Soil Biology and Biochemistry 222 (2026) 110284

Contents lists available at ScienceDirect

Soil Biology and Biochemistry

journal homepage: www.elsevier.com/locate/soilbio

Multitargeted metabarcoding and morphological identification reveal practice-specific soil biodiversity outcomes in Mediterranean cropping systems

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ARTICLE INFO

Keywords:
Amplicon sequencing
Bioinoculation
Cropping patterns
Organic amendments
Regenerative agriculture
Soil fauna

ABSTRACT

Meeting future food demands while preserving ecosystem integrity requires agricultural systems that sustain soil biodiversity. Despite its critical role in regulating soil fertility, crop resilience, and food production, the response of soil biodiversity to sustainable agricultural practices remains poorly understood, particularly across multiple groups of organisms. Here, we evaluated the effects of organic amendments, cropping pattern diversification, and microbial inoculation on soil biodiversity using 23 treatment-control pairs from 11 case studies across six Mediterranean countries and three different cropping systems. We employed an integrative methodology combining multitargeted DNA metabarcoding (16S rRNA, ITS, 18S rRNA, COI, Oligo01) and morphological identification (Macfadyen extractor and pitfall traps) to study the structural and functional diversity of soil microorganisms, microfauna, microarthropods, and macrofauna. Data from molecular methods differed between primers and from morphological data; specifically, 18S rRNA and COI yielded markedly different diversity estimates, and both underperformed morphological identification for soil fauna resolution, indicating the need for primer standardisation, increased soil input mass and methodological complementarity between molecular and morphological approaches. Overall, our results show that compost addition positively influenced prokaryotic chemoheterotrophs, epigeic Collembola, spider richness, and total macrofauna abundance, with mulching eliciting broader positive responses. Cropping pattern diversification (i.e., rotations, cover crops, intercropping) more frequently enhanced than reduced soil biodiversity metrics, with 32% of effect sizes being moderately positive compared to 24% moderately negative. Microbial inoculations had limited influence on biodiversity, consistent with their intended role of enhancing specific functions while minimising disruption to resident communities. Although positive responses to sustainable agricultural practices were more frequent than negative

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https://doi.org/10.1016/j.soilbio.2026.110284
Received 24 October 2025; Received in revised form 17 June 2026; Accepted 7 August 2026
Available online 8 August 2026
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ones, effects were often not statistically significant and were taxon- and context-dependent, highlighting the importance of site-specific assessments.

1. Introduction

Global food demand is projected to increase by 35–56% between 2010 and 2050 (van Dijk et al., 2021), while the majority of the global population remains dependent on soil-grown, plant-based diets for subsistence (FAOITPSGSBISCBDEC, 2020). Current agricultural practices, however, are unlikely to meet future food needs due to ongoing land degradation, declining soil fertility, climate change, and increasing frequency of pest outbreaks and extreme weather events (Newbery et al., 2016; Smith and Myers, 2018). Considering these multifaceted challenges, a more resilient agricultural model that incorporates sustainable management practices and makes sustainable use of natural resources is essential (Bange et al., 2016). Understanding the individual contribution of each of these practices to soil biodiversity is a necessary first step towards designing more integrated and context-appropriate agricultural systems.

Soil biodiversity plays a key role in maintaining soil fertility (Delgado-Baquerizo et al., 2020), enhancing crop resilience to pests and climate-related abiotic stressors (de Vries et al., 2020), and regulating food production (Romero et al., 2024). Agricultural diversification, the deliberate introduction of functional biodiversity across spatial and temporal scales, seeks to restore biotic interactions that sustain yield-supporting ecosystem services (Kremen et al., 2012). This can be achieved through organic amendments (e.g., manure, compost, crop residues) and reduced soil disturbance (e.g., minimal tillage) (Epelde et al., 2018; Kremen et al., 2012). Functional diversity can also be promoted by increasing crop diversity through intercropping and rotation schemes, or by enhancing non-crop biodiversity via features such as flower strips, hedgerows, and seminatural habitats (Tamburini et al., 2020). Inoculation with beneficial soil microorganisms (e.g., arbuscular mycorrhizal fungi, nitrogen-fixing and other plant growth-promoting bacteria) is another approach, with native strains often yielding the most consistent benefits for soil microbial communities (Li et al., 2024).

