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Soil microbiome and phytochemical responses to a decade of compost and biochar amendments in an olive orchard

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ABSTRACT

Long-term field experiments provide key insights into whether organic amendments can enhance soil health and the sustainability of Mediterranean perennial cropping systems. However, their persistent effects on soil microbial communities and crop quality remain insufficiently understood, especially under semiarid Mediterranean conditions. This research assessed the long-term effects of repeated compost and biochar applications on soil organic matter, microbial communities, and the nutritional and phytochemical status of a mature olive orchard (*Olea europaea* L. cv. Arbequina). Four treatments based on biennial organic amendments over a decade were evaluated: (i) control, (ii) compost, (iii) compost-biochar mixture (90:10, dry weight), and (iv) biochar. Soil microbiome analysis via high-throughput sequencing of the 16S rRNA and ITS regions revealed no lasting alterations after four applications over ten years compared with the control without amendment. However, high-resolution seasonal sampling after the fifth amendment indicated distinct, primarily fungal, community shifts. Yet recovery to the original state highlights the resilience of these microbial communities to anthropogenic inputs. In contrast, fruit quality and phytochemicals in olives were more sensitive to soil management practices, with organic amendments notably influencing oleuropein concentrations in olive fruits (up to 24% lower than the control; $p < 0.05$, $\eta^2 = 0.79$), while yields remained stable across treatments. This decade-long study reveals that established Mediterranean olive orchards possess a highly resilient soil microbiome that resists permanent shifts despite repeated organic amendments. However, the sensitivity of secondary metabolites—specifically oleuropein—to these practices suggests that adding phytochemical indicators provides a high-sensitivity metric for monitoring "soil health" in semi-arid perennial cropping systems. By simultaneously assessing soil, microbial, and phytochemical data over 11 years, this research provides a unique, system-level reference that captures management impacts often missed by traditional soil or yield metrics.

1. Introduction

Feeding a growing global population while safeguarding environmental integrity is one of the most pressing challenges of our time (Hunter et al., 2017). Climate change further exacerbates this challenge, threatening agricultural productivity in many regions. Meeting future food demands requires resilient soils that can support high yields while minimizing environmental costs. Recycling organic residues as soil amendments is a promising strategy to maintain soil organic matter, enhance nutrient cycling, buffer climate impacts, and address the challenge of managing large volumes of residues (Kaya et al., 2024; Okur et al., 2025). Compost application not only reduces and sanitizes

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Abbreviations: ASV, Amplicon Sequence Variant; CH, Carbohydrates; DOC, Dissolved Organic Carbon; EC, Electrical Conductivity; FA, Fatty Acids; LOD, Limit of Detection; LOQ, Limit of Quantification; MUFA, Monounsaturated Fatty Acids; PUFA, Polyunsaturated Fatty Acids; SFA, Saturated Fatty Acids; WSN, Water Soluble Nitrogen.

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organic waste but also improves soil quality and functionality (Zhang et al., 2019). Similarly, biochar - a carbon-rich material produced through the pyrolysis of organic feedstocks under oxygen-limited conditions - has emerged as a tool to enhance long-term soil carbon sequestration, mitigate greenhouse gas emissions, and potentially improve soil health (Lehmann et al., 2011; Xu et al., 2023).

Soil microbes are central to these processes, and their diversity and functional redundancy strongly influence the long-term sustainability of agroecosystems (Allison and Martiny, 2008; Okur et al., 2025). Organic amendments can substantially reshape these microbial communities (Barra Caracciolo et al., 2015; Gul et al., 2015), but the magnitude and direction of these responses depend heavily on soil type, organic amendment quality, and climatic context (Barra Caracciolo et al., 2015; Rodríguez-Berbel et al., 2020; Singh et al., 2022; Xu et al., 2023; Yang et al., 2025). Long-term field trials are crucial for understanding persistent changes in microbial networks, but they remain scarce, particularly in calcareous Mediterranean soils.

Mediterranean agriculture is strongly defined by the cultivation of the olive tree (*Olea europaea* L.), which accounts for over 80% of global olive-growing areas (FAOSTAT, 2023). Olive tree fruits and leaves are rich in bioactive phytochemicals, such as polyphenols and triterpenes, known for their nutritional and pharmaceutical value (Galmés et al., 2021). These health benefits arise largely from their antioxidant properties linked to reduced risks of cardiovascular disease and cancer (Tuck and Hayball, 2002). In plants, these compounds often accumulate in response to stress (Kishor et al., 2023; Sánchez-Rodríguez et al., 2011, 2019), and several studies indicate that high nutrient availability reduces the content of these compounds in plant tissues (Verardo et al., 2013). Notably, this metabolic plasticity is often more pronounced in fruits than in leaves, as they act as metabolic sinks highly sensitive to shifts in resource allocation (Schneider et al., 2021). Phenolic concentration in olive fruits varies with cultivar, pedoclimatic conditions, farming techniques, and ripening stage (Pierantozzi et al., 2025). However, little is known about how long-term compost or biochar amendments impact the accumulation of these compounds in plant tissues. At the same time, resilient microbial communities can sustain nutrient availability under stress, supporting stable olive yields and potentially shaping phytochemical expression. Valorizing these bioactive compounds adds an extra dimension to the sustainability of olive production systems.

Taking into consideration the potential for sustainable olive orchard production, a long-term field experiment was established in 2013 in a semiarid organic olive orchard. The study compares the application of compost, biochar, and their mixture, with a non-treated control. In previous studies, we observed significant short-term and mid-term effects on soil nutrient availability and microbial activity (Fornes et al., 2024; Sánchez-García et al., 2016), but only minor responses in the tree nutritional status and crop yield. This long-term evaluation bridges the gap left by shorter studies, capturing the slow-evolving physiological responses inherent to perennial woody systems. Unlike controlled microcosm studies, this in-situ research addresses the system-level dynamics of long-lived perennial systems. After a decade of biennial applications, we examined soil microbial communities (archaea, bacteria, fungi), crop yield, plant nutritional status, and the phytochemical composition of fruits and leaves. Our primary objectives were: (1) to assess the efficacy of organic amendments for sustainable management in calcareous Mediterranean soils; (2) to evaluate the impact of long-term nutrient shifts on olive fruit quality, specifically its bioactive profile; and (3) to advance the conceptual understanding of soil-plant-microbiome interactions and their resilience under dominant environmental constraints. We hypothesized that i) organic amendments would increase microbial diversity and alter community structure by providing nutrients and niches for specialized microbial taxa compared to unamended soil, ii) compost and the blend of compost with biochar would enhance productivity due to sustained improved nutrient availability, and iii) the concentration of bioactive compounds would decrease with increased nutrient availability because of the associated reduced plant stress.

2. Materials and methods

2.1. Experimental set-up and localization

A randomized field plot design was established in 2013 in an organic olive orchard (cv. Arberquina), comprising four treatments with three replicates each: (i) no amendment (control), (ii) compost, (iii) a mixture of compost and biochar at a 90:10 ratio (dw:dw), and (iv) biochar. Each experimental plot covered an area of 168 m^2. In total, 12 plots were arranged and separated by buffer rows of olive trees to avoid treatment interference. The experiment is located in the commercial organic olive grove farm “SAT Casa Pareja” in Jumilla, Spain (38°23′N; 1°22′W). At the onset of the experiment, the orchard had been under organic management for 15 years, and the olive trees were 20 years old. The long-term experimental timeline (2013–2024) is detailed in Figure S1. This area has a semi-arid Mediterranean climate characterized by low annual rainfall, mainly during autumn and spring, with maximum temperatures during the dry summer season. The soil is classified as a Haplic Calcisol (IUSS et al., 2022), composed of 57% sand, 16% clay, with 30% carbonate, 1.31% total organic carbon (TOC), and a pH of 8.12. More details about the experimental site are available in Sánchez-García et al., (2016).

The amendments were applied every two years, covering a 1-meter width on each side of the tree, along the irrigation pipelines, at a dose of 20 Mg ha^{-1} (equivalent to 6 Mg ha^{-1} considering the whole plot area). This involved five repeated applications over ten years of the experiment (in 2013, 2015, 2017, 2021, and 2023). An operational gap in soil amendments occurred between 2019 and 2020 as a consequence of the funding transition between projects and logistical constraints during the COVID-19 pandemic. The orchard is managed under certified organic standards, which preclude the use of synthetic pesticides or herbicides. It is maintained with minimum tillage (three times per year) and uses deficit irrigation (drip irrigation during summer). Pruning practices follow a three-year triennial cycle: in the first year, a heavy winter pruning is performed; in the second year, a lighter summer pruning is conducted to remove water sprouts (suckers); and in the third year, the trees are left unpruned to minimize intervention.

