



Research paper

Ecoenzymatic and respiration-based characterization of soil microbial function in vineyard systems

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ABSTRACT

Soil enzymatic and respiration profiling was applied to characterize the soil microbial functions of 20 vineyard sites across two major Slovenian wine-growing regions, Primorska and Podravje. Extracellular enzymatic activity (EEA) and multiple substrate-induced respiration (MSIR) assays were used to quantify microbial activity patterns and to link them to soil physicochemical properties, topography, and vineyard management practices. EEA and MSIR varied significantly among vineyard locations and between the two regions. Primorska sites generally exhibited higher enzymatic activity, particularly for nitrogen- and phosphorus-acquiring enzymes, and greater variability in respiration rates than Podravje. Microbial activity responded also to management practices, with higher respiration observed in organically managed soils, while enzymatic activity showed contrasting patterns with conventionally managed vineyards - exhibiting higher overall enzyme activity. However, variability among sites was high, and these trends should be interpreted within the broader environmental context. Management-, site-, and soil microbial- functional differences were translated into coherent multivariate patterns. Heatmaps, PCA, and t-SNE analyses consistently demonstrated a clear separation of samples by regional origin and additional differentiation at the microlocation level. Redundancy analysis revealed that soil chemical properties, notably pH, clay content, and total organic nitrogen, were the primary drivers of microbial function, whereas management and topography played secondary roles. These findings demonstrate that EEA and MSIR are powerful tools for assessing soil microbial functioning in vineyard soils. The observed microbial functional patterns were closely associated with environmental gradients, suggesting that these approaches provide biologically relevant information that may complement future *terroir*-related studies and support sustainable vineyard management.

1. Introduction

Soil is a fundamental component of terrestrial ecosystems that supports plant productivity, nutrient cycling, water regulation, and long-term carbon storage. These functions strongly depend on the activity of soil organisms, particularly microorganisms, which mediate key biogeochemical processes through their metabolic capabilities. Among these processes, extracellular enzymes and microbial respiration play central roles in decomposing organic matter and releasing nutrients that are essential for plant growth and ecosystem functioning. Soil microbial functioning is recognized as a core indicator of soil health and environmental sustainability (Sahu et al., 2017). In agricultural systems, including vineyards, land use intensification, climate variation and

management practices can modify microbial functioning by altering organic inputs and disturbance regimes (Yang et al., 2021). Beside these also soil physicochemical properties, including pH, moisture, and organic matter, are recognized to be an important drivers of microbial functioning.

In recent years, the importance of the soil microbial functional dimension of *terroir* has been increasingly recognized (Arif et al., 2020; Dries et al., 2021; Kaul et al., 2021). *Terroir*, a concept traditionally rooted in geology, climate, topography, and vineyard management practices, now includes the microbiological dimensions of soil, particularly the structure and function of the microbial communities (Gobbi et al., 2022). Soil microbial activity influences nutrient cycling, organic matter turnover, and the availability of key elements such as

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nitrogen and phosphorus, which indirectly affect vine physiology and grape composition (Griggs *et al.*, 2021). While our study does not directly assess wine characteristics, these microbial processes represent a potential mechanistic link between soil functioning and *terroir* expression in a glass of wine.

Despite this progress, important knowledge gaps remain in the literature. In particular, it is unclear whether low-cost functional assays can detect ecologically meaningful differences in microbial functioning across landscapes where natural soil variation and management practices overlap, such as vineyards. Few studies have evaluated whether these functional assays are reliable and sufficiently sensitive tools for distinguishing soil conditions relevant to viticultural management and soil microbial functional dimension of *terroir* expression (Riches *et al.*, 2013). In contrast, most current assessments of soil microbial communities rely on advanced molecular tools, such as 16S rRNA gene and ITS sequencing. While molecular approaches such as 16S rRNA and ITS sequencing provide detailed insights into microbial community composition, they do not directly measure functional activity and are often limited in their ability to capture real-time metabolic processes. In contrast, enzymatic and respiration-based assays reflect the integrated functional response of microbial communities to environmental conditions. However, these approaches also have limitations, including lower taxonomic resolution and potential sensitivity to environmental variability (Bell *et al.*, 2013; Gao *et al.*, 2022). Therefore, rather than replacing molecular methods, functional assays should be considered as complementary tools, particularly suitable for assessing ecosystem functions and management-related effects.

Functional assays, such as extracellular enzyme activity (EEA) and multiple substrate-induced respiration (MSIR) assays, have gained attention as simple, rapid, and cost-effective tools for assessing soil microbial functioning. Originally developed as basic indicators of microbial activity, these methods have evolved considerably over the past two decades. EEA focuses on the hydrolytic activity of enzymes involved in key nutrient cycles, including carbon, nitrogen, and phosphorus (Jackson *et al.*, 2013), whereas MSIR captures the microbial respiratory response of the soil to a range of carbon substrates (Chapman and Campbell, 2007). Recent methodological advances have significantly increased the analytical depth and ecological relevance of such assays. The foundational article that established ecoenzymatic stoichiometry by Sinsabaugh *et al.* (2009), published in *Nature*, initiated the development of ecoenzymatic stoichiometry, which enabled researchers to infer microbial nutrient limitations by calculating the ratios of enzyme activities associated with different elemental cycles. Recent research has continued to expand and refine this approach by developing new models and critical thresholds for identifying nutrient limitations and applying ecoenzymatic stoichiometry across diverse ecosystems (Cui *et al.*, 2021; Kunito *et al.*, 2024). Similarly, MSIR has advanced beyond bulk respiration measurements to support the calculation of microbial functional diversity using indices such as Shannon's diversity index and the metabolic quotient (qCO_2), which reflects microbial carbon use efficiency. Together, these extensions allow EEA and MSIR to provide multi-informational outputs that link microbial metabolism to ecological functions, resource use strategies, and environmental constraints. Despite their increasing sophistication and interpretive power, these methods remain more accessible and affordable than molecular sequencing, offering a valuable alternative for functional soil assessments in both research and practical contexts of land management. Despite their advantages, these methods remain underutilized in viticulture. There is a clear need to assess whether these accessible techniques can capture meaningful ecological patterns, particularly in distinguishing the influence of environmental conditions from management interventions on microbial function across different *terroirs*. Although studies on viticulture are still limited, similar functional assays have been used in other agronomic fields as decision-support tools for identifying optimal field locations and selecting suitable management practices. For example, extracellular enzyme activity and respiration-

based indicators have been applied in horticulture (Wang *et al.*, 2023), cereal cropping (Chen *et al.*, 2021), and tree plantation systems (Xu *et al.*, 2022) to guide site selection, determine organic input requirements, and monitor soil biological responses to tillage, fertilization, and plant restoration. While most published studies have focused on other cropping systems, principles and methodologies, such as using enzyme activity ratios to guide organic input needs or monitor biological responses, are directly transferable to vineyards (Kunito *et al.*, 2024). By measuring the ratios of key extracellular enzyme activities and soil respiration, researchers and practitioners can infer how vineyard soils respond to management practices such as organic amendments, tillage, and fertilization, and identify potential nutrient limitations that may affect vine growth and grape quality. Recent reviews have emphasized that ecoenzymatic stoichiometry provides a temporally integrated indicator of nutrient availability and can be combined with respiration-based measures for a comprehensive understanding of soil function, making these tools increasingly relevant for viticulture as interest in soil health and sustainable land management increases.

The present study addresses this gap by applying enzymatic and respiration-based functional profiling to soils from 20 vineyards across two climatically and topographically distinct Slovenian wine regions, Primorska and Podravje. We analyzed the correlation between microbial functioning, measured through EEA and MSIR assays, and environmental gradients or/and cultivation practices, including tillage and organic farming versus conventional farming. Our aim was to clarify the relative importance of environmental and agricultural factors in structuring vineyard soil microbial communities, evaluate how microbial functioning varies across vineyard soils within different environmental conditions, and assess whether cost-effective functional assays such as EEA and MSIR can reliably reflect these patterns. The novelty of this study lies in the validation of a practical methodological approach for assessing microbial functioning in vineyard soils and exploring its potential contribution to *terroir*-related research, with implications for both scientific understanding and vineyard management.

2. Materials and methods

2.1. Study sites and sampling design

Soil samples were collected from 20 vineyards across two Slovenian wine-growing regions, Primorska ($n = 11$) and Podravje ($n = 9$), encompassing nine microlocations (Fig. 1). At each vineyard, composite soil samples were obtained from 20 to 25 random points along the inter-row at two depths (0–10 cm and 11–20 cm). The samples were then cooled and processed within 6 h. The soil samples were sieved through a 2 mm sieve. Fresh subsamples were used for biochemical analyses, and air-dried fractions were reserved for physicochemical characterization.

Topographic variables (elevation, slope, and aspect) were derived from GIS data (MKGP-RKG, 2023), and short-term climatic conditions were obtained from nearby meteorological stations for the 7-day period preceding the sampling date. Data for the Primorska wine-growing region were obtained from the Bilje weather station and those for the Podravje region were sourced from the Maribor weather station. Vineyard management practices (organic vs. conventional, and tilled vs. non-tilled) were recorded for each site. The complete dataset is presented in Table S1.

2.2. Soil physicochemical analyses

The soils were fully characterized for texture, pH, total organic carbon (TOC), total nitrogen (TN), C/N ratio, available phosphorus (Olsen P), and inorganic nitrogen (NO_3^- and NH_4^+). Soil texture was measured in a Bouyoucos cylinder using an ASTM 152H hydrometer. The soil pH was measured in water (1:2.5, weight-to-volume ratio). Total organic carbon and total nitrogen (TOC and TN) were determined by automated thermal analysis, where carbon was converted to CO_2 by

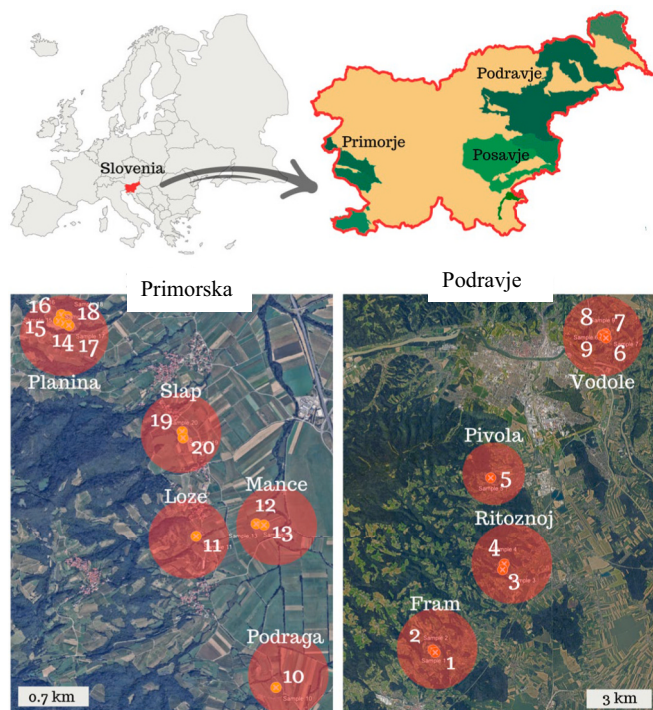


Fig. 1. Map of vineyard soil sampling sites across the two major Slovenian wine-growing regions: Primorska (west) and Podravje (northeast). The upper panel shows Slovenia's location within Europe and highlights the three official wine regions (Primorje, Podravje, and Posavje). The lower panels present detailed satellite maps of the selected vineyard micro-locations in each region. Red-shaded circles denote clustered micro-locations, while numbered points represent individual vineyard plots where soil samples were collected. Micro-locations include Planina, Slap, Loze, Mance, and Podraga in Primorje, and Fram, Ritoznoj, Pivola, and Vodole in Podravje. Scale bars indicate relative spatial distances. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

flash combustion at 1080 °C (Costech Elemental Combustion System 102 elemental analyzer; Costech Instruments). The carbonates were removed from 10 mg of the subsample by treatment with HCl in silver capsules. The C/N ratio was then calculated. Olsen P was measured using 2 g of soil sample extracted in 40 mL of 0.5 M NaHCO₃ (Olsen et al., 1954). After extraction (30 min, 250 RPM), the solution was filtered through (0.2 µm CA filter), and P was measured using ICP-AES. Inorganic nitrogen (NO₃⁻ and NH₄⁺) was measured after 60 min of extraction of 5 g of soil into 25 mL of 0.5 M KCl at 200 RPM (Mulvaney, 1996). The extraction solution was filtered through a 150 mm glass filter paper and analyzed using a Segmented Flow Injection Analyzer (Skalar San++).

