_Fungi	nmol g^{-1}	0.083	0.748	1.972	7.58 (±0.38)	7.58 (±0.20)	8.22 (±0.53)	7.61 (±0.56)	7.56 (±0.46)	7.53 (±0.49)	7.04 (±0.28)	8.97 (±0.85)
Urease	µg $NH4^+$ h^{-1} g^{-1}ds	0.48	0.91	1.64	136.03 (±23.89)	200.44 (±47.26)	163.40 (±21.67)	154.23 (±15.38)	105.58 (±10.69)	132.95 (±24.05)	112.38 (±17.66)	214.02 (±56.71)
α—glucosidase	µmol h^{-1} g^{-1} ds	2.87	2.43	0.31	47.39 (±4.44)	57.37 (±5.97)	95.67 (±42.84)	57.01 (±13.86)	140.67 (±39.88)	123.37 (±48.36)	85.86 (±35.23)	61.91 (±9.74)
β—glucosidase	µmol h^{-1} g^{-1} ds	0.10	1.62	0.68	309.84 (±149.77)	158.74 (±46.94)	161.02 (±36.31)	222.75 (±27.76)	202.59 (±61.59)	161.32 (±18.42)	259.41 (±75.45)	314.84 (±90.77)

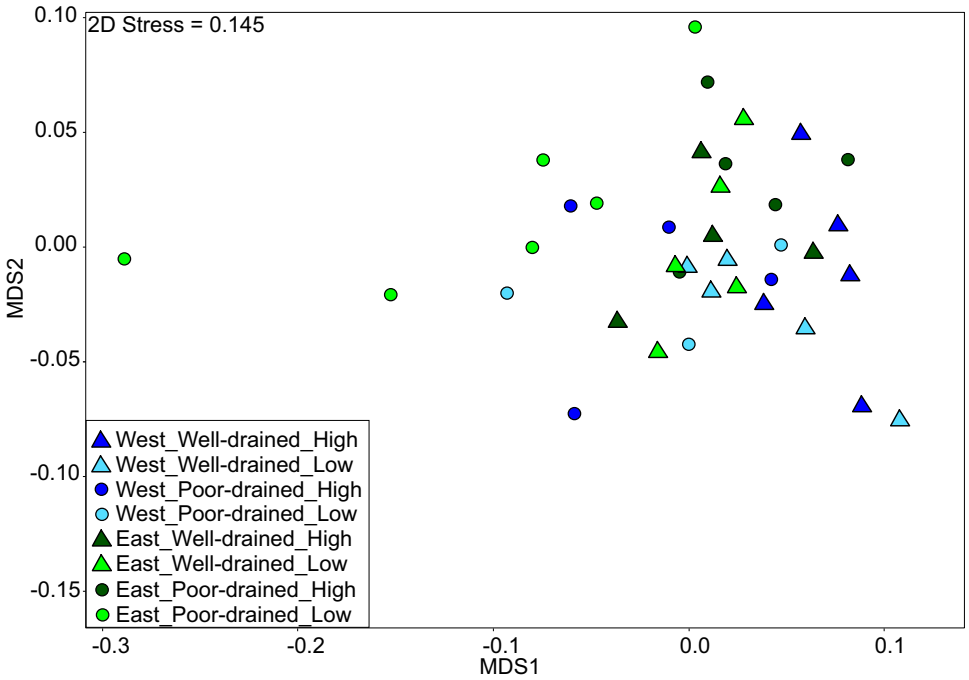

Fig. 2 Non-metric multi-dimensional scaling plot showing the effect of region (blue = West, green = East), drainage (triangle = well-drained, circle = poor-drained) and management (dark green/blue = high intensity, light green/blue = low intensity) on soil microbial community structure based on phospholipid fatty acid biomarkers

was significant within this factor. Thus, the study's first hypothesis was not supported.

Previous studies, using both PLFA and next-generation sequencing techniques, have reported that grassland management intensity is as strong a driver of fungal community structure as continental-scale geographic factors are (i.e., regional differences along a North–South European transect), while soil bacteria are more strongly driven by geographic factors (Barreiro et al. 2022; Fox et al. 2021). In line with these findings, this study did show that 'Region' significantly influenced soil microbial community structure, with soil pH being a significant driver of this variable in both the West and East regions, as documented in numerous studies (Cao et al. 2016; Kaiser et al. 2016; Wang et al. 2017). While soil pH was the sole driver of soil microbial community structure in the West region, other variables significantly explained the variation in the East region, including annual rainfall levels.