Composts were prepared annually on the farm by mixing two-phase olive mill waste (50% dw), sheep manure (25% dw), and olive tree pruning waste (25% dw) in windrows, with regular temperature and moisture monitoring over six months, following a standardized protocol. The compost composition showed minor interannual variability, ranging from 33% to 36% C and 2.3–2.4% N. Biochar used in 2013, 2015, and 2017 was provided by a local supplier from holm oak wood, under the framework of the EU project FERTIPLUS, with a 67.3% C and 0.84% N content. In 2021 and 2023, biochar was produced from vineyard pruning, with a 47.5% C and 0.54% N content, under the framework of the EU project EIT Food – Black to the Future. Both biochars were derived from woody species and produced by slow pyrolysis at 600–650°C, ensuring similar properties and functional consistency. A detailed description of the applied amendments is provided in previous studies (Fornes et al., 2024; Sánchez-García et al., 2016; Sánchez-Monedero et al., 2019).

2.2. Soil sampling and analyses

Soil and orchard conditions were assessed 10 years after the experiment began, following four applications of organic amendments. Soil was sampled at five time points: the first in May 2023, immediately before the fifth amendment, corresponding to Year 10, and then seasonally (every three months) over the subsequent year, capturing the short-term effects of the last amendment across key phenological stages (flowering, fruiting, and harvest). At each time point, four topsoil cores

(0–20 cm) were collected from the amended area of each plot, 1-meter width along the irrigation lines, composited, sieved (2 mm), homogenized, and stored at −20 °C until analysis. Depth was selected to target the topsoil layer, where the incorporated amendments had direct interaction with the irrigation-induced moisture.

2.2.1. *Physicochemical analyses*

Ball-milled subsamples were analyzed for C and N using a LECO CHNS-932 elemental analyzer. Soil organic C was measured after pretreatment with 2 N HCl to remove inorganic C. NO_3^--N, water soluble nitrogen (WSN) and water dissolved organic carbon (DOC) were determined in water extracts (1:10 dw/v), while NH_4^+-N was determined in 2 M KCl extracts (1:10 dw/v). All extracts were shaken for 2 h, centrifuged (15 min at 2740 x g), and filtered (0.45 µm). NO_3^- was quantified by ion chromatography (HPLC, model 861, Metrohm AG, Herisau, Switzerland). NH_4^+ was measured colorimetrically using Berthelot's reaction (Sommer et al., 1992). DOC and WSN content were quantified using an elemental liquid analyzer (TOC-VCSN, Shimadzu). Soil pH and electrical conductivity (EC) were determined in water extracts (1:10 dw:v) in a CRISON GLP21 pH meter and an Inolab 7110 conductimeter, respectively.

2.2.2. *DNA extraction and sequencing*

A total of 60 soil samples (a composite soil sample per treatment plot at each of five time points) were sent to IGA Technology Services S.R.L. (Udine, Italy) for DNA extraction and high-throughput sequencing of the marker gene (metabarcoding). DNA was extracted using the DNeasy PowerLyzer PowerSoil Kit (QIAGEN), following the manufacturer's instructions. The DNA concentration was then measured using a Qubit fluorometer. Library preparation followed the Illumina 16S Metagenomic Sequencing Library Preparation protocol, involving two amplification steps: an initial PCR with locus-specific primers, followed by a second PCR to add flow-cell adapters and unique indices (Nextera XT Index Kit, FC-131-1001/FC-131-1002). For the first PCR, 25 cycles were used for 16S rRNA gene amplification, and 30 cycles for ITS and archaeal targets. Thermal cycling followed the manufacturer's recommended protocol: initial denaturation at 95°C for 3 min; followed by cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s; and a final extension at 72°C for 5 min. The bacterial community was accessed with the primers 341 F (5′-CCTACGGGNBGCASCAG-3′) and 805 R (5′-GACTACNVGGGTATCTAATCC-3′). To profile the fungi, the primers ITS1F (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS2R (5′-GCTGCGTTCTTC ATCGATGC-3′) served as guides. The archaeal domain was targeted using Arch340wF (5′-CCCTAYGGGGYGCASCAG-3′) and Arch806R (5′-GGACTACVSGGGTATCTAAT-3′). Finally, libraries were sequenced on an Illumina NovaSeq 6000 platform using 250-bp paired-end reads. The generated 180 amplicon sequencing dataset was submitted to the NCBI Sequence Read Archive under BioProject numbers PRJNA1403626 (SAMN54684591-SAMN54684653), PRJNA1404231 (SAMN54706912-SAMN54706974), PRJNA1404282 (SAMN54710019-SAMN54710081), which can be found at https:// www. ncbi. nlm.nih. gov/ sra. The accession numbers for the soil samples are provided in Table S1.

2.2.3. *Bioinformatic analyses*

Primary bioinformatic processing was performed by the service provider (IGA Technology Services S.R.L), including base calling and demultiplexing via Bcl2Fastq v2.20 of the Illumina pipeline. Adapter sequences were masked using Cutadapt v1.16 with the following parameters: –anywhere (on both adapter sequences), –overlap 5, –times 2, – minimumlength 35, and mask-adapter (2). No quality clipping was applied to the raw data. Subsequently, the 250 bp sequence reads were processed as follows: (i) only reads retaining 250 bp length with Phred score > 20 were maintained, (ii) ambiguous or undefined bases (Ns) were not allowed, and (iii) reads with a quality Phred score < 20 were removed. The obtained sequences were processed using the "Dada2" package version 1.26.0 (Callahan et al., 2016) for quality filtering and chimaera removal, followed by amplicon sequence variant (ASV) inference in R software (R Core Team, 2025). For taxonomic classification, prokaryotic sequences were classified with the SILVA reference database (Release 138) (Quast et al., 2013) using the naïve Bayesian RDP classifier implemented in DADA2 with an 8-mer size, and 100 bootstrap replicates. Fungal sequences were classified using the Unite v8.3 database (Nilsson et al., 2019) with the same parameters.

2.3. *Sampling, preparation, and analysis of leaves and olives*

Leaf samples were collected in May and July 2023 for phytochemical analyses, while nutrient analyses were conducted on July-collected leaf samples following Fernández-Escobar, (2019). To ensure a representative sample, at least 100 leaves were harvested per plot (the experimental unit), collected from four branches per tree across all cardinal orientations (N, E, S, and W). Sampling was conducted across all six trees within each plot, and leaves were pooled to form a single composite sample per treatment replicate before further processing. After sampling, leaves were rinsed with deionized water and gently dried in paper envelopes. Subsamples of leaves for macro- and micronutrient determinations were oven-dried at 60°C for 72 h and milled.

While harvests were performed annually, the 2023 campaign was an extremely low-yield year due to the "year off" in the alternate bearing cycle (Figure S1). Consequently, olive sampling for phytochemical and nutrient profiling was scheduled for the 2024 harvest campaign (December 2024) to ensure representative data. At harvest, the yield per plot was recorded, and a representative pooled subsample of 1 kg of olives per plot was retained for later analyses. Manually cracked olives and leaf subsamples for phenolic extraction were frozen at −80°C, freeze-dried, and stored at −80°C until use. Olive subsamples for characterization and lipid profile determination were stored at −20°C until analysis.

2.3.1. *Leaf mineral analysis*

Total concentrations of macro and micronutrients in olive leaves were measured after HNO_3/H_2O_2 digestion by using inductively coupled plasma (GC/MS/QTOF 7250B, Agilent Technologies, Santa Clara, CA, USA). Total N was determined by automatic elemental analysis (automatic elemental analyzer CHNS-932, LECO, USA).

2.3.2. *Olive proximate composition and lipid profile*

Olive nutritional analyses were performed according to internal standard methods based on UNE-EN ISO protocols at the National Technological Center for Preserves and Food (CTNC, Molina de Segura, Murcia, Spain). Fatty acid (FA) composition [saturated (SFA), monounsaturated (MUFA), polyunsaturated (PUFA), and trans] was determined by gas chromatography with flame ionization detection (GC-FID). Total sugars and sodium chloride (NaCl) were quantified by ion chromatography. Moisture and ash contents were determined gravimetrically. Total fat was measured using the Soxhlet extraction method, and protein content was quantified by the Kjeldahl method. Carbohydrate (CH) content was determined by difference: CH $= 100–$(moisture$+$protein$+$fat$+$ash). The energy value was estimated using Atwater conversion factors (Atwater and Benedict, 1902).