The pseudo-total metal concentration in the soil was measured using the USEPA 3052 (1995) mineralization method and inductively coupled plasma atomic emission spectroscopy (ICP-AES, Agilent 5800). Bioavailable cationic macronutrients (Ca, Mg, and K) were analyzed by extraction with a 1 M BaCl₂ solution, whereas micronutrients (Cu, Zn, Fe, and Mn) were analyzed using the DTPA/TEA method developed by (Lindsay and Norvell, 1978). A certified soil sample (BCR-CRM 600) was also extracted to ensure quality control during the analyses. The extraction solutions were analyzed using ICP-AES (Agilent 5800). Calibration was performed using standard solutions (0.5, 1, 5, 10, 30, and 50 mg L⁻¹) prepared from an ICP-standard 23-element solution in 5% HNO₃ (Merck solution IV), with yttrium (Y) as the internal standard. Strict quality assurance and quality control (QA/QC) were performed, including blanks, analysis of the reference material (CRM 601), and laboratory control sample analysis.

2.3. Enzyme assays

Enzyme-activity assays were conducted following the protocol established by Bell et al. (2013), targeting the activities of six widely recognized extracellular enzymes: α-1,4-glucosidase (AG), β-1,4-glucosidase (BG), β-D-cellobiohydrolase (CB), L-leucine aminopeptidase (LAP), phosphatase (PHOS), β-xylosidase (XYL), and N-acetyl-β-D-glucosaminidase (NAG). These enzymes play key roles in soil nutrient cycling, specifically in carbon (BG, CB, AG, and XYL), nitrogen (LAP and NAG), and phosphorus (PHOS) cycles (Bell et al., 2013). The assay utilized methylumbelliferyl-(MUB)-linked substrates for β-D-cellobioside, phosphate, N-acetyl-β-D-glucosaminide, and β-D-glucopyranoside, whereas the hydrolytic enzyme LAP was assayed using a 7-amino-4-methylcoumarin-(MUC)-linked substrate L-leucine.

Briefly, 1.37 g of fresh soil was homogenized in 45 mL of sodium acetate buffer (50 mM, pH 6.5, and 8.0, consistent with soil pH). An aliquot of 800 µL of this homogenate was transferred to a 96-well microplate, followed by the addition of 200 µL of the respective substrate solution. The microplates were then incubated in the dark at 25 °C for 3 h. After incubation, the plates were centrifuged at 1500 RPM. Centrifugation was exclusively used to remove soil particles with strongly scattered fluorescence and impaired optical readings. Although many fluorometric EEA protocols measure fluorescence directly in slurry, preliminary tests on vineyard soils showed high turbidity and heterogeneous particulate loads, which caused nonlinear fluorescence responses. Briefly, low-speed spin allowed consistent optical clarity without significantly interrupting enzymatic reactions, as incubation was completed prior to centrifugation, and the supernatant was carefully transferred to a black, flat-bottomed 96-well plate. The fluorescence of the supernatants was measured using a microplate fluorometer (Infinite, TECAN Instruments, Crailsheim, Germany) with excitation and emission filters set at 365 and 460 nm, respectively.

Fluorescence data were converted to enzyme activity using standard curves generated from MUB or MUC standards. For each standard curve, 800 µL of soil slurry was mixed with 200 µL of varying concentrations of MUB or MUC standard solutions, incubated, and measured in parallel with the sample plates. The final enzyme activity was calculated as outlined by Bell et al. (2013) and expressed as nmol of substrate converted per hour per gram of dry soil (nmol h⁻¹ g⁻¹).

2.4. MicroResp assay

The methodology for evaluating multiple substrate-induced respiration (MSIR) in soil samples was adapted from the MicroResp™ assay as described by Campbell et al. (2003). The assay employed 14 distinct substrates that were categorized into four groups. All substrates were added at equimolar C concentrations, following the manufacturer's MicroResp guidelines, to avoid substrate-specific inhibition. The exact masses varied according to the substrate molecular weight; however, the resulting C input per well remained within the standard MicroResp working range and did not exceed the glucose-equivalent saturation dose used for microbial biomass determination. The list of categories and carbon substrates used in the assay was as follows: carbohydrates (L-arabinose (ARA), D-fructose (FRU), D-glucose (GLU), and D-trehalose (TRE)), amino acids (L-alanine (ALA), L-arginine (ARG), L-cysteine HCl (CYS), γ-amino butyric acid (GABA), and L-lysine (LYS)), carboxylic acids (citric acid (CIT), LL-malic acid (MAL), oxalic acid (OXA), and α-ketoglutaric acid (AKGA)), and aminocarbohydrate N-acetyl glucosamine (NAGA). The core principle of the MicroResp™ assay is based on inducing maximum microbial respiration through the addition of excess carbon sources, which is indicative of the fraction of soil microbial biomass that can metabolize a given substrate. This assay not only quantifies MSIR, but also provides insights into the microbial community-level physiological profile (CLPP), reflecting the functional capabilities of soil microorganisms involved in the carbon cycle.

For this assessment, carbon sources were grouped into four

categories: amino acids, aminocarbohydrates, carbohydrates, and carboxylic acids. To determine basal respiration (BR), pure water was used, and D-glucose was applied at a concentration of 30 mg C g⁻¹ soil to quantify microbial biomass (MB). This concentration follows the SIR approach of Anderson and Domsch (1978), in which glucose is supplied in excess to ensure that respiration reflects active microbial biomass rather than substrate limitation. Although lower concentrations are sometimes used in modern adaptations of SIR, our pre-tests showed that lower glucose doses produced unstable BR:MB ratios in coarse-textured low-C vineyard soils. This chosen concentration was selected based on preliminary dose-response testing, where lower concentrations resulted in non-linear and unstable respiration responses. Importantly, CO₂ production remained linear during the incubation period, indicating the absence of substrate inhibition within the measurement window. Before initiating MicroResp™, a pre-incubation step was required to equilibrate the moisture conditions, restore microbial activity after sieving and handling stress, and minimize artifactual respiration pulses. This step is recommended for soils with heterogeneous structures such as vineyard soils with high aggregate variability. Fresh soil samples were adjusted to a moisture content of 18% and then distributed into 96-deep well plates, in accordance with the manufacturer's instructions. The soil samples were pre-incubated for 5 d at 25 °C.

After pre-incubation, specific substrates were introduced into the soil samples and the deep-well plates were sealed with a detection plate. The detection plate contained a cresol red-dyed gel that changed colour in response to CO₂ emission. Microbial respiration, indicated by CO₂ release, was measured using a microplate reader (Infinite, TECAN Instruments, Crailsheim, Germany) at a wavelength of 590 nm before and after a 6-h incubation at 25 °C. The percentage of CO₂ was then converted to µg C-CO₂ · h⁻¹ · g⁻¹ of dry soil.

The metabolic quotient for CO₂ (qCO₂) was computed using the following formula proposed by Anderson and Domsch (1993): qCO₂ = BR/MB. Additionally, microbial functional diversity (H') was calculated using the Shannon-Weaver index ($H' = -\sum P_i (\ln P_i)$), where P_i represents the proportion of activity associated with a specific substrate relative to the total activity across all 14 substrates evaluated (Zak et al., 1994).

2.5. Data analysis

We assessed the soil enzymatic stoichiometry by calculating the ratios of soil enzyme activities: EEC (BG + CBH + BX):EEN (NAG + LAP), EEC:EEN (ALP), and EEN:EEN. These ratios were used to estimate microbial resource limitations, as described by Cui et al. (2021). Specifically, this method relies on a scatter plot of EEC:EEN against EEN:EEN, which is divided into four sections using 1 as the intersection of both axes. The resulting quadrants represent phosphorus limitation, nitrogen limitation, co-limitation of carbon and nitrogen, and co-limitation of carbon and phosphorus.

Based on enzymatic stoichiometry theory, microbial metabolic constraints were determined using a vector model that employed untransformed proportional activities (Moorhead et al., 2016). Vector length (adimensionless value) represents the magnitude of the relative carbon-to-nutrient limitation (Eq. 1). The vector angle (measured in degrees) indicates the relative nitrogen versus phosphorus limitation, calculated using Eq. 2. Vector angles less than 45° suggest that microbial metabolism is more constrained by nitrogen than phosphorus, with the degree of limitation increasing as the angle decreases. Conversely, vector angles greater than 45° suggest greater limitation by phosphorus than by nitrogen, with the extent of limitation increasing as the angle increases.

(1) Relative C limitations = Vector length (unitless) = $\sqrt{x^2 + y^2}$
 where x represents the relative enzymatic activity for carbon acquisition compared to phosphorus acquisition (i.e., [BG + XYL + CBH]/[BG + XYL + CBH + AP]) and y represents the relative enzymatic activity for carbon acquisition compared to nitrogen acquisition (i.e., [BG + XYL + CBH]/[BG + XYL + CBH + NAG + LAP]).

(2) N/P limitations = Vector angle (°) = DEGREES (ATAN2 (x, y))

2.6. Statistical analysis

All statistical analyses were conducted using a combination of software tools including RStudio (v4.3.3), IBM SPSS Statistics 25, SIMCA 14.1, Minitab 21, XLSTAT 2022, and Microsoft Excel. Analyses were performed on a Windows 10 system equipped with an Intel® Core™ i7-1355U CPU and 32 GB RAM.

The statistical dataset consisted of 40 soil samples collected from 20 vineyards, with two sampling depths (0–10 and 10–20 cm) per vineyard. Each soil sample was treated as an individual observation in the multivariate analyses (PCA, hierarchical clustering, t-SNE and RDA). Sampling depth was included as an explanatory variable where appropriate. The objective of the study was to evaluate patterns of microbial functioning across contrasting soil conditions rather than to model repeated measurements within individual vineyards.