The study of soil biodiversity is inherently challenging due to the immense abundance, taxonomic, and functional diversity, as well as the wide variability in the size and morphology of soil organisms (Anthony et al., 2023). It is common to classify soil organisms into four categories based on body length (Bagyaraj et al., 2016): macrofauna (500 μm–50 mm), mesofauna (80 μm–2 mm), microfauna (5–120 μm), and microorganisms (1–100 μm). All of these groups are embedded in a highly complex trophic network, where organic matter decomposition is primarily driven by microorganisms due to their metabolic versatility and wide array of enzymes (Epelde et al., 2020; Potapov, 2022). Soil microorganisms are, in turn, structured by a diverse community of invertebrates, with whom they interact through direct predator-prey relationships, as well as via indirect mechanisms such as competition for resources, transport of propagules, and habitat modification (Scheu et al., 2005). To accurately assess the impact of agricultural practices on soil biodiversity, it is essential to include multiple taxonomic groups, thereby capturing potential changes across the full spectrum of the soil trophic web (Neyret et al., 2024).

Identifying the wide range of taxonomic groups present in soil samples requires substantial taxonomic expertise (André et al., 2001), as well as considerable time and financial investment (Querner and Bruckner, 2010), limiting the scalability of biodiversity assessments. In response, the increasing adoption of molecular techniques in biodiversity research has begun to transform soil ecological studies by offering more efficient and comprehensive approaches to characterise soil communities (Griffiths et al., 2006; Oliverio et al., 2018). Among these techniques, environmental DNA (eDNA) approaches have become particularly prominent due to their cost-efficiency and scalability. Environmental DNA can be extracted from environmental samples, and then preserved, amplified, sequenced, and taxonomically assigned based on sequence data (Deiner et al., 2015; Ruppert et al., 2019). Nevertheless, these approaches often suffer from limited validation and ecological contextualization, which hinders their interpretability (Donhauser et al., 2023; Köninger et al., 2025).

Besides, selecting appropriate metrics to evaluate the impact of agricultural practices on soil biodiversity is intrinsically difficult, as biodiversity spans taxonomic, phylogenetic, and functional dimensions that cannot be fully captured by a single metric (Ferrier, 2002; Liu et al., 2018). Although species richness and Shannon's index are widely used, they provide only partial insights. Increasing attention is being given to functional trait-based metrics, as it is the ecological functions of organisms, rather than their taxonomic identities, that are most critical for sustaining agricultural systems (Epelde et al., 2025; Hedde et al., 2022; Violle et al., 2007).

Apart from that, agricultural management involves a multiple set of practices, complicating comparisons across farms and farming systems (van Rijssel et al., 2025). Although numerous studies have examined the effects of agricultural practices on soil biodiversity (Lori et al., 2025), a lack of methodological standardisation has hindered cross-site and cross-study comparisons.

Using standardised and harmonised sampling, multitargeted metabarcoding and morphological identification protocols, including analyses conducted in the same laboratories, this study aimed to determine how sustainable agricultural practices (organic amendments, cropping pattern modifications, and microbial inoculations) can influence the structural and functional diversity of soil microorganisms, microfauna, microarthropods, and macrofauna in a variety of Mediterranean cropping systems. To this end, we analysed 23 treatment-control pairs from 11 case studies across six countries and three crop types. We hypothesised that sustainable agricultural practices would generally exert positive effects on soil biodiversity, although the magnitude and direction of these responses would vary among taxa, functional groups and management interventions. As a secondary objective, we compared multitargeted metabarcoding and morphological identification approaches to evaluate their consistency and complementarity in detecting biodiversity patterns and responses to agricultural management. We hypothesised that morphological identification would offer higher taxonomic resolution, whereas multitargeted metabarcoding would be sufficient to detect biodiversity responses to the sustainable agricultural practices.