2.3.3. *Phenolic profiling of leaves and olives*

Lyophilized leaf powder (100 mg) or olive pulp powder (200 mg) were extracted with 1.5 mL of methanol:water:formic acid (70:29:1, v:v:v). The mixtures were vortexed and subsequently sonicated in an ultrasonic bath for 60 min. Then, extracts were stored overnight at 4 °C and centrifuged at 9500 x g for 15 min to separate the supernatant. The resulting supernatant was filtered through a 0.22 µm polyvinylidene fluoride (PVDF) filter before HPLC-DAD-MS^n/ESI analysis.

Chromatographic analyses were performed on an Agilent 1100 LC system (Agilent Technologies, Palo Alto, CA, USA) equipped with a Phenomenex Kinetex C18 (5 µm, 2 × 250 mm). Separation was carried out at 25°C using a gradient elution at a flow rate of 0.8 mL/min. The mobile phases consisted of water plus 1% Formic acid (A) and acetonitrile (B). A multistep linear gradient was applied: 0 min, 5% B; 20 min, 40% B; 28 min, 60% B; 30 min, 95% B; followed by a column wash and re-equilibration to initial conditions for 5 min. The injection volume in the HPLC system was 20 µL.

Quantification was performed via external standard calibration using five concentration levels ($R^2 > 0.99$), following the validated procedures described by Gironés-Vilaplana et al. (2014) and Migues et al. (2018). Glycosylated flavonoids were quantified as quercetin equivalents (0.015–1 mM), anthocyanins as cyanidin equivalents (0.030 – 1 mM), and secoiridoids as oleuropein equivalents (0.5 – 2 mM). All standards were of analytical grade (purity ≥ 95%, Sigma Aldrich – Merck). The Limit of Detection (LOD) and Limit of Quantification (LOQ) were determined based on signal-to-noise ratios of 3 and 10, respectively. To ensure reproducibility and instrument stability, solvent blanks and midpoint calibration checks were run between batches to monitor carryover and response drift. Phenolic compounds were identified based on their elution order, retention times, and ultraviolet-visible and mass spectra features compared to the authentic standards analyzed under the same conditions and with reference to previously published data (Abaza et al., 2017; Kabbash et al., 2023; Quirantes-Piné et al., 2013) (Tables S2, S3).

2.4. *Statistical analysis and data visualization*

All raw data and R scripts used for the statistical analyses and visualizations are available (https://digital.csic.es/handle/10261/428687). Statistical analyses and data visualization were conducted in R (version 4.4) (R Core Team, 2025) using RStudio (version 2023) (Posit Team, 2023). The plot was defined as the experimental unit ($n = 3$ per treatment). To ensure a representative characterization of each unit and minimize intra-plot spatial heterogeneity, a systematic pooling strategy was employed: soil cores, leaves, and fruits were collected across all six trees per plot and combined into a single composite sample before analysis. While the study involved repeated sampling over time, initial testing with a linear mixed-effects model (treating "plot" as a random effect to account for hierarchical structure) resulted in singular fits with zero variance component for the random effect, indicating that plot-level variability was negligible relative to the residual error; thus, a fixed-effects ANOVA framework was adopted.

Soil, leaf, and olive physicochemical data and yield measurements were analyzed using a one-way analysis of variance (ANOVA). Assumptions were checked using the *leveneTest()* function from the car R package (Fox et al., 2022) (homogeneity) and the Shapiro-Wilk test (normality); data were natural log-transformed when assumptions were not met. Pairwise comparisons were conducted using the Tukey HSD test. To quantify the effect magnitude and statistical robustness, Eta-squared (η^2) and Post-hoc Power Analysis were calculated using the *effectsize* and *pwr* packages, respectively (Ben-Shachar et al., 2020; Champely et al., 2020).

Bacterial, archaeal, and fungal community compositions were presented as relative abundances at the phylum level. Before analysis, ASV tables were rarefied to the minimum sequencing depth for bacteria (31,493), archaea (22,741), and fungi (3,584 counts per sample). Alpha diversity (Shannon, Simpson, and Pielou's evenness) was calculated from ASV data using the *diversity()* and *specnumber*() functions in the vegan R package (Oksanen et al., 2025). Pielou's evenness was calculated as $J = H'/\ln(S)$, where H' is Shannon diversity and S is the observed richness. Differences among treatments were tested with one-way ANOVA followed by Tukey's adjustment ($\alpha = 0.05$) using *emmeans*. To further characterize the magnitude and precision of treatment effects, we calculated Eta-squared (η^2) as an effect size measure using the *effectsize* package (Ben-Shachar et al., 2020). Beta diversity was evaluated using Bray–Curtis dissimilarities from square-root transformed and Wisconsin-standardized ASV data. Community differences across treatments and sampling times were evaluated using permutational multivariate analysis of variance (PERMANOVA, 999 permutations) via the *adonis2* function. Homogeneity of multivariate dispersions were assessed using the *betadisper* and *permutest* functions. Given that multivariate dispersions were not consistently homogeneous across all subsets, ordinations were generated by Non-Metric Multidimensional Scaling (NMDS) with the *metaMDS* function in vegan (Oksanen et al., 2025). NMDS is a non-parametric, rank-based approach that is less sensitive to heteroscedasticity than metric methods, providing a more faithful representation of community distances when group variances differ. A two-dimensional solution was selected based on stress value inspection (<0.2), indicating a good fit of the ordination.

To explore the mechanistic links between soil chemical drivers, microbial diversity, and plant quality traits, a correlation analysis was performed using Spearman's rank correlation (ρ) via the *stats* and *ggcorrplot* packages (Kassambara, 2023). Hierarchical clustering was applied to the correlation matrix to group variables with similar response patterns.

Statistical significance was considered at $p < 0.05$, with $0.05 \leq p < 0.10$ considered as a trend. Figures for data visualization were generated using the R package ggplot2 (Wickham, 2016).

3. Results and discussion

3.1. *Soil physicochemical status and seasonal C and N availability*

Ten years after the experiment started, all the organic amendments resulted in increases in soil C concentration (Table S4). Relative to the unamended control, compost, mixture, and biochar treatments increased soil organic C in the upper soil layer (0–20 cm) by 0.78, 1.04, and 1.24%, respectively. Total soil N remained comparable between the control and biochar treatments (0.16–0.18%), whereas compost and mixture treatments registered higher N contents (0.27% in both treatments). The increase in total soil C under biochar amendment is attributed to the recalcitrance of its aromatic structures, which resist microbial mineralization (Harvey et al., 2012). Conversely, the rise in soil N with compost resulted from direct and repeated nutrient addition. These differential accumulation patterns resulted in significant shifts in soil C/N ratios after ten years (Table S4), which underscores the importance of amendment selection: biochar acts primarily as a stable carbon sink, while compost functions as a reservoir for soil fertility. DOC and WSN were consistently higher in compost- and mixture-amended plots. These increases were evident before the fifth amendment in May 2023 (10-Year Status; $p < 0.01$, $\eta^2 \sim 0.75$) and remained consistent across all subsequent seasonal samplings (Year 11; $p < 0.001$, $\eta^2 \geq 0.90$) (Fig. 1, Table S5). The pronounced increases in soluble and total organic C under compost and mixture treatments highlight their potential to improve soil health. Soil mineral N was very low in all the treatments, amended or not, in all the sampling events (Table S6), conditions that favor microbial N acquisition. Under N-limited conditions, organic N must be depolymerized into free amino acids and further mineralized to ammonium to become available for plant uptake (L'Espérance et al., 2024). Enhanced C and N availability is expected to foster microbial diversity and resilience to climate-related stresses by alleviating nutrient competition. The relation between available DOC and WSN was similar among treatments ($p > 0.05$, $\eta^2 \leq 0.40$; Table S5), despite the higher total C/N ratio in the biochar-amended plots (Fig. 1, Table S4). The water-soluble C and N concentrations in biochar-treated plots were similar to the unamended control, reflecting the stability of the added biochar. Soil pH remained stable at 8.7 ± 0.1 across all treatments (Table S4), likely due to the buffering provided by the native soil carbonates.