2.6.1. Descriptive and univariate analyses

Descriptive statistics (mean and standard deviation) were calculated for all soil physicochemical properties, microbial respiration data (MSIR), and enzymatic activities (EEA). One-way and two-way ANOVA were applied to assess the effects of vineyard region (Primorska, Podravje), micro-location, management type (organic vs. conventional), and tillage on microbial and soil variables. When the assumptions of normality and homogeneity of variances (tested via Shapiro-Wilk and Levene's tests) were violated, non-parametric Kruskal-Wallis tests were employed. Tukey's honest significant difference test was used for post hoc comparisons.

2.6.2. Multivariate analyses

To identify compositional patterns among samples, multivariate analyses were performed as follows: (1) Heatmap Clustering: A total of 40 vineyard soil samples and 34 variables (soil properties, MSIR rates, enzymatic activities, and topographic features) were included. Both column-wise (variable standardization) and row-wise (sample-wise normalization) transformations were used. Hierarchical clustering was performed using the Euclidean distance and complete linkage. Clusters were visualized using dendrograms, indicating similarities in compositional profiles and aiding the identification of clusters influenced by region, land use, and microbial structure. (2) Principal Component Analysis (PCA): PCA was applied to assess the variation and dimensionality of the dataset. (3) t-distributed Stochastic Neighbor Embedding (t-SNE) with k-means clustering: t-SNE was used to visualize nonlinear patterns in sample similarity based on a full multivariate dataset. It combines k-means clustering and convex hulls. (4) Redundancy Analysis (RDA): Multiple RDA models were employed to relate microbial activity to soil and environmental predictors; RDA 1: topographic features and soil properties (e.g., pH, TOC, NH₄⁺, NO₃⁻, Olsen-P, clay, silt, and sand) were used to explain the variation in respiration rates (15 carbon substrates). RDA 2: Soil site descriptors (depth, status, and tillage), topographic features, and soil properties were used to explain the variability in respiration rate. RDA 3: Topographic and soil properties (EAG, EBG, PHOS, NAG, XYL, CB, and LAP) were used to explain enzymatic activity. RDA 4 (integrated): A combined model including respiration and enzymatic traits as response variables and all environmental predictors (site descriptors, topography, and soil properties).

2.6.3. Microbial stoichiometry and diversity

Ecoenzymatic stoichiometry theory was applied to assess nutrient limitation using enzyme ratios (C:N:P), vector length (acquisition intensity), and vector angle (imbalance). Microbial biomass (GIR), metabolic quotient (qCO₂), and Shannon diversity index (H') were calculated from MSIR profiles using the MicroResp™ system.

Statistical significance was set at $p < 0.05$. Visualizations were produced in R, SPSS, and Excel, including PCA biplots, RDA triplots, t-SNE

projections, and annotated heat maps, to aid the interpretation of microbial and soil interactions.

3. Results

3.1. Environmental and agricultural practices variability of studied vineyards

To better understand the environmental and management-related factors associated with soil microbial functioning in Slovenian vineyards, it is essential to consider the heterogeneity among the studied sites. This section presents a detailed characterization of the 20 vineyard soils sampled from two distinct wine-growing regions (Fig. 1), Primorska and Podravje, which differ markedly in terms of climate, topography, and land management.

Regional contrasts were evident, although air temperatures during the sampling period were comparable between regions, soil temperatures at 10 and 20 cm depths were, on average, higher in Primorska. In contrast, Podravje experienced substantially higher precipitation levels (Table S1). The vineyards in Primorska were generally situated at lower elevations, whereas those in Podravje were located at higher altitudes and steeper slopes (see Fig. S1). Vineyard exposition was distributed across all major aspect classes, with Podravje vineyards more frequently oriented toward southern and western exposures, and Primorska vineyards more commonly oriented toward northern and eastern exposures. Nevertheless, each vineyard had a unique topographical profile in terms of elevation, slope, and aspect, as detailed in Table S1.

The selected vineyards also exhibited substantial variability in soil physicochemical properties (Table S2). The soil pH ranged from acidic to alkaline, with neutral to alkaline soils predominating in Primorska and more acidic soils characteristic of the Podravje region. Phosphorus availability (Olsen P) varied significantly, ranging from phosphorus depletion at 4.26 mg kg⁻¹ to excessively high availability levels of 118 mg kg⁻¹ across the regions. Similarly, total organic carbon (TOC) varied markedly among vineyards, from 1.03 up to 11.47% in individual vineyards. The total organic nitrogen and mineral nitrogen pools (NH₄⁺ and NO₃⁻) also showed pronounced spatial variability, reflecting differences in the soil fertility status and management history (Table S2).

In addition to the standard soil properties, the pseudo-total concentrations of the selected heavy metals revealed evidence of legacy contamination of several vineyards (Table S3). Copper concentrations frequently exceeded national threshold values (Official Gazette of the Republic of Slovenia, No. 68/96, 1996), particularly in surface soils, consistent with long-term fungicide use, whereas nickel concentrations were elevated at several sites, likely reflecting geogenic sources. In contrast, the zinc and lead concentrations remained below the critical thresholds across all vineyards.

Agricultural management practices also contribute to the site heterogeneity. Among the vineyards studied, both organic and conventional systems were represented, with organic management maintained for at least five consecutive years. Tillage practices varied within both systems: tillage was practiced in three organic and four conventional vineyards, allowing us the assessment of their combined and individual effects. Organically managed soils generally exhibited 35% higher total organic carbon and 30% higher ammonium availability but 43% lower nitrate concentrations than conventionally managed soils (Table S4). Tillage was associated with reduced organic matter content (30%) and lower ammonium availability (37%), with depth-dependent effects on nutrient distribution, particularly for Olsen P. Notably, tillage in organically managed soils was not associated with substantial differences in Olsen P availability (Fig. S2). Copper concentrations tended to be highest in organically managed topsoils, whereas tilled vineyards showed a greater proportion of Cu in deeper soil layers. In contrast, nickel (Ni) concentrations remained stable across different soil depths and management systems (Fig. S2).

Collectively, these results demonstrate substantial environmental,

pedological, and management-related variability among the studied vineyards, providing an important framework for interpreting patterns of soil microbial functioning.

3.2. Extracellular enzymatic activities (EEA) and stoichiometric characteristics of soil carbon, nitrogen, and phosphorus metabolizing enzymes

Potential extracellular enzymatic activities (EEA) varied widely across the studied vineyard soils, spanning more than three orders of magnitude (2 to 1478 nmol g⁻¹ h⁻¹) and showed a log-normal distribution of the data (Fig. S3, f). Among the measured enzymes, those involved in nitrogen and phosphorus acquisition generally exhibited higher activities than those of carbon-acquiring enzymes (Fig. S26, a). Leucine aminopeptidase (LAP) showed the highest mean activity (368 nmol g⁻¹ h⁻¹), followed by phosphatase (PHOS, 307 nmol g⁻¹ h⁻¹), and *N*-acetyl-β-D-glucosaminidase (NAG, 272 nmol g⁻¹ h⁻¹). In contrast, enzymes related to cellulose, hemicellulose, and simple carbohydrate degradation (BG, CB, AG, and XYL) displayed comparatively low activities.

3.2.1. Effect of sampling location on extracellular enzymatic activity

Clear regional patterns emerged, with soils from the Primorska wine-growing region exhibiting higher activities of carbon (33%), nitrogen (28%), and phosphorus-acquiring enzymes (30%) than Podravje soils and vineyard microlocations (Fig. 2). These regional contrasts were consistently observed across individual enzymes and aggregated enzyme groups, reflecting broader differences in environmental and edaphic conditions between the wine-growing regions. Within the regions, enzymatic activities further diverged at the microlocation scale, demonstrating strong site-specific functional heterogeneity (Fig. 2, c-d). Certain microlocations, such as Planina and Ritoznoj, were characterized by markedly elevated EEA, whereas others, including Loze and Mance, exhibited substantially lower activity levels. These patterns indicate structured functional heterogeneity within regions and suggest that local site conditions are associated with distinct enzymatic activity profiles. Together, the regional contrasts and distinct microlocation-level profiles indicate that EEA reflects spatial variation in soil microbial functioning across the studied vineyard soils.

Ecoenzymatic stoichiometric analysis revealed pronounced imbalances in microbial resource acquisition. Across microlocations, the activities of carbon-, nitrogen-, and phosphorus-acquiring enzymes (EEC, EEN, and EEP, respectively) showed clear contrasts. On a regional scale, Podravje soils consistently displayed EEC:EEN values below the threshold line (ratio = 1), indicating a higher nitrogen demand, whereas several Primorska microlocations exhibited higher EEC:EEP ratios, reflecting a comparatively greater carbon demand than phosphorus demand. The EEN:EEP ratios aligned with this pattern, with most vineyards across both regions exhibiting values above 1, confirming that the demand for nitrogen generally exceeded that for phosphorus (Fig. S6).

At the microlocation level, the ratios exhibit site-specific patterns. In the Podravje region, the distribution of the stoichiometric ratios was highly homogeneous among Ritoznoj, Fram, Vodole, and Pivola (Fig. S6). All microlocations were characterized by low EEC:EEN values (below 1), combined with consistently high EEN:EEP and elevated EEC:EEP ratios, indicating a uniform pattern of strong nitrogen demand relative to both carbon and phosphorus. This homogeneous behavior at the microlocation scale in Podravje contrasts with the higher variability observed among the microlocations in Primorska. The Slap, Planina, and Podraga microlocations exhibited a ratio pattern similar to that observed in Podravje, indicating consistent nitrogen-dominated limitation. In contrast, the Loze and Mance microlocations showed that all ratios were clustered within a narrow range between 1 and 1.5. This balanced positioning around the threshold suggests that microbial communities at these sites experience co-limitations, where carbon,