A significant effect of 'Drainage' on soil microbial community structure in Irish managed grasslands was seen in this study. Higher levels of the fatty acid biomarker for AMF was seen in well-drained sites than in the poor-drained sites in both the West and East regions. This finding can be linked to the influence that Drainage has on the physicochemical conditions of the soil, namely water holding capacity, soil aeration, soil nutrient levels and plant growth conditions (Richter et al. 2018; Coyle et al. 2016). AMF have previously been reported to have a higher abundance in aerobic conditions (Vallino et al. 2014), consistent with the strictly aerobic characteristics of AMF (Chareesri et al. 2020), which would explain the higher abundance in well-drained soils. The same response was observed for the fatty acid biomarker for SF, but only in the East region. This result highlights that natural drainage condition is an important factor to consider when assessing soil biological conditions within regions that share similar climatic conditions.

While there is growing evidence in the literature to support that soil fungi are more sensitive to agricultural grassland management intensity than soil bacteria (Barreiro et al. 2022; Fox et al. 2021; Li et al. 2022; Richter et al. 2024), only bacterial biomarkers significantly responded to the factor 'Manage' in this study. The fatty acid biomarkers for Gram positive bacteria had a higher abundance in the high management sites, compared to the low management sites in the East region, which is a region with more favourable agronomic conditions. High proportions of Gram positive bacteria, relative to Gram negative, are usually associated with soils intensively managed with low soil organic carbon, such as intensive grasslands or arable soils (Francisco et al. 2016; Bossio et al. 2005; Herman et al. 2012), however the Gram positive: Gram negative ratio was not significantly influenced by the factor 'Manage' in this study. The poor-drained, high management sites had the highest abundance of Gram positive bacteria in the East region. Agricultural management intensity would be inherently higher in this region, due to more favourable climatic conditions leading to higher grassland production (O'Donnell et al. 2021; Collins and Cummins 1996). The poor-drained, high management sites had also the lowest levels of SOC, indicating the higher management pressure in these sites. The carbon utilization

preferences of the Gram positive and Gram negative bacteria have been reported to differ, with the former using more SOM-derived recalcitrant organic matter and the latter utilizing more simple, plant-derived sources of C (Fanin et al. 2019).

Extracellular enzymatic activities were largely not affected by 'Drainage', thus the study's second hypothesis was not supported. There was no effect of 'Drainage' on the level of activity of five of the six enzymes tested as part of this study. The only enzyme in which 'Drainage' did have a significant effect on was acid phosphatase, which catalyses the hydrolysis of phosphate esters and anhydrides (Nannipieri et al. 2011), but this factor had a smaller effect than that of 'Region'. The effect of 'Drainage' on the activity of acid phosphatase was seen only in the East region, with higher levels of activity being seen in the well-drained sites than the poor-drained sites. Well-drained soils likely have more O_2 availability than poorly drained soils, allowing for the heterotrophic decomposition of soil organic matter by soil microbes. A previous study (Graça et al. 2021), did not report a significant effect of drainage on the activity of acid phosphatase, it did however observe significantly higher levels of alkaline phosphatase in well-drained Irish grassland sites than poorly drained sites. This enzyme was not examined as part of this study. On the other hand, extracellular enzymatic activity exhibited a strong sensitivity mainly to 'Region'. The overriding effect of 'Region' on soil extracellular enzymatic activity, as well as the C:N and C:P enzyme stoichiometric ratio, contrasts with that observed with microbial community structure (i.e., a more balanced influence of the three examined factors), indicating that there may be differing underlying drivers for microbial community structure and activity. This research avenue however, will require further study, with a more in-depth analysis of the impact of these factors on soil microbial-mediated nutrient cycling potential. This will likely require a molecular