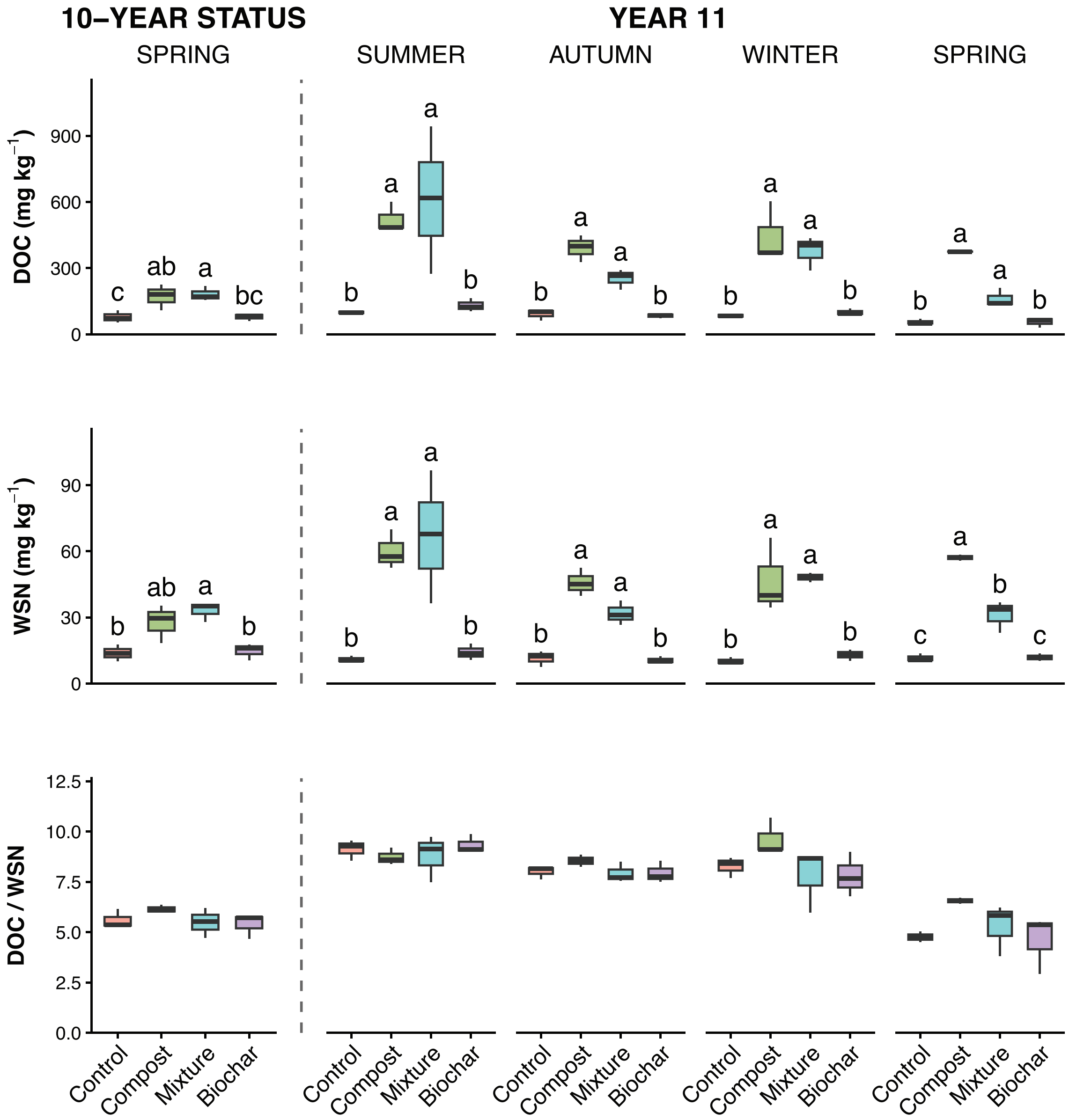

Fig. 1. Water dissolved organic C (DOC) and water-soluble N (WSN) after a decade of biennial amendments. Dash line indicates the fifth application of the amendments (Spring 2023). Boxplots display the median (horizontal line), the interquartile range (box), and the full range of observations (n = 3). Letters indicate significant differences according to the Tukey test ($p < 0.05$), whereas the absence of letters shows no differences. For detailed ANOVA parameters, effect sizes (η^2) and statistical power, see Table S5 in the Supplementary Material.

3.2. Decadal legacy of biennial amendments and post-amendment microbial dynamics

Bacterial, archaeal, and fungal soil communities showed the usual composition of a Mediterranean semiarid agricultural soil (Miralles et al., 2021; Siles et al., 2024) (Figures S2, S3, and S4). Left panels in Figs. 2A, 3A, and 4A show the legacy of a decade of biennial amendments in the alpha diversity indices of the bacterial, archaeal, and fungal groups, respectively. Bray-Curtis dissimilarities in community composition of these microbial groups are depicted in Figs. 2B, 3B, and 4B.

Despite the consistent differences in total and soluble soil organic C among treatments, the diversity indices showed no significant differences ten years after the experiment began. This outcome contrasts with our initial hypothesis that increased nutrient availability would enhance microbial growth and diversity by alleviating N and organic-C limitations. Despite higher DOC and WSN levels, microbial diversity was not affected. While these results indicate a high degree of taxonomic stability, it is important to distinguish this from functional resilience. It is possible that the microbial communities underwent physiological or metabolic shifts that are not reflected in their taxonomic composition. Similar results in semi-arid soils have been attributed to a large proportion of dormant or inactive microorganisms that require little DOC-derived energy for metabolic activity (Bastida et al., 2016). It has also been suggested that functional redundancy within microbial communities may decouple diversity from soil C availability (Bastida et al., 2016; Wertz et al., 2007). In our study, the experimental field had been organically managed – with biennial compost applications - for a decade before the initiation of the experiment, and even the control plots, which have not received any amendments during the past decade, exhibited remarkably high diversity indices across all three microbial groups. This already elevated baseline, reflected in high alpha diversity indicators, may have masked potential treatment effects. Furthermore, biochar

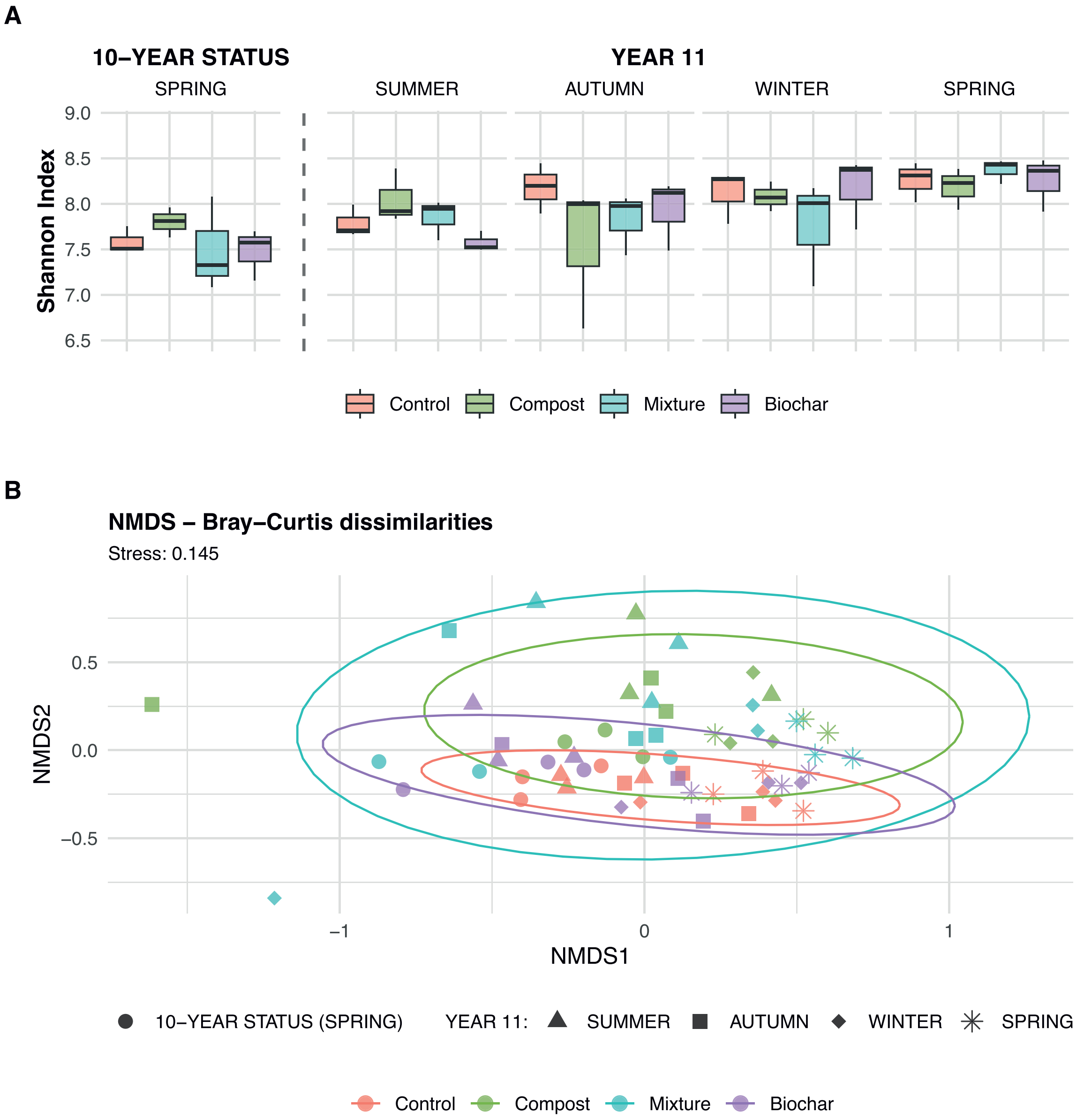

Fig. 2. Alpha (A) and beta (B) diversity of bacterial communities based on ASV-level composition after a decade of biennial amendments. (A) Shannon index. Boxplots display the median (horizontal line), the interquartile range (box), and the full range of observations. Dash line indicates the fifth application of the amendments (Spring 2023). The absence of letters shows no differences between treatments ($p > 0.05$). (B) NMDS ordination of bacterial community composition (Stress = 0.145) with 95% confidence ellipses for treatment groups. A detailed summary including Shannon, Simpson, and Pielou's evenness, ANOVA parameters, effect sizes (η^2), statistical power, and PERMANOVA results (including dispersion p-values) is available in the Supplementary Material (Table S6).