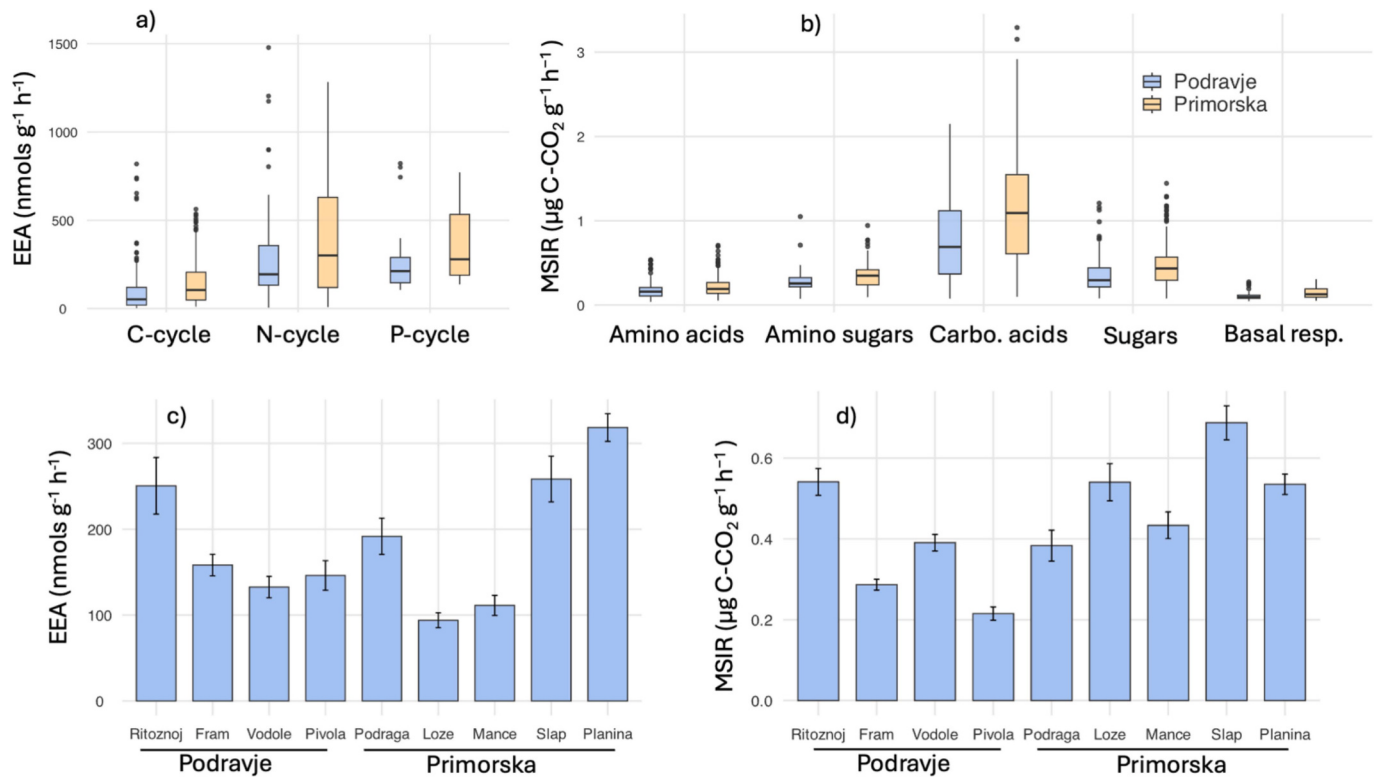


Fig. 2. Boxplots and microlocation means for extracellular enzymatic activities (EEA) and multiple substrate-induced respiration (MSIR) across vineyard soils in the Primorska and Podravje regions. (a) EEA grouped by nutrient-cycling categories (C-, N-, and P-acquiring enzymes). (b) MSIR responses grouped by substrate functional classes. (c–d) Mean EEA and MSIR values for vineyard microlocations, illustrating fine-scale spatial variation in microbial functioning.

nitrogen, and phosphorus demands are more evenly expressed, and no single nutrient strongly dominates metabolic constraints. Vector analysis supports these pattern shifts. Vector angles were below 45° for nearly all vineyards, indicating nitrogen limitation, except for Loze and Mance, where the angles were close to 45°, indicating a more balanced nutrient demand. Overall, the stoichiometric ratios across microlocations indicated predominant nitrogen or combined carbon and nitrogen limitations in vineyard soils.

3.2.2. Effect of topographic factors on extracellular enzymatic activity

Topographic variables showed only weak associations with overall enzymatic activity. Elevation and aspect showed negligible correlations with EEA, whereas slope displayed a statistically significant but weak relationship (Pearson coefficients = −0.05 and 0.02, respectively; Fig. S16). Nevertheless, the enzyme group responses varied along topographic gradients, with carbon-acquiring enzymes peaking at intermediate elevations and phosphorus-acquiring enzymes declining at higher altitudes (Fig. S7). Stoichiometric analysis also revealed that the 15°–25° slope category represented the most challenging conditions for microbial resource acquisition (Fig. S8). Nitrogen limitation was particularly strong, as reflected by a 23% reduction in the EEC:EEN ratio and a 14% reduction in the EEC/(EEC + EEP + EEN) ratio relative to both gentler (<15°) and steeper slopes (> 25°). Finally, vineyard aspects also influenced EEAs. Southwestern and eastern orientations supported 20–40% higher EEAs than the western and northern orientations (Fig. S9), specifically EEC and EEN activities. Vineyards with northern exposure showed signatures of combined carbon and phosphorus limitations, consistent with the distribution patterns observed in the EEC:EEN versus EEN:EEP scatter plots (Fig. S9).

3.2.3. Effect of soil chemical properties on extracellular enzymatic activity

Soil physicochemical properties were strongly associated with extracellular enzymatic activity. The highest EEA values were observed

in soils with near-neutral pH (Fig. S11) and moderate to high clay content (Fig. S10) and elevated total organic nitrogen (Fig. S12). Acidic, highly alkaline, and coarse-textured soils exhibited reduced activity. Phosphorus availability, expressed as Olsen P, exhibited only a weak relationship with phosphatase activity (Fig. S11), suggesting that microbial phosphorus acquisition remained relatively stable across a wide range of soil P conditions. Total organic carbon varied substantially among the studied soils; however, stoichiometric ratios indicated persistent nutrient limitations across the TOC classes. The EEC:EEP and EEN:EEP ratios were consistently greater than one across all TOC classes (Fig. S11). These patterns indicate that nitrogen and carbon limitations remained strong regardless of the TON and TOC content, reflecting the high microbial metabolic demand for these nutrients in vineyard soils.

3.2.4. Effect of heavy metal content on extracellular enzymatic activity

Heavy metal concentrations were also associated with differences in enzymatic patterns. Elevated copper (Cu) and nickel (Ni) concentrations showed weak positive correlation with the overall EEA (Pearson correlation coefficients: 0.13 and 0.12, respectively). Across the concentration classes, intermediate Cu concentrations (61–100 mg kg⁻¹) were associated with higher enzyme activities and lower vector angles, indicating stronger nitrogen demand (Fig. S13). whereas Ni concentrations were associated with more balanced carbon and nitrogen acquisition. These patterns were supported by vector angle analysis; angles declined from 44° in non-polluted soils to 41° in Cu-polluted soils, reflecting intensified N demand, whereas no comparable trend was observed for Ni. Because these correlations were weak, the observed patterns should be interpreted as associations rather than evidence of direct effects of heavy metal concentrations on microbial functioning. In contrast, zinc (Zn) and lead (Pb), which were present at concentrations below regulatory thresholds, showed no clear association with enzymatic functioning.

3.2.5. Effect of viticultural management on extracellular enzymatic activity

Management practices was associated with secondary but detectable effect on EEA. Conventionally managed vineyards exhibited a 20% higher overall enzyme activity than organically managed vineyards (Fig. S14); however, differences associated with tillage were more pronounced than those associated with farming system alone. Enzymatic activity was consistently highest in the non-tilled- topsoils (0–10 cm) and lowest in the tilled subsoils (Fig. 3). Notably, β -glucosidase (BG), a key enzyme in cellulose degradation, exhibited the greatest variation, with its activity more than doubling in the upper non-tilled layer compared to that in the tilled subsoil. Carbon- and nitrogen-acquiring-enzymes showed the greatest sensitivity to soil disturbance, whereas phosphatase (PHOS) activity followed the same downward trend, albeit to a slightly lesser extent, suggesting a milder response of P-cycling enzymes to soil disturbance. The reduction in EEA associated with tillage was smaller in organic than in conventional vineyards. In conventional vineyards, tilled soils showed a significant reduction in EEAs (by 56%) compared with non-tilled soils, whereas in organic vineyards,

this decrease was less pronounced (by 18%). Nevertheless, the highest microbial N limitation was recorded in the organic non-tilled vineyard soils (Fig. S14). The vector angle was significantly lower (41° , $p = 0.004$). Similarly, the $EEC/(EEC + EEP + EEN)$ ratio was reduced in organic vineyard soils, indicating that N limitations in these soils were more pronounced than C limitations.

3.2.6. Enzymatic activity response to soil properties, topographic features and vineyard management

Multivariate modelling indicated that enzymatic patterns were associated with combined edaphic and site-related gradients rather than with a single dominant variable. In redundancy analysis (RDA) using enzyme activities as response variables and topography and soil properties as predictors, the constrained model explained $\sim 58\%$ of the enzymatic variability (Fig. 4, b), with the first canonical axis capturing the vast majority of the constrained inertia and the second axis contributing only a small additional fraction. In this framework, total organic nitrogen, clay content, and pH displayed the strongest

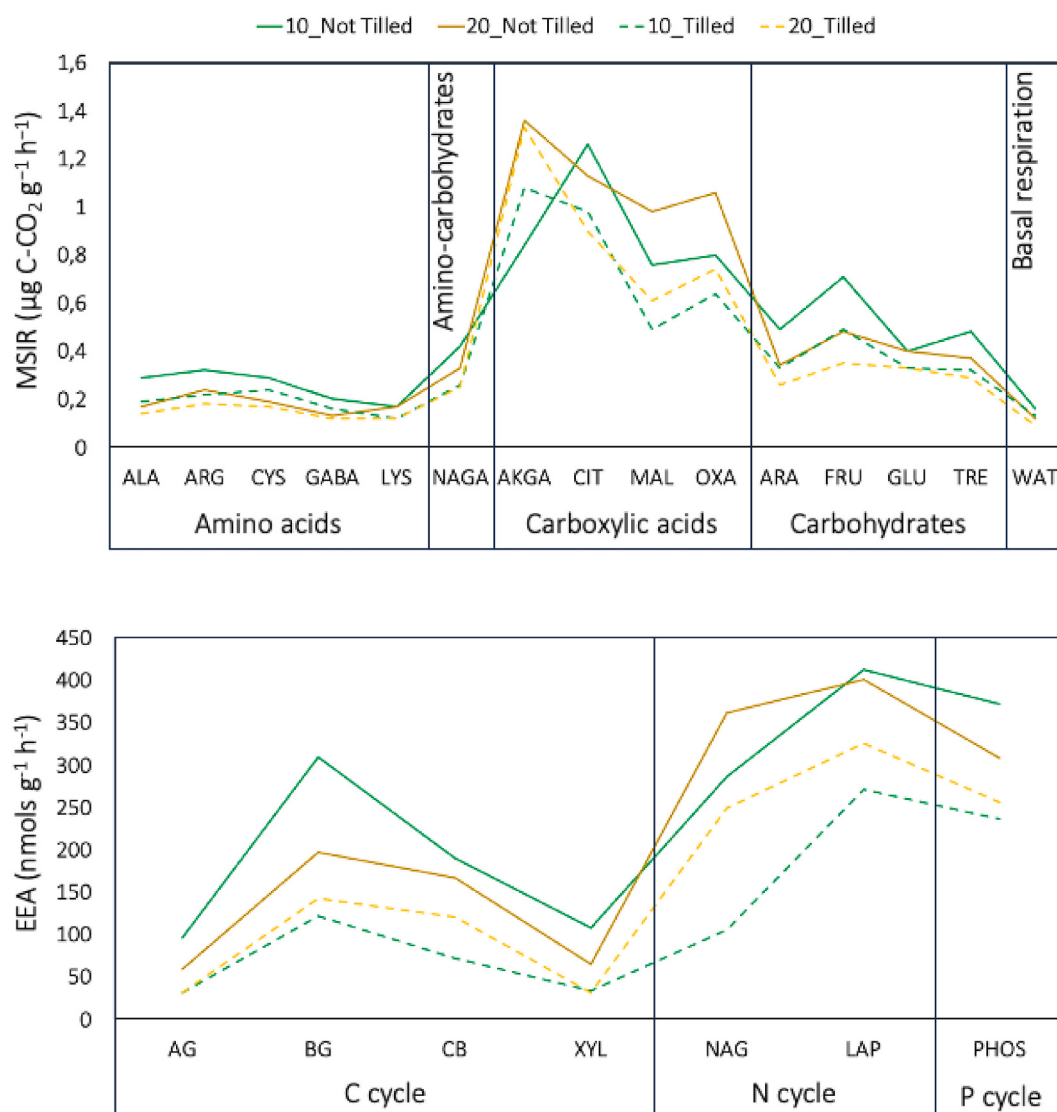


Fig. 3. Effects of soil depth and tillage on microbial functional activity in vineyard soils. (Top) Multiple substrate-induced respiration (MSIR) rates for 15 carbon substrates, grouped by biochemical category (amino acids, amino-carbohydrates, carboxylic acids, and carbohydrates), as well as basal respiration (WAT), in soils sampled at two depths (10 cm and 20 cm) and under two management regimes (tilled vs. non-tilled). (Bottom) Potential extracellular enzymatic activities (EEA) for seven enzymes associated with the carbon (AG, BG, CB, XYL), nitrogen (NAG, LAP), and phosphorus (PHOS) cycles across the same depth and tillage conditions. Non-tilled soils at 10 cm generally exhibited the highest MSIR and EEA, while tilled soils at 20 cm had the lowest activity, indicating that reduced soil disturbance and surface layers support greater microbial functional potential.