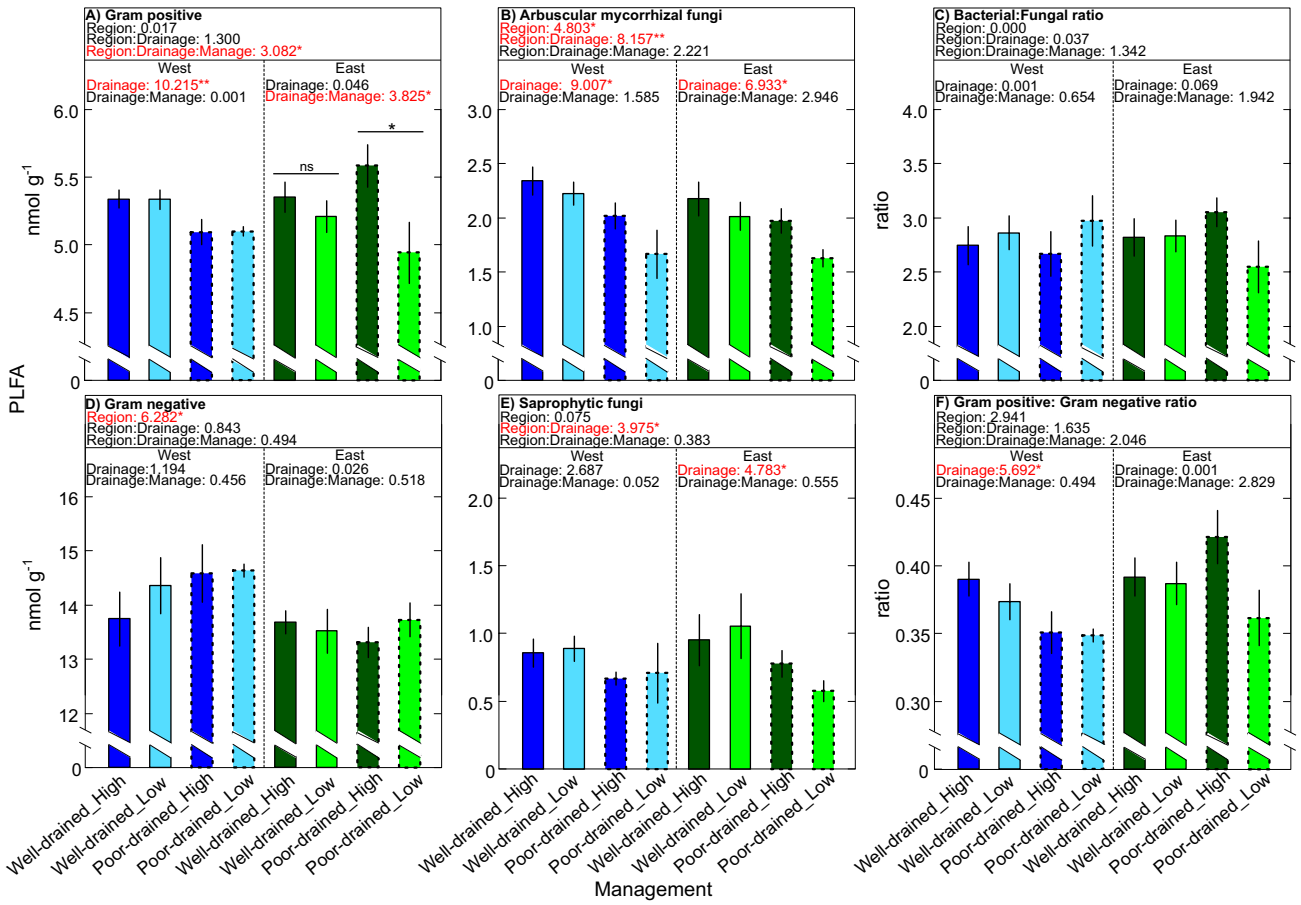

Fig. 3 Boxplot showing the effect of region (blue = West, green = East), drainage and management on phospholipid fatty acid biomarkers A) gram positive bacteria (sum of i14:0, i15:0, i16:0 PLFA), B) fungal arbuscular mycorrhizal fungi (AMF, 16:1ω5c), C) bacterial:fungal PLFA ratio, D) gram negative bacteria (sum of 16:1ω7c, cy17:0, 18:1ω7, cy19:0 PLFA), E) saprophytic fungi (SF, 18:2ω6c) and F) gram positive:gram negative PLFA ratio. Also shown as an insert panel is the F – value for each experimental variable (Region, Drainage, Manage), with significant effects highlighted in red. significance codes: '***' $p \leq 0.001$ '**' $p \leq 0.01$ '*' $p \leq 0.05$ 'ns' $P > 0.05$ from an analysis of variance model. Bars represent means and error bars represent standard error