effects on microbial communities are generally weaker in calcareous soils than in neutral or acidic soils (Xu et al., 2023), which may have further contributed to the observed stability in microbial diversity. Seasonal sampling following the fifth amendment captured transient responses to the different amendments during the subsequent year, which varied depending on the microbial group.

For all treatments, the bacterial community was primarily composed of Actinobacteria and Proteobacteria. Following the fifth amendment, compost- and mixture-amended plots showed a decreased proportion of Actinobacteria, which may be linked to the introduction of microbes through the organic amendments as well as to the faster proliferation of organic-matter decomposers belonging to other phyla such as the Proteobacteria and Bacteroidota (Fierer et al., 2007; Rodríguez-Berbel et al., 2020) (Figure S2). However, we did not observe a consistent effect of the treatments on bacterial alpha diversity throughout the seasonal monitoring. We observed a transient effect three months after the fifth amendment (Summer 2023), when the Shannon index showed a trend with substantial effect size (p = 0.1264, $\eta^2 = 0.492$), a shift in Pielou's evenness (p = 0.0557, $\eta^2 = 0.592$), which was significant in the Simpson's Index (p = 0.0441, $\eta^2 = 0.617$). However, these differences dissipated in subsequent seasons, with no significant differences between treatments (Fig. 2A, Table S6). Beta diversity analysis, based on Bray-Curtis distances of bacterial community composition, revealed a significant effect of the treatments ($p = 0.001$) and a higher impact of the seasonal influence ($p = 0.001$), although the effect size is relatively small (7.4% and 14.2% of the variability explained by the treatment and sampling timepoint, respectively). Compost and Mixture treatments show higher community dispersion, with shared taxa among all treatments (Fig. 2B). For bacterial communities, dispersions had a marginal effect across treatments ($p = 0.047$) and timepoints ($p = 0.094$), indicating caution with the interpretation of the PERMANOVA results (Table S6). However, the high significance of the PERMANOVA model ($p = 0.001$) relative to the marginal dispersion effect suggests a genuine shift in bacteria composition.

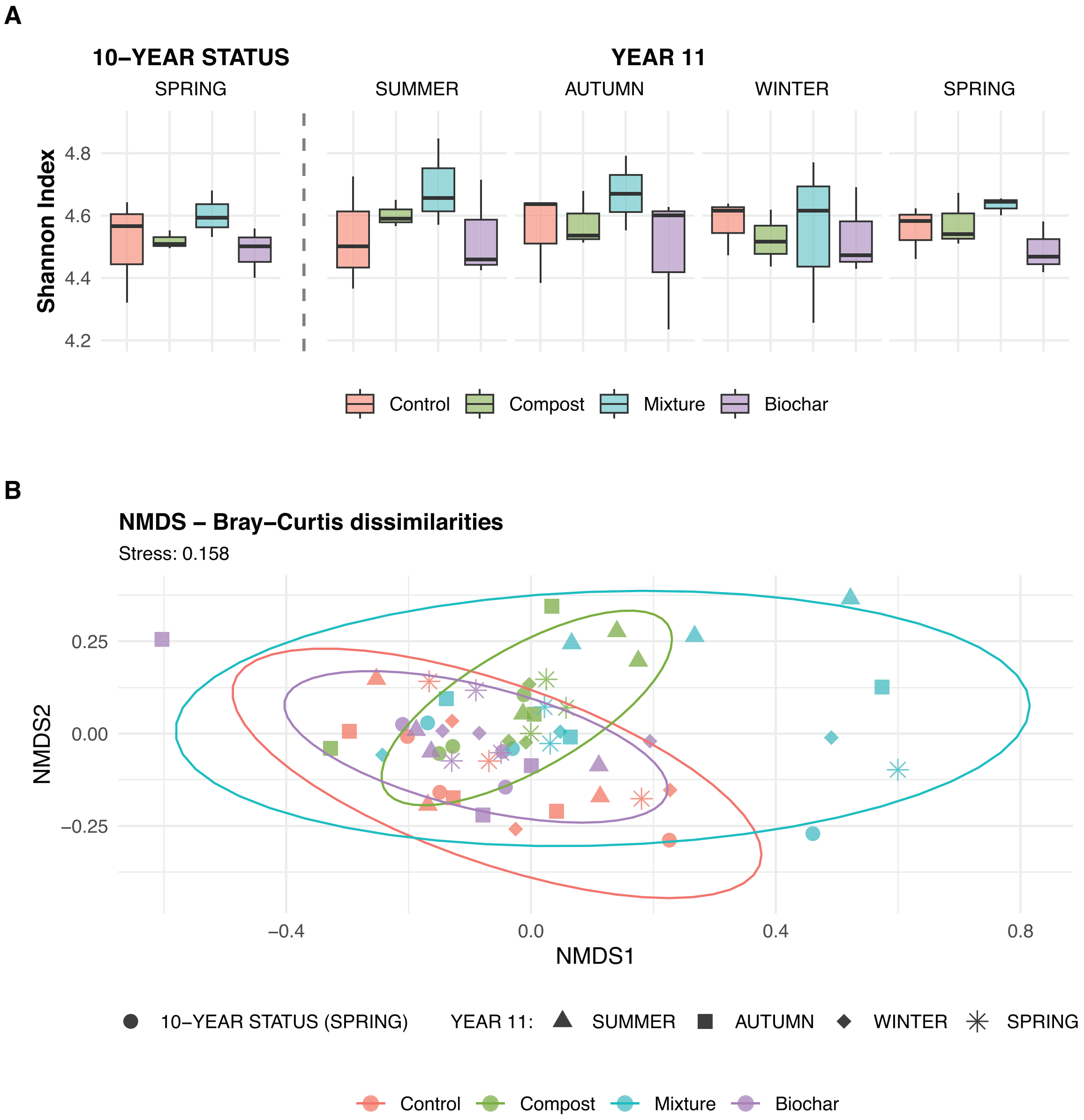

Fig. 3. Alpha (A) and beta (B) diversity of archaeal communities based on ASV-level composition after a decade of biennial amendments. (A) Shannon index. Boxplots display the median (horizontal line), the interquartile range (box), and the full range of observations. Dash line indicates the fifth application of the amendments (Spring 2023). The absence of letters above boxplots shows no differences among treatments ($p > 0.05$). (B) NMDS ordination of archaeal community composition (Stress = 0.158) with 95% confidence ellipses for treatment groups. A detailed summary including Shannon, Simpson, and Pielou's evenness, ANOVA parameters, effect sizes (η^2), statistical power, and PERMANOVA results (including dispersion p-values) is available in the Supplementary Material (Table S7).

Archaeal communities were dominated by the phylum Crenarchaeota, which accounted for more than 95% of the total archaeal relative abundance (Figure S3). Following the fifth amendment, we observed transient shifts in phylum-level relative abundances, but the community composition stabilized during the second half of the year. Fig. 3 illustrates treatment effects on the diversity of archaeal communities across all sampling timepoints. The transient effect observed in the phylum relative abundance did not reflect a significant impact on the Shannon and Simpson diversity indexes at the ASV level. In contrast to the bacteria domain, compost and mixture treatments showed a slight decrease of Pielou's evenness three months after the amendment, although this effect was not significant ($p = 0.453$, $\eta^2 = 0.267$). Bray-Curtis dissimilarities revealed significant effects of both treatment and sampling timepoint on archaeal community composition, with relatively small effect sizes (14.0% and 9.7% of explained variability, respectively, Table S7). For archaeal communities, dispersions differed significantly among treatments ($p < 0.05$) but not among timepoints ($p > 0.05$). This indicates that treatment-related differences in community composition should be interpreted cautiously, as they may be partly influenced by differences in within-group variability. Mixture-treated plots showed the highest dispersion, followed by compost-treated plots.