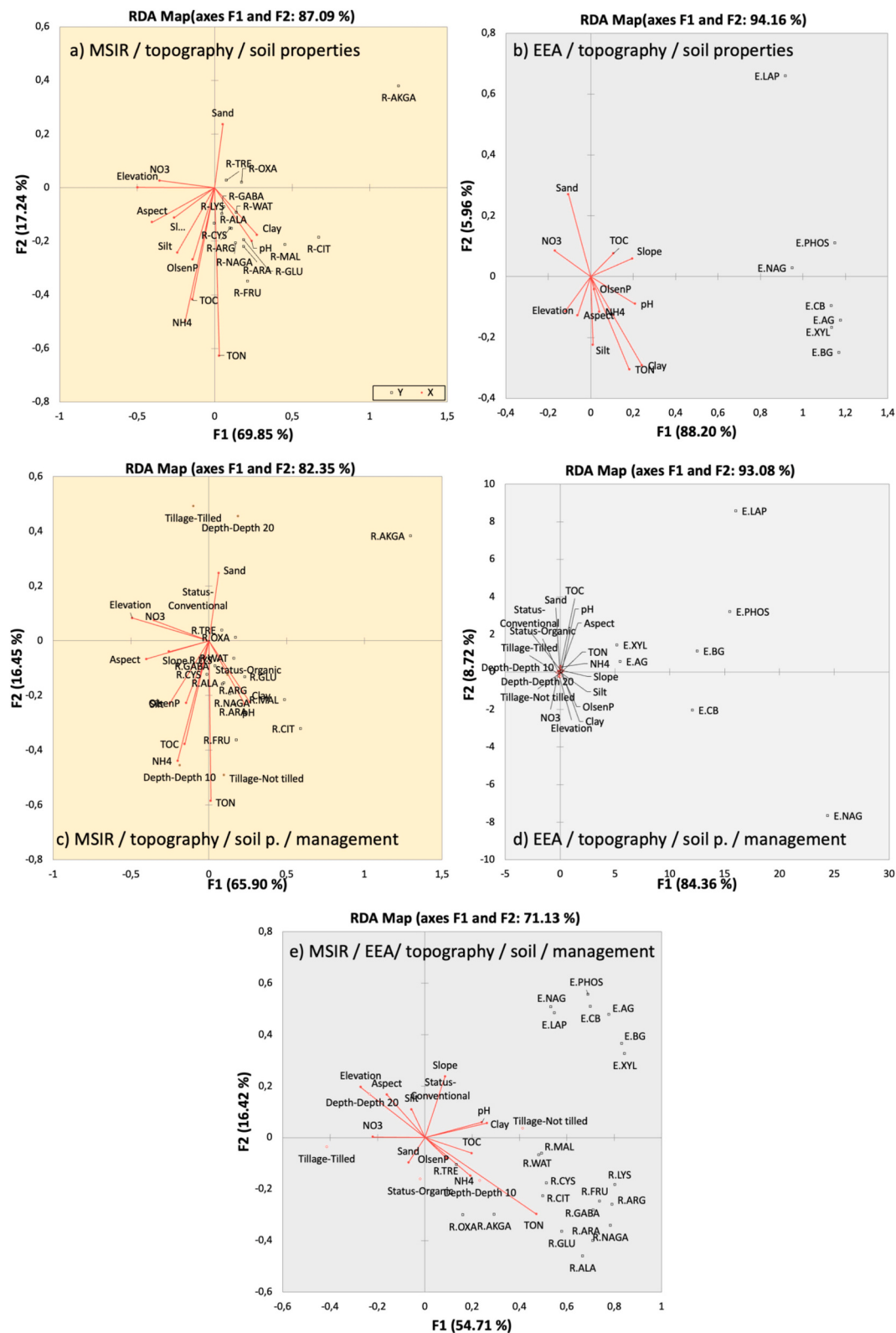


Fig. 4. Redundancy Analysis (RDA) ordination diagrams illustrating the relationships between microbial functional traits (extracellular enzymatic activity and substrate-induced respiration) and environmental variables across 40 vineyard soil samples from 9 micro-locations in two Slovenian wine-growing regions.

(a) and (c) display MSIR data (respiration on 15C substrates) in relation to topographic and soil physicochemical parameters (a). Additional site descriptors, including soil depth, vineyard management status (organic vs. conventional), and tillage regime (tilled vs. non-tilled) (c).

(b) and (d) illustrate the distribution of extracellular enzyme activities (EEA) with respect to topographic and soil physicochemical parameters (b). Additional site descriptors, including soil depth, vineyard management status (organic vs. conventional), and tillage regime (tilled vs. non-tilled) (d).

(e) integrates MSIR and EEA data with the full suite of environmental predictors, highlighting the relative influence of edaphic and management factors. Arrows represent significant explanatory variables ($p < 0.05$) with vector length proportional to their contribution to the explained variance on axes F1 and F2.

alignment with the primary gradient, whereas carbon-acquiring enzymes (BG, AG, XYL, and CB) were tightly clustered and separated from nitrogen- and phosphorus-acquiring enzymes (LAP, NAG, and PHOS), indicating coordinated responses within the elemental acquisition groups. In an extended RDA (Fig. 4, d) that additionally included management descriptors (depth, farming system, tillage), the overall explanatory power remained high (~58%), but no single predictor emerged as strongly directional. Most soil, topographic, and management vectors were short and positioned near the ordination origin, indicating that the enzyme variability was distributed across multiple weak gradients. Consistent with this, farming systems, tillage, and depth did not produce pronounced directional separation, suggesting that management variables were associated primarily with local variation within broader environmental gradients.

3.3. Multiple substrate induced respiration (MSIR), catabolic activity and functional diversity of soil microorganisms in vineyards

Multiple substrate-induced respiration (MSIR) revealed pronounced variability in microbial catabolic activity across vineyard soils, with values ranging from 0.04 to 3.29 $\mu\text{g C-CO}_2 \text{ g}^{-1} \text{ h}^{-1}$ (Fig. S3). Basal respiration (BR) and glucose-induced respiration (GIR) span a broad range and follow log-normal distributions, indicating a strong heterogeneity in microbial activity and biomass. The values of the metabolic coefficient ($q\text{CO}_2$) ranged from 0.12 to 1.88 $\mu\text{g C-CO}_2 \text{ g}^{-1} \text{ h}^{-1}$ similarly showed a log-normal distribution, while Shannon's functional diversity index (H') ranged between 2.15 and 2.70 and was normally distributed across vineyard soils (Fig. S3). These indicators provide complementary information on microbial physiological efficiency and functional versatility.

The catabolic physiological profile (CLPP) based on the amino acids, aminocarbohydrates, carbohydrates, and carboxylic acids utilization patterns demonstrated that carboxylic acids generated the highest CO_2 production rates, with AKGA and CIT showing mean activities of 1.13 ± 0.75 and $1.10 \pm 0.46 \mu\text{g C-CO}_2 \text{ g}^{-1} \text{ h}^{-1}$, respectively. In contrast, the utilization rates of carbohydrates, aminocarbohydrates, and amino acids were more uniform and markedly lower across vineyard soils (Fig. 2). These results indicate stronger respiratory responses of soil microbial functioning to carboxylic acid substrates than to the other substrate classes.

3.3.1. Effect of vineyard location on catabolic activity and functional diversity of soil microorganisms

In this study, clear spatial patterns emerged in soil microbial catabolic activity at both regional and microlocation scales. Vineyard soils from the Primorska wine-growing region showed, on average, 27% higher respiratory activity than soils from Podravje. This pattern was consistent with higher basal respiration and microbial biomass (GIR) measured in Primorska soils (Fig. S4). Within the regions, microlocation-specific differences were evident, with some sites displaying consistently elevated respiration across substrate groups, whereas others showed more constrained activity (Fig. 2). Despite these differences, higher microbial biomass and respiration at vineyard microlocations did not necessarily correspond to higher functional diversity or metabolic efficiency. In several microlocations, elevated BR and GIR coincided with lower $q\text{CO}_2$ values, indicating a more efficient microbial carbon use, whereas sites with moderate activity often exhibited the highest functional diversity. These patterns suggest that the microbial functional diversity is more closely associated with local environmental heterogeneity than with microbial biomass or catabolic activity.

3.3.2. Effect of topographic factors on catabolic activity and functional diversity of soil microorganisms

Topographic variables (elevation, slope, and aspect) were associated with measurable, but moderate, differences in microbial respiration.

Elevation and aspect were weakly and negatively correlated with MSIR and BR, respectively, indicating reduced catabolic activity at higher elevations and in west-facing vineyards (Table S6). In contrast, steeper slopes and northeast- or southwest-facing aspects were associated with higher respiratory activity (Fig. S15). Substrate-specific responses were largely stable across topographic classes, although carboxylic acid utilization declined with increasing elevation and slope. Functional diversity exhibited an opposite statistically significant trend (ANOVA, $p < 0.01$): H' increased with elevation, steeper slopes, and west-facing aspects, whereas $q\text{CO}_2$ declined, suggesting that microbial communities in more topographically constrained environments operate with greater metabolic efficiency and broader substrate use capacity despite lower overall activity. These results indicate a trade-off between the intensity of microbial activity and functional diversity along topographic gradients.