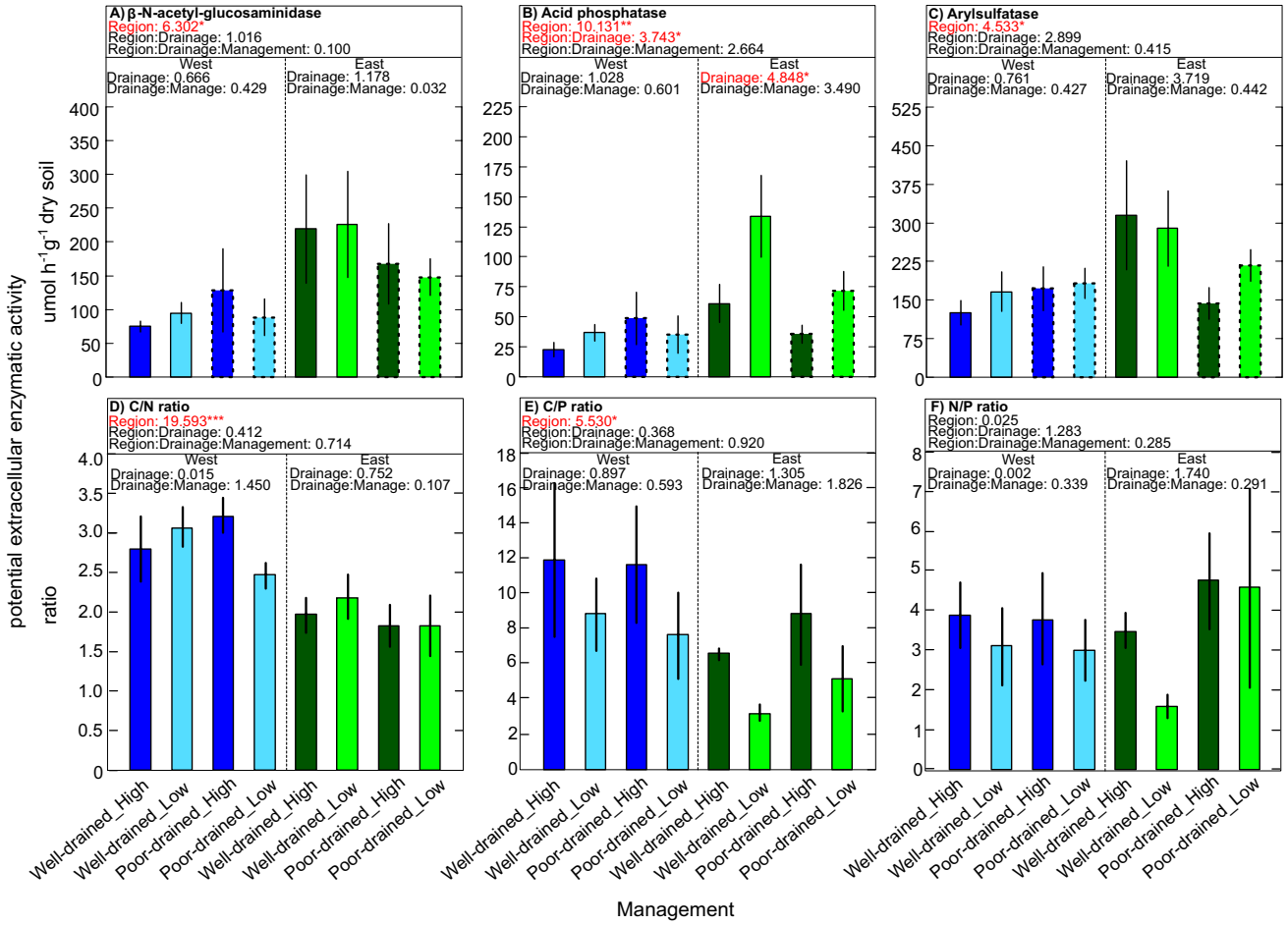

Fig. 4 Boxplot showing the effect of region (blue = West, green = East), drainage and management on A) β-N-acetyl-glucosaminidase, B) Acid phosphatase and C) Arylsulfatase. Also shown as an insert panel is the F – value for each experimental variable (Region, Drainage, Manage) across the two regions, and the F value for each experimental variable within the West and East region, with significant effects highlighted in red. Significance codes: '***' $p \leq 0.001$ '**' $p \leq 0.01$ '*' $p \leq 0.05$ from an analysis of variance model. Bars represent means and error bars represent standard error

approach to quantify the abundance of genes involved in the various nutrient cycles, through both a qPCR and/or metagenomics approach.