Ascomycota was the dominant fungal phylum across all treatments and seasons (Figure S4). Although fungal communities were comparable among treatments in the 10-year spring sampling (before the fifth amendment), the effects of compost and mixture application persisted throughout the subsequent year. Specifically, the treatment type emerged as the primary source of variation for fungal alpha diversity ($\eta^2 = 0.62$–0.96; Table S8). The high statistical power (0.83–1.00) associated with these indices confirms a strong amendment-driven response. Contrary to our first hypothesis, these amendments reduced fungal

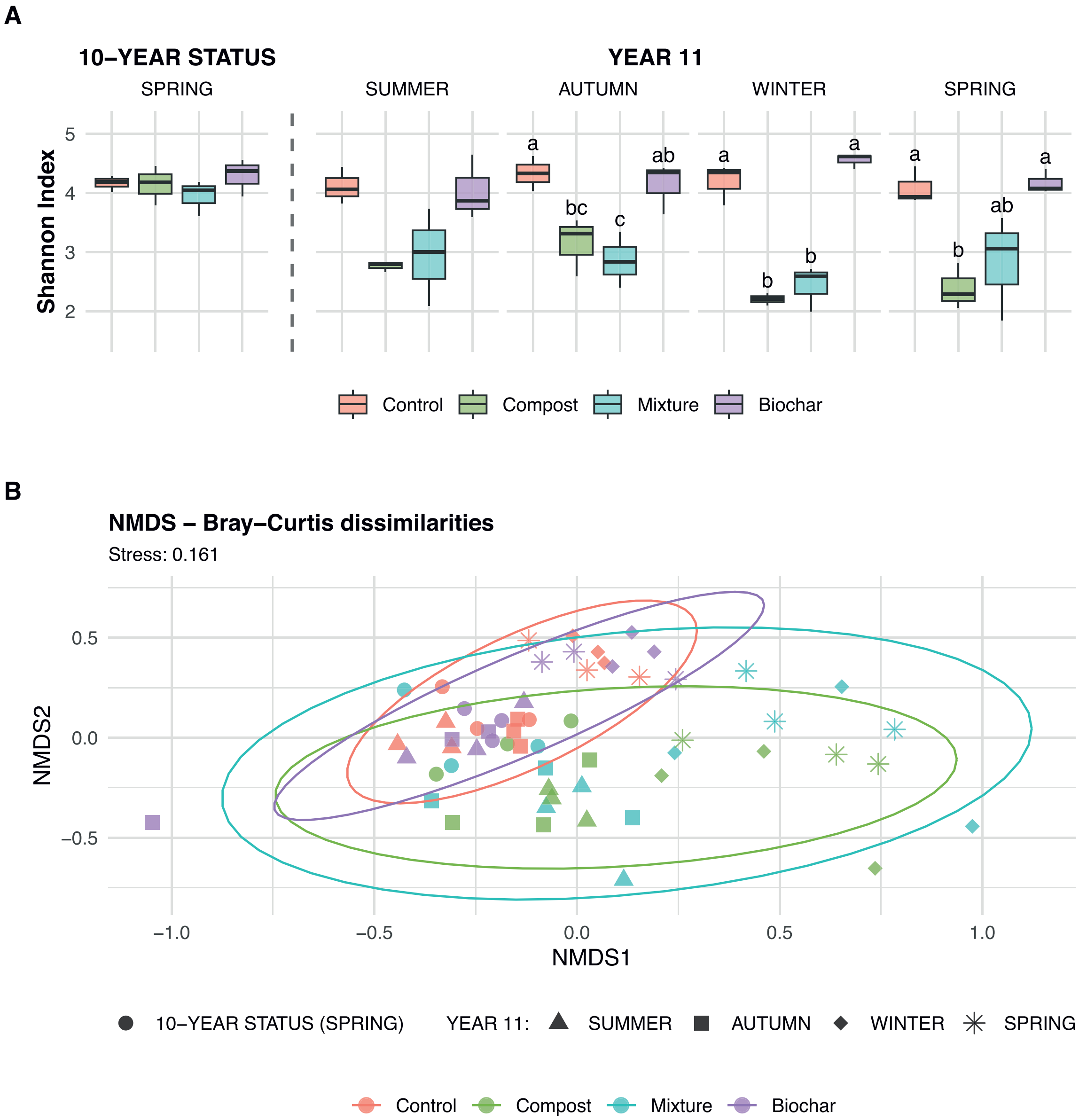

Fig. 4. Alpha (A) and beta (B) diversity of fungal communities based on ASV-level composition after a decade of biennial amendments. (A) Shannon index. Boxplots display the median (horizontal line), the interquartile range (box), and the full range of observations. Dash line indicates the fifth application of the amendments (Spring 2023). Letters above boxplots indicate significant differences among treatments (Tukey test, $p < 0.05$), whereas the absence of letters shows no differences. (B) NMDS ordination of fungal community composition (Stress = 0.161) with 95% confidence ellipses for treatment groups. A detailed summary including Shannon, Simpson, and Pielou's evenness, ANOVA parameters, effect sizes (η^2), statistical power, and PERMANOVA results (including dispersion p-values) is available in the Supplementary Material (Table S8).

diversity and evenness, as indicated by the alpha diversity indices (Table S8, Fig. 4A), which is consistent with the lower relative abundance of minor taxa (Figure S4). This reduction may reflect the dominance of a few competitive saprotrophic groups favored by the nutrient-rich amendments (Clocchiatti et al., 2020), which outcompeted minor taxa and reduced overall community evenness. Beta diversity analysis further showed more tightly clustered fungal communities in the control and biochar treatments, which overlap with the wider-dispersed communities of compost and mixture treatments (Fig. 4B). Treatment and timepoint impacts were significant ($p < 0.01$, Table S8) but explained a low percentage of the variability (6.8% and 13.1%, respectively). For fungal communities, dispersion among treatments was homogeneous ($p > 0.05$), but not among timepoints ($p < 0.05$).

The effects on fungal communities were the most persistent; however, by year 10 (two years after the fourth amendment), differences between treatments were no longer detectable. In contrast, bacterial responses were more transient, and the diversity indices displayed an opposite pattern (i.e. while amendments increased bacterial diversity, they decreased fungal diversity). This divergence likely reflects the distinct roles of these groups: the fungal community's sensitivity may reflect its role as primary decomposer of complex organic inputs (Okur et al., 2025), whereas the bacterial community exhibits higher structural resilience due to the mature status of the orchard and the soil's mineral properties (Rodríguez-Berbel et al., 2020). Previous studies have shown that the addition of organic amendments can strengthen bacterial-fungal correlations, particularly increasing negative correlations between the two groups (Luo et al., 2022). Compost-borne microorganisms may be related to the temporary shift in community composition that were outcompeted by the soil microorganisms after the amendment application (Saison et al., 2006). Overall, the soil microbial community was highly stable, especially the archaea domain, which may reflect a combination of factors. The long-term legacy of organic amendments may have established a high-diversity baseline close to biotic saturation, limiting further gains in diversity indices. Additionally, the environmental constraints of the Mediterranean climate, such as aridity and high soil pH, likely exert a stronger selective pressure than nutrient inputs, favoring a pre-adapted and resilient core microbiome. Despite this structural robustness, these communities probably underwent physiological or metabolic shifts that were not captured by taxonomic profiling but may align with the observed changes in soil nutrient cycling.