3.3.3. Effect of soil chemical properties on catabolic activity and functional diversity of soil microorganisms

Soil chemical properties were associated with differences in microbial catabolic activity and functional diversity. Across the texture classes, differences in basal respiration (BR) and the utilization of C substrates were modest (Fig. S17). In contrast, MSIR and microbial biomass increased with near-neutral pH. The extended neutral pH range (6.5–8.5) was associated with the highest MSIR and metabolic quotient ($q\text{CO}_2$ was 10% lower than that of vineyards with acidic soils ($\text{pH} < 6.5$). Within this pH range, the average Shannon's functional diversity index was significantly higher (ANOVA, $p < 0.001$) (Fig. S18). Similarly, the availability of Olsen P also had a range window (10–70 mg kg^{-1}), where soil MSIR, BR, and GIR were maximized (Fig. S18).

Higher total organic carbon (TOC) and elevated total organic nitrogen (TON) supported higher BR, MSIR, GIR, and H' , indicating that the microbial communities responded positively to improved nutrient and carbon resources. However, the AKGA response declined substantially with increasing TON and TOC for carboxylic acid substrates, suggesting that not all substrate groups responded uniformly to enhanced nutrient conditions. Regarding mineral nitrogen, varying concentrations of ammonium (NH_4^+) did not significantly affect BR or microbial biomass values, except for the carboxylic acid substrate AKGA, which showed a significant 74% decrease from the lowest to the highest NH_4^+ levels in vineyard soils. In contrast, elevated nitrate (NO_3^-) levels significantly increased $q\text{CO}_2$ while reducing microbial biomass (GIR) and MSIR in vineyard soils (Fig. S19). Reductions of 33–75% were observed across AKGA, OXA, MAL, and CIT, indicating that nitrate enrichment imposes a pronounced metabolic burden on microbial communities. Despite this, functional diversity increased with NO_3^- concentration (Pearson's $r = 0.31$, $p < 0.001$), suggesting that nitrate-rich vineyard soils support a broader, although less efficient, microbial substrate-use repertoire.

3.3.4. Effect of heavy metal content on activity and functional diversity of soil microorganisms

Elevated concentrations of Cu and Ni (Table S3) were associated with distinct microbial respiratory responses. Increased Cu levels were associated with a clear decline in the metabolic quotient ($q\text{CO}_2$) and a concurrent increase in functional diversity (H'), indicating that microbial communities exposed to elevated Cu levels had higher metabolic efficiency and broader functional capabilities. Basal respiration (BR) was 35% higher in soils with Cu concentrations above 60 mg kg^{-1} than in those with lower Cu levels (Fig. S22), and this pattern extended to MSIR responses across the carbon substrate groups. Carbohydrates and aminocarbohydrates showed particularly strong increases, with glucose and fructose utilization increasing by 40% and 37%, respectively, in Cu-contaminated soils. Carboxylic acids also exhibited enhanced utilization at Cu concentrations of approximately 100 mg kg^{-1} , particularly for AKGA (28%), MAL (19%), and OXA (14%). However, when Cu concentrations exceeded 150 mg kg^{-1} , MSIR levels decreased significantly across all carboxylic acid substrates (Fig. S22).

Similarly, Ni contamination (samples exceeding 70 mg kg⁻¹) was associated with higher microbial biomass (by 35%) and overall respiration but was associated with increased qCO₂ and 10% reduced functional diversity. Elevated BR and enhanced utilization of all substrate groups indicated that Ni exposure stimulated the overall microbial activity. The strongest increases were observed for carboxylic acids, such as AKGA, CIT, and MAL, and carbohydrates, such as ARA, FRU, and GLU. Several amino acids (ARG and LYS) showed increased utilization at elevated Ni concentrations (Fig. S22). Because these relationships were observed under field conditions, they should be interpreted as associations rather than evidence of direct effects of heavy metal concentrations on microbial functioning.

Zinc and lead, present at concentrations below regulatory thresholds, showed no consistent effects on microbial catabolic activity.

3.3.5. Effect of viticultural management on activity and functional diversity of soil microorganisms

Viticultural management practices was associated with measurable differences on microbial activity and functional organization in vineyard soils. Viticultural management was associated with differences in microbial respiration primarily through tillage and soil depth rather than through farming systems alone. Basal respiration did not differ markedly between organic and conventional vineyards, whereas the MSIR was higher in organically managed soils, particularly for carboxylic acid substrates. Notably, OXA, CIT, and MAL utilization increased by 32%, 18%, and 17%, respectively, in organic vineyards, indicating higher respiration responses to these C sources (Table S7).

Non-tilled soils consistently exhibited higher BR and MSIR, particularly for carboxylic acids (MAL, OXA, and CIT), particularly in the topsoil (Fig. 3). Despite tillage, qCO₂ differed by only 8% between treatments. However, the functional diversity of soil microbes (H') in tilled soils decreased significantly by 2% compared to that in non-tilled soils. The soil depth was also associated with differences in microbial responses. Both BR and MSIR declined substantially in the 10–20 cm layer, where the overall catabolic activity decreased by approximately 40% compared with that in the topsoil. Nonetheless, several carboxylic acids showed increased utilization in deeper soil horizons; AKGA, MAL, and OXA increased by 47%, 29%, and 27%, respectively. Despite these substrate-specific increases, functional diversity declined from 2.53 to 2.44 (H') with depth, indicating a reduction in microbial functional diversity in deeper soil profiles, even when individual substrates showed increased utilization.

3.3.6. Multiple substrate induced respiration (MSIR) response to soil properties and vineyard management

Redundancy analysis (RDA) was used to evaluate the combined associations of soil physicochemical properties, topographic factors, and vineyard management on microbial respiration profiles across carbon substrates (Fig. 4). The constrained model explained approximately half (49%) of the total variability in the MSIR responses, indicating that a substantial proportion of the variation in MSIR responses was associated with the measured environmental variables. The first canonical axis accounted for the majority of constrained inertia, whereas the second axis contributed a substantially smaller proportion, providing a robust low-dimensional representation of respiration patterns.

Within the ordination space, respiration responses to most substrates clustered tightly in the center-right, reflecting the overlapping patterns of microbial carbon utilization. In contrast, respiration induced by α -ketoglutaric acid (AKGA) was distinctly separated along the primary axis, indicating a unique response pattern that was weakly aligned with the measured variables. Among the explanatory variables, total organic nitrogen, soil pH, and clay content showed the strongest associations with the primary gradient expressing the strongest associations in regulating microbial respiration potential. Respiration responses were generally higher in soils with neutral pH and finer texture. In contrast, variables such as Olsen P, TOC, and NH₄⁺ contributed less strongly to the

constrained variation but still showed moderate alignment with microbial respiration to more labile compounds (e.g., FRU, NAGA, and ARG). However, nitrate availability and topographic variables showed weaker and less directional associations, respectively.

When vineyard management variables were included in the extended RDA model (Fig. 4, c), overall explanatory power increased only marginally. Soil depth, farming system, and tillage displayed short vectors and clustered near the origin of ordination, indicating a limited independent influence on respiration patterns. Nevertheless, non-tilled and organically managed soils tended to align with higher respiration rates for nitrogen- and carbon-rich substrates (e.g., R-NAGA, R-ARG, R-FRU, and R-GLU), suggesting a potential enhancement of microbial functional diversity under less-disturbed, organically managed conditions. Collectively, these results indicate that variation in MSIR was more strongly associated with soil physicochemical properties, particularly total organic nitrogen, pH and clay content, than with vineyard management variables. Management variables showed comparatively weaker associations with microbial respiration patterns.

3.4. Comparative analysis of origin versus vineyard management on soil biological activity

To synthesize the results obtained from individual enzymatic (EEA) and respiration-based (MSIR) analyses, an integrated multivariate approach was applied to evaluate how soil physicochemical properties, topographic characteristics, vineyard management, and soil biochemical functioning jointly structure vineyard soils. In the first step, the analyses aimed to identify which groups of environmental and management-related variables (topography, soil properties, or management) were most strongly associated with the observed microbial functional patterns. In the second step, the same integrated dataset was used to test whether vineyard soils could be grouped according to shared regional or microlocational origin by jointly considering soil properties, management descriptors, and microbial functional traits.

3.4.1. Integrated enzymatic and respiration profiling highlights stronger associations with edaphic and topographic variables than with vineyard management

In redundancy analysis (RDA) integrating both enzymatic and respiration-based variables, the constrained models captured a substantial proportion of microbial functional variability (Fig. 4, e). The first canonical axis explained the majority of the constrained inertia and was primarily associated with soil pH, total organic nitrogen (TON), total organic carbon (TOC), and clay content. Enzymatic activities related to carbon, nitrogen, and phosphorus acquisition (AG, BG, CB, XYL, LAP, NAG, and PHOS) were closely aligned with this gradient, along with respiration responses to several labile carbon substrates (e.g., ALA, ARA, GABA, GLU, and LYS). In contrast, management descriptors (organic vs. conventional, tilled vs. non-tilled, and soil depth) were represented by shorter vectors and weaker directional alignments, suggesting comparatively lower explanatory power than the measured soil physicochemical variables. However, their modest separation along the F2 axis suggests differentiation in the microbial responses. Organic management was associated with lower NO₃⁻ and moderate Olsen P levels. These variables were also associated with differences in microbial functional patterns, although their relationships were weaker than those observed for soil pH, TON and clay content. Tillage vectors (e.g., “not tilled”) pointed toward the enzyme and respiration groups, but showed weaker associations with the overall ordination structure than the main soil physicochemical variables.

These multivariate analyses identified variables that were most strongly associated with microbial functional variation across the studied vineyard soils; however, because the study was observational, these relationships should not be interpreted as direct causal effects.

3.4.2. Multivariate clustering reveals consistent patterns of microbial functioning among vineyard soils

Hierarchical clustering of standardized variables via heatmaps revealed the presence of four major soil sample clusters, each characterized by distinct biochemical profiles (Fig. S23). Although some clusters included samples from both wine-growing regions, the dominant pattern reflected differences associated with environmental conditions across vineyard sites. Cluster 4 consisted exclusively of samples from Primorska, many of which were organically managed. Overall, the clustering indicates that microbial functional profiles varied systematically among vineyard soils, while management practices appeared to contribute additional local-scale variability.

Principal component analysis (PCA) provided complementary support for these patterns (Fig. 5, a). The first two principal components together accounted for a substantial proportion of the total variance and clearly separated the vineyards from Primorska and Podravje, although partial overlap remained. This conclusion is further supported by the 3D PCA score scatter plot (Fig. S24), which adds PC3 (13.0% of the variance) to the rotated space. In this 3D representation, the samples from Podravje and Primorska formed two clearly distinguishable clusters. The third dimension offers improved spatial resolution, emphasizing the differences in sample positioning that may be less apparent in 2D images. The tighter regional grouping observed in the 3D plot, along with the minimal overlap between the origins, suggests a strong underlying structure in the data driven by both microbial function and soil characteristics. Together, these multivariate analyses indicate that EEA and MSIR measurements, when interpreted alongside soil physicochemical and topographic variables, provide complementary information for differentiating vineyard soils across environmental gradients.

To further explore the relationships among variables, we examined a PCA correlation circle plot (Fig. S25). Microbial functional indicators, including extracellular enzyme activities (e.g. PHOS, AG and XYL) and respiration responses to selected substrates (e.g. ARG, ARA and NAGA), were strongly associated with the first principal component, which explained 30.9% of the total variance. Their high $\cos^2$ values (>0.7) indicate that these variables contributed substantially to the primary axis of variation among vineyard soils. In contrast, classical edaphic variables, such as elevation, slope, texture components, and nutrient levels (e.g., NO_3 and Olsen P), were more aligned with PC2, which explained a smaller proportion (14.6%) of the total variance. This orthogonal orientation between microbial and edaphic vectors suggests that, while soil microbial functional indicators are partly influenced by site conditions, they also capture distinct ecological information that traditional soil analyses may overlook.