Overall our findings highlight that microbial community structure is shaped by the combined effect of management, drainage, and climatic region, while the abundance of AMF is driven by drainage, and microbial extracellular enzymatic activity varies by climatic region alone. These regional differences underscore the need for a targeted soil biology monitoring scheme in Ireland, prioritizing selected sampling regions over nationwide coverage to ensure efficiency and representativeness.

4.2 Implications for national soil sampling campaigns and study limitations

This study highlights the importance of considering regional/climatic, soil drainage and agricultural management differences in national sampling campaigns examining soil biological parameters. A deep understanding of biological indicators that takes into account their broader ecological context is essential when looking at monitoring schemes for soil biology.

In practical terms, when assessing soil biology at the landscape level, where soil drainage status and soil type vary, PLFA proved to be an effective tool. It captures both overall microbial abundance and individual fatty acid biomarkers, making it well-suited for detecting localized changes in microbial communities. PLFA may be an adequate tool for assessing landscape patterns within long-term sampling campaigns, due to the fact that there is an ISO standard for the technique, and that the analysis is comparatively simple and cost-effective. This approach seems to be preferable to next generation sequencing in such a scenario, as even though that technique would provide a more detailed analysis of soil microbial community structure and

its composition, as it still lacks an ISO standard (Manter et al. 2024), which may hamper its use for comparative purposes in long-term national soil monitoring campaigns.

Potential soil extracellular enzymatic activity showed minimal response to soil drainage status but was significantly influenced by region. This finding would contrast with that previously reported in a European scale study which examined potential extracellular enzymatic activity across a broad range of climatic regions, which did not report an effect of either climatic region or land-use (Hendriksen et al. 2016). It may be the case that the reliability of this assay type decreases at larger scales, particularly when measurements are taken only at single time points. Thus, a large-scale monitoring program of soil biology should avoid relying solely on enzymatic assays to assess soil microbial community functionality across a wide geographical range, as they may not effectively capture differences.

A greater understanding of the temporal variation in soil microbial community structure, abundance and microbial extracellular enzymatic activity, especially across climatic regions, is a key research consideration to determine the biodiversity and functional potential of soils at a national scale (Geisen 2021; Kostin et al. 2021). Future studies should include a broader range of land-use types to provide a more nationally representative understanding of the influence of land-use type on soil biological and physicochemical parameters (Froger et al. 2024).

5 Conclusions

This study highlights the need for considering national and localized environmental drivers of soil physicochemistry and biology in national soil sampling studies. While the factor 'Region' did emerge as the predominant factor driving soil physicochemical and extracellular enzymatic activity, the factor 'Drainage' did exert the strongest influence on the abundance of AMF, a key soil fungal group. No one factor was the main driver of soil microbial community structure, with 'Region', 'Drainage' and 'Manage' having a comparable influence. Given the strong impact of soil drainage on specific components of the soil microbiome, PLFA proved to be an effective tool for detecting localized changes, supporting a more targeted approach to soil biology monitoring rather than a uniform, nationwide strategy. Accounting for these environmental drivers in national monitoring schemes and soil biodiversity conservation efforts will enable more effective, context-specific strategies.

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Ana Barreiro: Data curation (supporting); Writing original draft (supporting).

David Wall: Conceptualization (equal); Funding acquisition (lead); Investigation (equal); Methodology (equal); Project administration (equal); Visualization (equal); Writing original draft (supporting).

Giulia Bondi: Conceptualization (equal); Investigation (equal); Methodology (equal); Project administration (equal); Supervision (lead); Data curation (lead); Visualization (equal); Writing original draft (equal).

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Data availability The data that support the findings of this study are available from the last listed author upon reasonable request.

Declarations

Competing interest The authors wish to state that there are no competing financial interest or personal relationships which would influence the work reported in this paper.

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