2. Materials and methods

2.1. Agricultural practices

As part of the ReCROP project, we monitored soil biodiversity across 11 case studies in six countries: France, Italy, Morocco, Portugal, Spain, and Tunisia (Fig. 1). These case studies focused on three cropping systems: aromatic plant fields, maize fields, and vineyards. For this study, only treatment-control pairs differing by a single management factor were included, ensuring that observed differences could be attributed to the agricultural practice under investigation rather than to differences in site conditions, crop type or overall management system. The implemented practices fell into three categories: (i) organic amendments, including compost or mulch application (6 treated–control pairs); (ii) cropping pattern modifications, such as crop rotation, intercropping, or cover cropping (7 treated–control pairs); and (iii) microbial inoculations (bioinoculations) involving bacteria and fungi applied individually or in

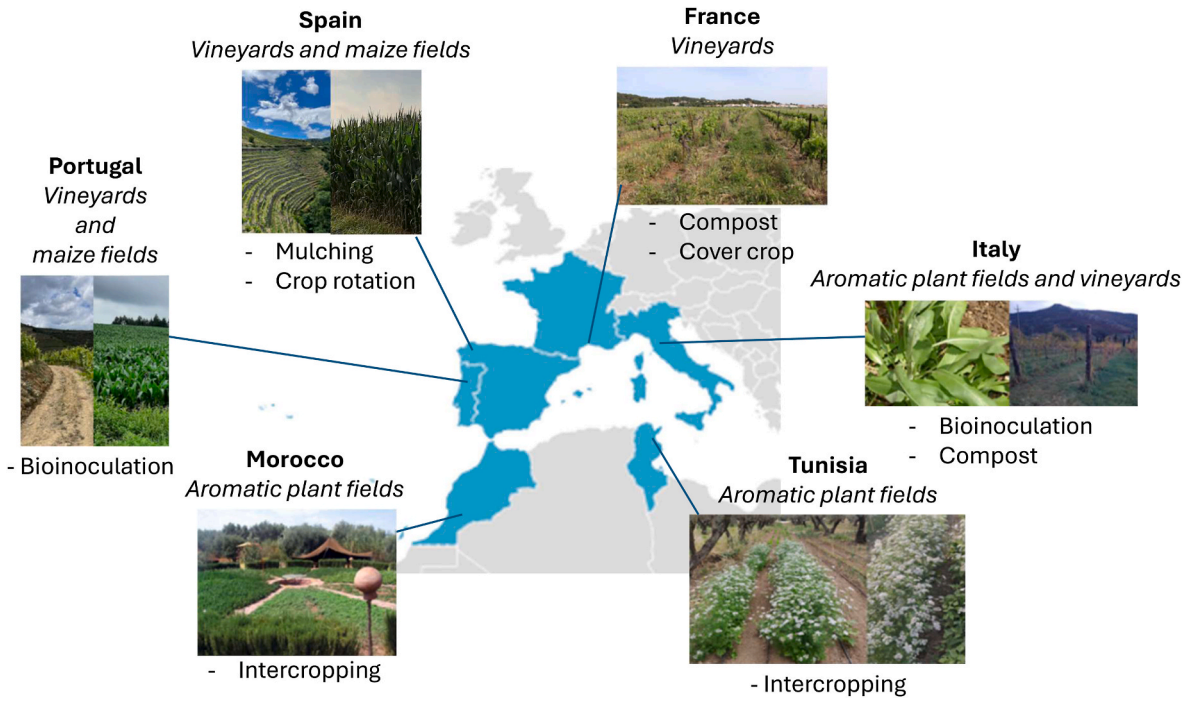

Fig. 1. Map showing the locations of the ReCROP case studies where soil biodiversity was measured. Created with Datawrapper.

consortia (10 treated–control pairs) (Fig. 2). Further site-specific details and descriptions of the implemented practices are available in Supplementary Table 1. Soil samplings were conducted between May 2022 and February 2024, exclusively during periods of mild climatic conditions, which are generally associated with increased activity and diversity of soil biota (Singh et al., 2021; Hillgén et al., 2024).

2.2. Multitargeted metabarcoding

In each case study, one composite soil sample was collected from each experimental plot (treatment and control). Each composite sample consisted of 20 randomly collected soil cores (0-10 cm depth, where microbial diversity is concentrated; Eilers et al., 2012) obtained using a soil core sampler and subsequently pooled. The number of replicate treatment and control plots varied among case studies, ranging from three to six replicates per treatment according to the experimental design implemented at each site (see Supplementary Table 1). All soil samples were stored at 4 °C and immediately sent to NEIKER Institute for analysis. Once in the laboratory, soils were sieved to < 2 mm. The DNeasy PowerSoil Pro kit (Qiagen, Hilden, Germany) was used to extract DNA from 0.25 g of soil in triplicate, to better capture the inherent spatial heterogeneity of soil microbial and faunal communities. Following extraction, DNA quantity and quality were determined using a Qubit fluorometer (Thermo Fisher Scientific) and a NanoDrop™ One spectrophotometer (Thermo Fisher Scientific), respectively. Extraction replicates were pooled and the resulting samples stored at −20 °C.