To visualize similarities among samples, we further applied t-distributed Stochastic Neighbor Embedding (t-SNE) combined with k-means clustering and convex hull visualization (Fig. 5b). This technique projected all 40 samples (20 vineyards, sampled at 10 and 20 cm depths) into a two-dimensional space, preserving the local neighborhood structure. The analysis identified five groups of vineyard soils with similar multivariate characteristics. Samples from Podravje generally formed more compact groups, whereas those from Primorska were more widely dispersed, reflecting greater environmental heterogeneity among the studied vineyard sites. As t-SNE is an exploratory visualization method, these results should be interpreted as supporting the observed multivariate patterns rather than as independent statistical confirmation of discrete soil groups.

Overall, the combined multivariate analyses demonstrate that extracellular enzyme activities and substrate-induced respiration capture consistent patterns of microbial functioning across vineyard soils. When interpreted together with soil physicochemical and topographic characteristics, these functional indicators provide biologically relevant information that may contribute to future *terroir*-related studies, while primarily reflecting differences in soil microbial functioning across environmental gradients.

4. Discussion

The results of this study revealed pronounced topographic, edaphic, and management-related variability across vineyard soils from the two major Slovenian wine-growing regions, Podravje and Primorska. Using rapid and cost-effective assays of extracellular enzymatic activity (EEA) and multiple substrate-induced respiration (MSIR), we identified consistent patterns of microbial functioning associated with contrasting environmental conditions. These findings highlight the potential of functional profiling as a practical approach for assessing soil microbial functioning and its response to environmental and vineyard management gradients.

4.1. Environmental factors

Environmental gradients in the studied vineyard sites were strongly associated with patterns of microbial functioning in the investigated soils. The sampling design included two climatically and topographically distinct Slovenian wine regions, Primorska and Podravje, which provided an opportunity to examine spatial variation in microbial functioning across contrasting environmental conditions. Vineyards in Primorska, located at lower elevations and characterized by warmer Mediterranean conditions and gentler slopes, contrasted sharply with Podravje sites, which were positioned higher, featured steeper gradients, and experienced more rainfall. Because these environmental characteristics co-varied between the two wine-growing regions, the observed differences should be interpreted as reflecting multiple interacting environmental gradients rather than regional identity alone. This broad environmental variability was further enriched by soil heterogeneity, ranging from acidic to alkaline pH, diverse phosphorus status, and variable organic matter content. Occasionally elevated concentrations of heavy metals, such as copper and nickel, particularly in older or intensively managed vineyards, introduce additional complexity to microbial habitat conditions.

Patterns of microbial functioning associated with these environmental gradients were reflected in both enzymatic activity (EEA) and multiple substrate-induced respiration (MSIR) rates. Primorska soils exhibited significantly higher average EEAs than Podravje soils, particularly for the carbon-acquiring enzymes (xylanase (XYL) and α -glucosidase (AG)). Similarly, the MSIR rates were 27% higher in Primorska soils. The elevated enzymatic and respiratory activity aligns with patterns in enzyme stoichiometry, which revealed widespread nitrogen limitation (mean C-acquiring to N-acquiring enzyme ratio, EEC:EEN, < 1). This trend has been observed in other studies, where organic matter enrichment often results in carbon-rich but nitrogen-poor substrates that trigger microbial investment in nitrogen-acquiring enzymes (Chen et al., 2022; Cui et al., 2023; Sinsabaugh et al., 2009). Enzyme stoichiometry further revealed phosphorus limitation emerged predominantly at higher elevations or in soils with low Olsen P. Such stoichiometric trends are consistent with established observations in soil microbial communities, where increased C:P enzyme acquisition ratios, comparable to the C:N imbalances reported here, often coincide with nutrient constraints (i.e., elevated enzyme acquisition of P relative to C and N content). Global analyses have shown that enzyme C:P ratios increase in ecosystems where phosphorus becomes limiting (Sinsabaugh et al., 2008). While such stoichiometric imbalances are commonly observed in systems with organic matter enrichment, another possible explanation for the elevated microbial activity in Primorska is the region's higher mean soil temperatures and longer growing season, which may prolong microbial metabolic windows and the turnover of extracellular enzymes, microbial metabolism, and extracellular enzyme production by enhancing enzymatic kinetics and substrate turnover (German et al., 2012; He et al., 2025; Schneckner et al., 2023). Additionally, gentler topography and reduced erosion risk may help maintain more stable microsite conditions and substrate availability, further stimulating microbial function (Qiu et al., 2021; Wang et al., 2024b; Yang et al., 2024).



Fig. 5. Multivariate clustering of vineyard soil samples based on microbial, topographic and physicochemical properties.

(a) Principal Component Analysis (PCA) illustrating the distribution of vineyard soil samples across environmental gradients associated with the two Slovenian wine-growing regions (Podravje = red, Primorska = green), with PC1 (30.9% of variance) was primarily associated with microbial functional indicators, whereas PC2 (14.6%) reflected variation related to topographic and soil physicochemical properties.

(b) t-distributed Stochastic Neighbor Embedding (t-SNE) plot with k-means clustering and convex hulls visualizing five data groupings identified by k-means clustering among the 40 vineyard soil samples. Cluster patterns reflect the strong influence of regional origin and underline the differentiation of microbiological and soil characteristics between Podravje and Primorska regions. Variables with high $\cos^2$ values (e.g., PHOS, AG, XYL, NAG, GLU, ARG) contributed most to sample separation. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

These environmental advantages may partially mask or compensate for nutrient limitations, resulting in elevated EEA and MSIR despite signs of stoichiometric imbalance. Interestingly, steeper slopes were associated with higher microbial activity in the present study, potentially reflecting nutrient redistribution through erosion or runoff, or greater microsite heterogeneity that may favour niche differentiation, as reported for mountain ecosystems (Massaccesi *et al.*, 2023).

Multivariate analyses further supported the observed associations between microbial functioning and environmental gradients. Both enzyme activity (EEA) and respiration rate (MSIR) were strongly associated with soil total nitrogen, organic carbon, clay content, slope (>25%), and pH, which is consistent with previous findings in agricultural systems (Amarasinghe *et al.*, 2024; Bublit *et al.*, 2025; Martin and Bolstad, 2009; Xu *et al.*, 2020). Higher clay content may provide an enhanced surface area for microbial colonization and better moisture retention, facilitating enzyme stability and substrate diffusion (Islamzade *et al.*, 2025). Meanwhile, microbial functioning showed a non-linear relationship with soil pH - optimal microbial activity occurring near neutral pH and a decline observed in both acidic and strongly alkaline soils, in line with the pH sensitivity of microbial biomass and enzyme kinetics reported in the soil ecology literature (Tripathi *et al.*, 2018; Wang and Kuzyakov, 2024).

Overall, the RDA results indicate that microbial functioning was closely associated with multiple interacting topographic and edaphic variables. Because these environmental characteristics co-vary across vineyard sites, the observed regional patterns likely reflect the combined influence of several environmental gradients rather than geographic location alone.

4.2. Vineyard management practices

Although their influence appeared secondary to broader environmental gradients, vineyard management practices were associated with measurable differences in soil microbial functioning. In this study, a comparison between organic and conventional vineyards revealed nuanced patterns of microbial respiration, enzyme activity, and diversity indices. Although basal respiration (BR) did not differ significantly between management systems, microbial communities in organically managed soils exhibited higher overall respiratory potential when evaluated using multiple substrate-induced respiration (MSIR), particularly in the case of carboxylic acids. While this may indicate that organic practices support microbial functioning with broader substrate utilization potential (Wang *et al.*, 2017; Wang *et al.*, 2024a), elevated carboxylic acid respiration could also reflect microbial responses to mild stress or nutrient imbalances (MacLean *et al.*, 2023; Yuan *et al.*, 2019). Carboxylic acids are among the first substrates utilized during microbial adaptation to environmental stress. Previous studies have shown that organic farming can enhance microbial abundance, diversity, and activity, and these communities are better equipped to adapt to stress by shifting toward immediate energy acquisition and resource use, particularly when organic inputs provide diverse and readily available substrates (Breitkreuz *et al.*, 2021; Mäder *et al.*, 2017). Thus, both enhanced microbial functional potential and subtle signs of stress adaptation may underlie the observed patterns.

Interestingly, microbial functional diversity, as measured by the Shannon index (H'), was marginally, but significantly, higher in conventionally managed soils. Similarly, overall enzymatic activity was also higher in conventional vineyards, indicating that conventional management did not suppress microbial functioning uniformly. This pattern may partly reflect differences in nutrient availability between systems, as mineral fertilization can increase the availability of labile nutrients and stimulate extracellular enzyme production involved in C, N, and P cycling (Solangi *et al.*, 2024). In addition, pesticide residues and other agrochemical inputs may alter microbial community structure and functional traits, potentially selecting for stress-tolerant or opportunistic microorganisms with broader metabolic capabilities (Königer

et al., 2026). Under such conditions, increased enzyme activity may also represent a stress-induced functional response rather than an unambiguous indicator of improved soil biological quality. One explanation for this finding could be that conventional systems, which often experience more frequent disturbances and chemical amendments, may favour broader but less stable community structures composed of opportunistic taxa (Srou *et al.*, 2020). Moreover, conventional management may provide a variety of substrates (fertilizers, crop residues, and root exudates) that support a broader range of microbial metabolic functions, as reflected by a higher Shannon index (Seitz *et al.*, 2021; Tang *et al.*, 2020). This is supported by the observation that qCO_2 , an indicator of microbial metabolic efficiency, remained similar across systems, suggesting that differences in microbial functional characteristics, rather than overall activity levels, contributed to the observed diversity patterns.

In contrast, tillage appeared to have a stronger association with microbial functioning than farming system alone. Both MSIR and enzymatic activity decreased markedly in tilled soils, particularly in deeper soil layers. Tillage disrupts soil aggregates, alters porosity, and disturbs microbial habitats, thereby reducing the continuity of organic matter and substrate availability (Mondal and Chakraborty, 2022; Six *et al.*, 1999). The more pronounced suppression of enzyme activity in conventionally managed tilled soils compared to their organic counterparts highlights the compounding impact of physical disturbance combined with lower organic matter input. In contrast, organic systems appeared more resilient, showing smaller functional losses under tillage, likely because of increased organic carbon availability and lower mineral nitrate concentrations, as observed in organically managed soils. Similar results were reported by (Wang *et al.* (2017), Bongiorno *et al.* (2020), and Kovács *et al.* (2024), who found that organic management supported more resilient microbial communities.