3.3. Nutrient and phenolic compounds of interest in leaves and mature olives

3.3.1. Nutrient profile of leaves and fruits

Differences in soil nutrient availability did not significantly affect leaf nutrient concentrations (Table S9). However, the lowest leaf N concentration was recorded in the control with values close to deficiency thresholds (Fernández-Escobar, 2019). The low impact of fertilization

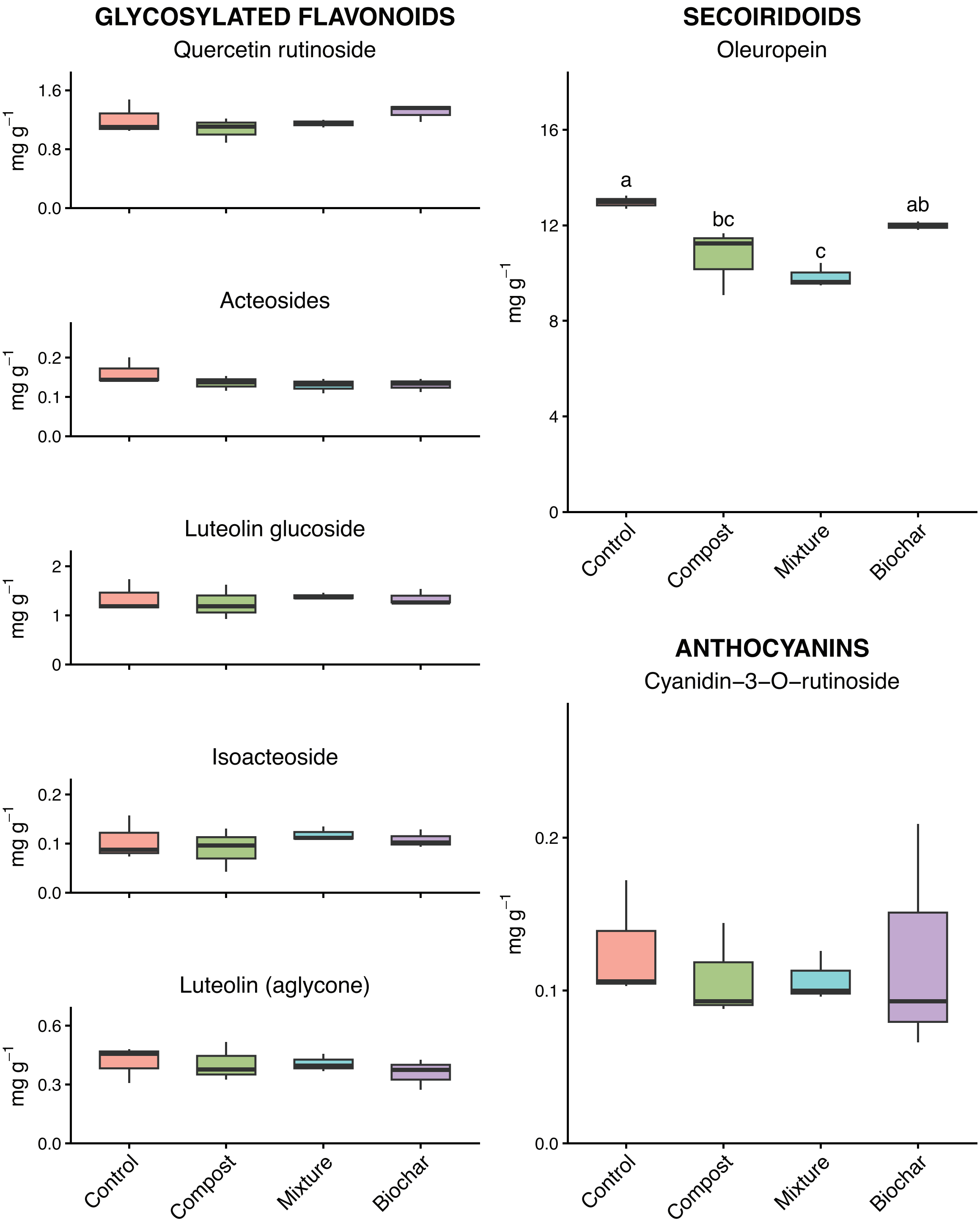

Fig. 5. Phenolic compounds in the mature olives' pulp after the fifth amendment, year 11 of the experiment. Left panel: Glycosylated flavonoids; Right panels: Secoiridoids (top) and Anthocyanins (bottom). Boxplots display the median (horizontal line), the interquartile range (box), and the full range of observations (n = 3). Letters indicate significant differences among treatments (Tukey test, $p < 0.05$), whereas the absence of letters shows no differences. For detailed ANOVA parameters, effect sizes (η^2) and statistical power, see Table S12 in the Supplementary Material.

on the nutritional status of well-established olive trees has also been previously observed (Fernández-Escobar, 2019; Fernández-Escobar et al., 2009). This response is attributed to the tree's efficient internal nutrient remobilization and low annual removal rates, which are readily sustained by native soil fertility. Likewise, crop yield did not differ among treatments (Table S9), with production in the 2024 campaign ranging from 7.2 to 7.8 Mg ha^{-1}. These results are consistent with those from previous campaigns (Fornes et al., 2024; Sánchez-García et al., 2016), indicating that variation in crop performance was more influenced by campaign-related factors (e.g., weather conditions and alternate bearing) than by the applied amendments.

In contrast, some effects of the treatments were observed in the nutrient profile of the mature fruits. Total FA content of the drupes ranged from 25.4% to 30.7%, with MUFA being the main group, ranging from 17.0% to 20.2% (Table S10). The lowest FA and MUFA concentrations were found in olives collected from the control plots, while organic amendments enhanced the lipid nutritional value of the olives. Given the role of N in photosynthesis, the reduced leaf N content in the control treatment may have limited nutrient translocation to the drupes in non-amended trees (Verardo et al., 2013). PUFA and SFA accounted for approximately 4 and 5% of total fruit nutrients, respectively, across all treatments. The complete nutrient profile of the mature fruits - including FA composition and the contents of CH, ash, sugar, protein, NaCl, moisture, and energy - is provided in the Supporting Information (Table S10).

3.3.2. *Phenolic content in leaves and fruits*

In line with the observed patterns for leaf nutrient content, leaf phenolic concentrations were scarcely affected by the amendments and primarily driven by the sampling season (Table S11). Phenolic compounds were quantified in olive leaves collected in May and July 2023, corresponding to the flowering and fruit growth stages, respectively. We identified 14 phenolic compounds already described on olive leaves (Kabbash et al., 2023; Quirantes-Piné et al., 2013) (Table S2). The inherent high variability of field experiments limited the ability to detect treatment effects on the leaf phenolic content. Nevertheless, a consistent shift in the concentration of most phenolic compounds was evident between phenological stages, with higher levels during flowering and markedly reduced levels during fruiting (Table S11). This observation is consistent with the role of leaf phenolics in structural reinforcement and oxidative protection (Kishor et al., 2023), and likely reflects a developmental shift in carbon allocation from leaf structural phenolics toward reproductive tissues during summer (Verardo et al., 2013).

The phenolic concentration of the mature olives was more responsive to management practices than the leaves (Fig. 5). The main phenolic compounds identified in the samples of mature olives are listed in the Supporting Information (Table S3). The general composition of the olives agreed with previously described results (Abaza et al., 2017), with 5 glycosylated flavonoids, 1 secoiridoid, and 1 anthocyanin clearly identified. The phenolic contents in the mature olives are also in agreement with previous findings by other researchers (Damak et al., 2008; Morelló et al., 2004; Vinha et al., 2005). The applied amendments did not influence the concentrations of glycosylated flavonoids in mature olives. The predominant glycosylated flavonoids detected were quercetin rutinoside and luteolin glucoside, followed in abundance by the luteolin aglycone. Acteosides and isoacteoside were present at low levels ($<$ 0.20 mg g^{-1}) across all treatments. Anthocyanin accumulation is associated with fruit color development during maturation, which increases during this phase, whereas secoiridoid concentration typically declines (Morelló et al., 2004). Olives were collected at full maturity, which in the Arberquina cultivar corresponds to a developed dark coloration, and no differences among treatments were observed in the concentration of cyanidin–3-o–rutinoside. In contrast, oleuropeine - the most prominent individual phenolic component of olive fruits (Damak et al., 2008) - showed lower concentrations under the compost and mixture treatments. The oleuropein levels in the present study were higher than those reported by Morelló and colleagues (2004) for the Arbequina olives grown in the Ebro Valley (Spain), but were comparable to the concentrations reported by Damak et al., (2008) for the Chétoui cultivar grown in the Sfax region (southern Tunisia). Both studies were conducted in Mediterranean environments, although the Sfax region is more arid, a climatic condition more similar to our study area. Vinha et al., (2005) determined the phenolic profile of 29 samples of mature olive fruits collected from 18 different cultivars and found that oleuropein content widely ranged from 0.4 to 22 mg g^{-1}. They found that cultivar affected the phenolic profile, but geographical origin had a stronger influence. In our case, differences in oleuropein content are likely related to long-term differences in soil nutrient availability. Previous studies have reported negative relationships between olive leaf N content and olive fruit phenolic content (Erel et al., 2013), consistent with the role of these compounds in stress response. A similar effect was found in the biochar-treated plots, with reduced soil nutrient availability and higher oleuropein content than in the compost and mixture treatments.

From an agronomic standpoint, the sensitivity of oleuropein to organic amendments is highly relevant for the production of high-stability oils. While increases in polyphenols enhance the oil’s shelf-life and health-promoting properties, they also modulate the organoleptic profile (Mateos et al., 2004). Our results suggest that organic amendments provide a tool for tailoring the phytochemical profile of the fruit, potentially increasing the market value of the olive oil without compromising yield stability.