Library preparation and Illumina paired end sequencing (250 bp, 30K tags per sample) was carried out by Novogene (Cambridge, UK). The regions chosen for amplicon sequencing were the following: 16S rRNA (V4) 515F-806R (5′-GTGCCAGCMGCCGCGGTAA-3′, 5′-GGACTACHVGGGTWTCTAAT-3′) for prokaryotes (Apprill et al., 2015; Parada et al., 2016); ITS (ITS1) 1 F F-1FR (5′-CTTGGTCATTTAGAGGAAGTAA-3′, 5′-GCTGCGTTCTTCATCGATGC-3′) for fungi (White et al., 1990); 18S rRNA (V9) 1380F-1510R (CCCTGCCHTTTGTACACAC, CCTTCYGCAGGTTCACCTAC) for total eukaryotes (Amaral-Zettler et al., 2009); COI (5-GGWACWGGWTGAACWGTWTAYCCYCC-3′, 5′- TAAACTTCAGGGTGACCAAARAAYCA-3′) for metazoa (Leray et al., 2013); and Oligo01 (5′-CAAGAAGACCCTATAGAGCTT-3′, 5′-CCTGTTATCCCTAAGGTARCT-3′) for Oligochaeta (Bloor et al., 2021). PCR amplifications were performed in 15 μL reactions containing Phusion High-Fidelity PCR Master Mix (Thermo Fisher Scientific), 0.2 μM each of forward and reverse primers, and approximately 10 ng of template DNA. Thermal cycling conditions consisted of an initial denaturation at 98 °C for 1 min, followed by 30 cycles of denaturation at 98 °C for 10 s, annealing at 50 °C for 30 s, and extension at 72 °C for 30 s, with a final extension at 72 °C for 5 min. The molecular data from this study have been deposited in Zenodo (10.5281/zenodo.15640853).

Quality control of the reads was performed using FASTQC software (Andrews, 2010). Then, different bioinformatic workflows were applied to the amplicon datasets according to marker-specific analytical pipelines established by the respective project working groups. For 16S rRNA and ITS, PCR primers were removed from the sequences using Cutadapt (Martin, 2011). The resulting FASTQ files were further analysed via *QIIME2* (Bolyen et al., 2019) as follows: sequences were imported into QIIME2 as *PairedEndSequencesWithQuality*, after which both reads were joined by the *qiime vsearch join-pairs* plugin. Then, low-quality reads were filtered using the *QIIME qualityfilter Q-score-joined* command with the default options. In the next step, deblur (Amir et al., 2017) was used for denoising (qiime deblur denoise-16S), after which the resulting reads were classified using the *qiime feature-classifier classifysklearn* and *silva-132-99-nb-classifier.qza* command as the reference model for assigning taxonomical classification to amplicon sequence variants (ASVs). The singletons were removed by the *QIIME feature-table filter-features* command, and contaminant and unclassified ASVs were also removed by the *QIIME taxa filter-table*. A table summarising the ASVs was created using a *qiime feature-table summarize* command.

Sequence read pairs resulting from 18S rRNA, COI and Oligo01 amplicons were first overlapped using *vsearch* v2.7.1 (Rognes et al., 2016; allowing up to 40 mismatches). Primers were then removed using *cutadapt* (discarding all sequences differing more than two bp to

Fig. 2. Summary of sustainable agricultural practices tested in ReCROP. Created with Biorender.