In terms of nutrient cycling, the microbial enzyme profile revealed the potential for nutrient limitation depending on the management regime. Carbon limitation was most evident in organically tilled soils, as indicated by the low ratios of carbon-acquiring enzymes relative to nitrogen- and phosphorus-acquiring enzymes. Conversely, nitrogen limitation dominated in organic no-till soils, possibly reflecting the slow release of organic nitrogen sources that are typical in these systems (Niedziński *et al.*, 2021). These patterns suggest differences in microbial resource allocation strategies wherein communities modulate their enzymatic investment in response to nutrient availability and demand. Similar findings have been reported in other perennial systems (Cui *et al.*, 2018; Li *et al.*, 2016; Seitz *et al.*, 2021), where enzyme allocation shifts according to the stoichiometric imbalances induced by management intensity.

While agricultural practices (e.g., tillage and farming status) did exhibit some influence, particularly for certain nitrogen- and carbon-related respiration traits, their explanatory power was modest and largely secondary to intrinsic soil conditions. The inclusion of management variables in the extended RDA models added clarity to the ordination but contributed only incrementally to the total variance. Management variables were associated primarily with local differences in microbial functional responses rather than override the dominant environmental constraints imposed by soil formation and regional climate. This observation is in contrast with the findings of the broader literature, including the review by Visconti *et al.* (2024), which emphasizes that sustainable agricultural practices, such as reduced tillage, cover cropping, compost application, crop rotation, and organic fertilization, can enhance soil microbial abundance, diversity, and activity by improving nutrient availability, organic matter content, and soil structure, while reducing chemical disturbances. However, our study indicates that Slovenian organic vineyards, despite being under organic management for at least five years, do not follow this trend. Multivariate analysis did not reveal a substantial increase in microbial activity attributable to the organic status. This discrepancy may be attributed to regional environmental constraints or legacy effects from prior vineyard

management practices, highlighting the complexity of translating the generalized benefits of organic agriculture into consistent improvements in microbial functioning across contrasting vineyard environments. Overall, the results support the view that reduced tillage and organic practices create a more favorable environment for sustaining microbial activity, resilience, and nutrient-processing capacity. Although conventional systems may temporarily support high enzymatic potential under certain conditions, these results do not necessarily indicate greater soil biological quality and may instead reflect stress-related functional responses. These insights are consistent with the broader literature, indicating that conservation-oriented practices such as cover cropping, organic amendments, and minimal tillage enhance microbial biomass, enzyme activity, and soil health in vineyards and other agroecosystems (Huber et al., 2024; Rathore et al., 2023; Wen et al., 2023).

4.3. Soil microbial functioning within the *terroir* framework

Understanding the concept of *terroir* in viticulture (Van Leeuwen and Seguin, 2006) increasingly requires attention to the biological dimensions of soil, particularly the role of soil microbial communities. Traditionally, the concept of *terroir* have been explained by a combination of soil physicochemical properties, topography, and climatic factors. However, recent studies have highlighted that soil microorganisms and their functional characteristics contribute significantly to the distinctiveness of vineyard ecosystems (Knight et al., 2020). This discussion explores how simple microbiological assays, specifically ecoenzymatic activity (EEA) and substrate-induced respiration (MSIR), can be used to capture patterns of microbial functioning associated with contrasting vineyard environments.

Using multivariate analyses, including PCA, hierarchical clustering, and t-SNE projection, we found that microbial functional variables derived from EEA and MSIR consistently differentiated vineyard soils across contrasting environmental conditions. The consistent grouping observed across several exploratory multivariate analyses of vineyard soils suggests that microbial functioning patterns are closely aligned the combined environmental characteristics of the vineyard sites. Although soil texture, slope, and nutrient availability influence microbial function (as discussed above), microbial data provides complementary biological information that may contribute to *terroir*-related studies (Flörl et al., 2025). This is especially clear when examining PCA correlation structures, where microbial indicators, such as phosphatase, α -glucosidase, and glucose- and arginine-induced respiration, are more strongly associated with the dominant axes of variation than with many classical soil parameters. Similarly, Fernandez et al. (2016) reported that differences in soil function could be better explained using both soil physicochemical test values and microbial structure data than using soil tests alone. In our study, microbial functional variables with high $\cos^2$ values underscored their contribution to explaining variability and highlighted their value as sensitive indicators of site specificity. Microbial functioning may contribute to feedbacks that modify soil conditions through biochemical and biophysical mechanisms, creating feedback loops that further differentiate soil environments from those measured using classical parameters (Philippot et al., 2024).

One of the most important findings of this study was that simple functional assays consistently differentiated vineyard soils across both regional and local environmental gradients. Enzymatic profiles (EEA) and respiration responses (MSIR) varied consistently between the Primorska and Podravje regions, and were further differentiated at the microlocation level. These spatial patterns in microbial functioning were consistently visualized across PCA, hierarchical clustering, and t-SNE analyses and were further supported by redundancy analysis, indicate that microbial functioning captures biologically meaningful variation among vineyard soils, even without molecular community analyses. Studies using advanced sequencing and machine learning approaches have similarly revealed that microbial signatures, especially those of fungi and bacteria, can distinguish vineyards at both regional and local

scales and that these microbial patterns are linked to differences in grape and wine chemistry, as well as sensory profiles (Flörl et al., 2025). For example, research using long-read sequencing in Portuguese vineyards has established unique microbiome signatures for each *terroir*, identifying indicator species and functional profiles that serve as microbial fingerprints to distinguish one site from another (Cruz-Silva et al., 2023). Our results complement these sequencing-based studies by demonstrating that functional assays capture biologically meaningful variation among vineyard soils, although they do not resolve microbial community composition or taxonomic diversity. Vineyard microlocations with higher functional potential (e.g., Planina, Ritoznoj) exhibited enhanced ecoenzymatic stoichiometry and respiration across a wide range of C substrates, whereas others (e.g., Loze, Mance) reflected severe microbial nutrient constraints. Such metabolic signatures mirror the 'microbial fingerprints' reported in sequencing-based *terroir* studies and support the view that microbial functioning represents an important biological component of *terroir*.

Functional assays provide a practical approach for assessing the biological component of *terroir* through microbial functioning. Their integration into *terroir*-related studies provides not only a clearer picture of how soils differ across regions, but also evidence that microbial dynamics are not secondary to *terroir* but are a fundamental part of it. Recognizing this, future *terroir* frameworks could benefit from incorporating microbial functioning alongside traditional soil and topographic descriptors to capture the biological identity of vineyard landscapes more fully, as proposed by Franco et al. (2024). By focusing on functional assays such as EEA and MSIR, we can capture meaningful ecological differences without relying on expensive molecular tools for analysis. This makes soil microbial analysis accessible for routine vineyard monitoring and sustainable viticulture management.

The present study has several potential limitations that should be acknowledged. Although EEA and MSIR effectively characterize soil microbial functioning, these methods do not provide information on microbial taxonomic composition or community diversity and therefore cannot replace molecular approaches such as amplicon sequencing or metagenomics. Moreover, functionally similar microorganisms may perform comparable ecological roles (functional redundancy), meaning that similar functional profiles may arise from different microbial communities (Ramond et al., 2025). Furthermore, extracellular enzyme activities and substrate-induced respiration represent potential microbial functioning measured under standardized laboratory conditions and therefore do not necessarily reflect in situ ecosystem process rates. Finally, because this study was observational the reported relationships should be interpreted as associations rather than causal relationships, and the generality of these findings should be validated across additional viticultural regions encompassing contrasting environmental conditions and management systems. Future studies integrating functional assays with molecular approaches across broader geographic scales will further strengthen our understanding of the biological component of vineyard *terroir*.

5. Conclusion

This study demonstrated that ecoenzymatic activity (EEA) and substrate-induced respiration (MSIR) assays are practical and robust tools, for assessing soil microbial functioning across contrasting vineyard soils. Using low-cost and rapid enzymatic activity assays and respiration profiling, we captured consistent patterns of microbial functioning across contrasting vineyard environments. The EEA and MSIR results were closely associated with environmental gradients across vineyard soils. Soils with a higher pH, clay content, and total organic nitrogen consistently exhibited higher enzymatic activity and microbial respiration rates. For example, EEA was the highest in neutral to slightly alkaline soils, and MSIR responses were elevated in clay-rich profiles with higher nutrient availability. Topographic factors, such as slope and elevation, influence microbial functional diversity, with

steeper and elevated sites showing lower biomass and higher substrate-use diversity. Management effects were also evident; tillage led to a 56% decline in EEA in conventional systems, whereas organic systems exhibited only an 18% reduction. Organic vineyards also had 12% higher MSIR rates and greater stability in the microbial response across microlocations. The ability of EEA and MSIR to capture spatial patterns of microbial functioning provides complementary biological information that may support *terroir*-related studies alongside traditional soil, climatic and topographic assessments.

Our results showed that soil physicochemical properties were more strongly associated with microbial functioning than vineyard management practices. Redundancy analysis revealed that pH, TON, and clay content explained up to 58% of the variation in enzymatic activity and 56% of the variation in substrate-induced respiration (SIR). Topographic variables, such as elevation and slope, contributed significantly, but to a lesser degree, to influencing patterns, particularly at upland sites. Management practices, including tillage and organic versus conventional systems, affected microbial activity (e.g., MSIR rates were 12% higher in organic soils and EEA declined by 56% in tilled conventional soils); however, their influence was secondary to soil properties and terrain. These findings are consistent with sequencing-based studies reporting that soil pH, texture and nutrient availability are closely associated with microbial community composition and function. While our study did not assess microbial taxonomic composition, the observed functional patterns are consistent with these broader ecological relationships.

As modern viticulture increasingly demands precision, sustainability and authenticity, there is a pressing need for reliable tools that can guide vineyard decision making. Accessible microbiological assays, such as EEA and MSIR, meet this need by providing clear functional indicators of soil microbial health and activity. These methods allow vineyard managers to assess site-specific microbiological processes that underpin *terroir* expression, offering insights into nutrient cycling efficiency, soil function, and ecological responses of microbial communities to environmental and management conditions. Eoenzymatic stoichiometry and respiration-based functional diversity offer practical alternatives for wineries where financial, technical, or infrastructural constraints may limit access to advanced techniques. This approach highlights the value of functional microbial indicators for soil health assessment and sustainable vineyard management while providing complementary biological information for future *terroir*-related research. Although EEA and MSIR do not replace molecular approaches for characterizing microbial community composition, their simplicity, affordability, and sensitivity make them valuable tools for routine soil monitoring and for integrating biological information into vineyard management and future *terroir*-related studies.

CRedit authorship contribution statement

Erika JEZ: Writing – original draft, Visualization, Methodology, Funding acquisition, Data curation, Conceptualization. **Jan RESCIC:** Writing – original draft, Methodology, Formal analysis, Data curation. **Lorena BUTINAR:** Writing – review & editing, Supervision, Methodology, Data curation. **Adrian HERMES:** Writing – original draft, Visualization, Software, Data curation. **Elisa PELLEGRINI:** Writing – review & editing, Visualization. **Marco CONTIN:** Writing – review & editing, Supervision, Methodology, Funding acquisition, Conceptualization.

Consent for publication

Not applicable.

Ethics approval and consent to participate

Not applicable.

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Declaration of competing interest

The authors declare that they have no affiliations with or involvement in any organization or entity with any financial interest in the subject matter or materials discussed in this manuscript.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.apsoil.2026.107331>.

Data availability

Data will be made available on request.

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