To synthesize the complex interactions between soil, microbes, and crop quality, we performed a targeted multivariate analysis. Variables were selected for this synthesis based on their sensitivity to the organic amendments, using a threshold of significance ($p < 0.05$) or marginal significance ($p < 0.1$) coupled with substantial effect sizes ($\eta^2 > 0.4$). Within the soil compartment, we focused on the labile fractions (DOC and WSN) rather than total C and N pools, as the former better reflect the immediate metabolic availability and seasonal dynamics influenced by the treatments; specifically, we integrated the annual average of seasonal measurements from Year 11. Regarding crop status, leaf P content was included due to its high significance. Leaf N content exhibited marginal significance ($p = 0.17$, $\eta^2 = 0.44$) and was included as it is a critical indicator of nutritional shifts. In the microbial domain, we prioritized fungal diversity, as this group demonstrated the most consistent treatment responses throughout the year; specifically, the annual averages from Year 11 of the Fungal Shannon and Evenness indices were included to represent stable community shifts. Finally, for fruit quality domains, we selected primary metabolites (fatty acids and oleuropein) that demonstrated robust responses to the decadal legacy of the amendments.

3.4. *Mechanistic links between soil drivers, microbial diversity, and crop quality*

Following the identification of key responsive variables, a Spearman’s rank correlation (ρ) was performed to explore mechanistic links. Hierarchical clustering of the correlation matrix (Fig. 6) revealed strong positive correlations between labile soil nutrients and olive fatty acids. Soil DOC and WSN were robustly associated (ρ =0.93) and positively correlated with higher levels of MUFA ($\rho = 0.68$–0.72).

An inverse relationship was observed between soil nutrient enrichment and fungal community structure. Both fungal Shannon and Pielou’s evenness showed strong negative correlations with soil DOC ($\rho = -$ 0.74 and $\rho = -$ 0.64, respectively) and soil WSN ($\rho = -$ 0.80 and $\rho = -$ 0.71, respectively). This statistical trend supports our earlier observation that nutrient-rich amendments favor specific, competitive saprotrophic groups (Clocchiatti et al., 2020).

Oleuropein accumulation displayed a strong negative correlation with soil nutrient availability (Soil DOC $\rho = -$ 0.74, soil WSN $\rho = -$ 0.69). It was also negatively correlated with leaf N content ($\rho = -$ 0.54). However, leaf N and P content displayed weak correlations with other

	Fungal Shannon	Fungal Evenness	Leaf N	Leaf P	Soil DOC	Soil WSN	PUFA	MUFA	SFA
MUFA									0.88
PUFA								0.76	0.86
Soil WSN							0.53	0.68	0.59
Soil DOC						0.93	0.61	0.72	0.59
Leaf P					0.09	0.17	0.36	0.26	0.35
Leaf N				−0.1	0.38	0.51	−0.04	0.36	0.3
Fungal Evenness			−0.38	−0.23	−0.64	−0.71	−0.42	−0.4	−0.46
Fungal Shannon		0.95	−0.33	−0.14	−0.74	−0.8	−0.45	−0.38	−0.44
Oleuropein	0.76	0.74	−0.54	0.18	−0.74	−0.69	−0.48	−0.61	−0.6

Fig. 6. Spearman's rank correlation matrix for significant soil drivers and microbial/plant outputs. The heatmap illustrates the monotonic relationships between soil drivers (DOC and WSN) and output variables (oleuropein, Fungal Shannon and Evenness index, Leaf N and P content, MUFA, PUFA, and SFA). Each cell displays the Spearman correlation coefficient (ρ). The color scale indicates correlation direction: green denotes a positive correlation, red denotes a negative correlation, and white indicates no correlation (n = 3). DOC: Dissolved Organic Carbon; WSN: Water Soluble Nitrogen; MUFA: Monounsaturated fatty acids; PUFA: Polyunsaturated fatty acids; SFA: Saturated fatty acids.

variables ($\rho < |0.51|$). These results are consistent with previous studies reporting the higher sensitivity of fruit quality than leaf responses to changes in management practices (Schneider et al., 2021).

Overall, our results demonstrate a significant chemical responsiveness of the fruit to organic management. The observed decoupling between soil microbial stability and fruit phytochemical shifts provides field-based evidence of the resource-allocation dynamics in this Mediterranean perennial agroecosystem. It indicates that while soil ecological architecture remains robust after a decade of biennial amendments, the tree's physiological allocation is highly plastic. For growers, this in-situ evidence implies that organic amendments can enhance the lipid-related nutritional quality of the olives while simultaneously modifying the concentration of functional compounds like oleuropein. These findings highlight the need for practical field management strategies recognizing the potential trade-offs between primary yield quality and secondary health-promoting metabolites in mature, long-lived olive orchards.

3.5. Study considerations and future perspectives

While this decadal study provides robust evidence of soil-plant interactions in a Mediterranean context, several inherent limitations of long-term field experimentation should be considered. This single-orchard study ensures agronomic relevance but is limited by its specific calcareous soil and climate. Additionally, previous organic management may have narrowed the baseline contrast between treatments, potentially contributing to the high microbial resilience observed. Regarding the experimental scale, the systematic pooling of samples and moderate replication – standard in long-term field experiments due to prohibitive logistical demands - may mask the detection of subtle intra-plot spatial variability and increase the risk of false negatives (Type II errors). To mitigate this, we incorporated effect sizes (η^2) to highlight relevant trends that traditional *p*-values might overlook. Furthermore, while taxonomic profiling reveals structural stability in the soil microbiome, it does not directly capture functional processes such as nutrient mineralization rates or enzymatic activities. Despite these constraints, the comprehensive nature of this 11-year dataset – integrating soil, microbial, and phytochemical parameters – establishes a rigorous reference for future studies evaluating sustainable management in perennial Mediterranean systems. Finally, although this decadal study captures long-term trends, future research should incorporate interannual climate modeling to resolve how extreme weather events influence soil-plant dynamics. Additionally, to fully resolve underlying mechanisms, such studies should integrate metagenomics or direct enzymatic assays.

4. Conclusions

Our results demonstrate that differences in soil C and N availability among treatments were consistent and persisted throughout the entire experimental period and across seasons. However, contrary to our first hypothesis, the sustained differences in nutrient availability were associated with minor and transient responses in the soil microbial community, despite a decade of biennial amendments and some variation among specific microbial groups.

Our results suggest a high degree of microbial resilience in organically managed olive orchards. This advances our conceptual understanding of soil-plant microbiome interactions in these systems, suggesting that community structure is likely moderated by dominant environmental constraints such as aridity, which may exert a stronger selective pressure than nutrient inputs. The stability of crop yield despite contrasting management practices underscores the robustness of the system.

The higher sensitivity of fruit quality – particularly oleuropein content – which displayed a clear inverse relationship with soil nutrient availability, highlights a significant metabolic plasticity of the olive tree. Oleuropein appears to be a sensitive indicator of the physiological balance between primary growth and secondary metabolism. Recognizing these metabolic shifts provides valuable insights for management strategies that aim to optimize both yield stability and the health-promoting qualities of olive oil. This decadal integration of soil, microbial, and phytochemical data provides a comprehensive system-level reference for sustainable management in Mediterranean perennial crops.

CRediT authorship contribution statement

Calderon Martha: Writing – review & editing, Data curation. **María Luz Cayuela:** Writing – review & editing, Supervision, Resources, Project administration, Investigation, Funding acquisition, Conceptualization. **María Sánchez-García:** Writing – review & editing, Writing – original draft, Visualization, Investigation, Formal analysis, Data curation, Conceptualization. **Sánchez-Monedero Miguel:** Writing – review & editing, Supervision, Resources, Investigation, Funding acquisition, Conceptualization. **Moreno Diego:** Writing – review & editing, Resources, Investigation, Funding acquisition. **Bustamante Danilo:** Writing – review & editing, Data curation.

Declaration of Generative AI and AI-assisted technologies in the writing process

During the preparation of this work the author(s) used Gemini (Google) to refine the linguistic clarity of the manuscript and to optimize R scripts for the public repository, specifically to streamline statistical functions, reduce code redundancy, and assist in debugging. ChatGPT

(OpenAI) was used as a secondary tool to explore alternative debugging options and generate specific illustrative elements for the graphical abstract. After using this tool/service, the author(s) reviewed and edited the content as needed and take(s) full responsibility for the content of the published article.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.eja.2026.128175.

Data availability

Raw data and R scripts supporting this study are available in a public repository at https://digital.csic.es/handle/10261/428687.

Data and Rscripts for "Soil microbiome and phytochemical responses to a decade of compost and biochar amendments in an olive orchard" (Digital CSIC)

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