the correct primer sequence or being incomplete). Using *vsearch,* we also discarded all sequences with more than one expected error or outside of the expected amplicon sequence lengths: 100–180 bp for 18S, 274–333 bp for COI, and 100–150 bp for Oligo01. The remaining sequences were de-replicated and sorted by abundance using *vsearch* and clustered into sequence variants (SVs) using SWARM v2 (Mahé et al., 2015). Abundances of unique sequences across samples were retained to construct an SV contingency table, using the scripts *fasta_merging.py* and *matrix_creation.py*, of SLIM (Dufresne et al., 2019), discarding SVs with a total abundance across samples of one (singletons). Putative chimeras were identified and removed using *vsearch*, in reference-based mode (using the same taxonomic databases as later used for classification), followed by *de novo* mode. To correct remaining PCR and sequencing artefacts and merge intra-specific or intra-genomic SVs, we also applied LULU post-clustering curation (Frøslev et al., 2017) with default parameters except for increasing minimum similarity to 97% for 18S. Curated SVs were aligned to marker-specific reference databases using blastn v2.6.0+ and taxonomically classified using CREST v3.1.0 (Lanzén et al., 2012; https://github.com/lanzen/CREST). SilvamodPR2 v138 was used as reference database for 18S rRNA SVs, Midori v253 for COI, and a custom database for Oligo01. The later was built by summarising and curating the Genbank taxonomy annotation from 18,974 sequences annotated as Oligochaeta and with the text "rRNA" or "ribosomal" appearing in the sequence name (search term: "(Oligochaeta[Organism]) AND (ribosomal OR rRNA)" using the NCBI web portal), and producing CREST-specific database files as described in the GitHub repository (https://github.com/lanzen/CREST/wiki/CREST-User-guide).

Functional prediction of amplicon sequencing data was performed using Faprotax (Louca et al., 2016) and Funguild (Nguyen et al., 2016) for prokaryotes and fungi, respectively. Trophic group assignment of protists was conducted following Singer et al. (2021).

2.3. Soil fauna sampling and morphological identification

2.3.1. Endogeic fauna

In each plot, three intact surface soil cores (5 cm depth, 6 cm diameter) were collected and transported to the laboratory for microarthropods extraction using a Macfadyen-type high thermal gradient device (Macfadyen, 1961), according to ISO 23611-2 (2006). Collembola and Acari were counted. Acari were separated into three groups: Oribatida, non-uropod Gamasida (GNU), and other Acari. Collembola were isolated and initially identified to the morpho-species level under a stereomicroscope (Kyowa Optical, SDZ-TR-PL, 7–45× magnification), and to the species level when possible. Specimens were cleared in Marc André solution I and mounted in Marc André solution II (André, 1946). Species identification was verified using phase-contrast microscopy (Zeiss Axio Scope A1, 200–630× magnification) and based on several dichotomous keys (Bretfeld, 1999; Dunger and Schlitt, 2011; Jordana, 2012; Potapov, 2001; Thibaud et al., 2004). Identified species were assigned to one of three ecomorphological categories (epedaphic, hemiedaphic, or euedaphic) according to Gisin's (1943) life-form classification, based on habitat preferences and morphological traits. Additionally, an ecomorphological index was calculated following the method of Joimel et al. (2017).

Collembola were isolated and then identified at morpho-species level under a stereo-microscope (Kyowa optical, SDZ-TR-PL, 7 to 45× magnification). Collembola were identified at species level when possible. Collembola specimens were clarified in Marc André solution I and mounted in Marc André solution II (André, 1946). Species identification was checked using phase-contrast microscopy (Zeiss, Axio Scope A1, 200 to 630× magnification) according to various dichotomous keys (Bretfeld, 1999; Dunger and Schlitt, 2011; Jordana, 2012; Potapov, 2001; Thibaud et al., 2004). Collembola species were assigned to one of three ecomorphological categories (epedaphic, hemiedaphic, euedaphic) corresponding to life forms based on habitat preferences defined

by Gisin (1943), using morphological traits. An ecomorphological index was subsequently calculated following Joimel et al. (2017). Body length was also measured.

2.3.2. Epigeic fauna

Arthropods were captured using pitfall traps of 8 cm deep (100 ml), half-filled with a non-attractive and non-toxic preservative (monopropylene glycol) (Dubs et al., 2018). In each plot, 5 pitfall traps were placed randomly, with a minimum distance of 3 m between each trap. Pitfall traps were collected 8 days after their installation. Organisms collected in the pitfall were sorted in the laboratory and transferred to 90% ethanol for conservation. In each sample, all trapped individuals were counted. For subsequent analyses, we focused on the total abundance of beetles (Coleoptera), epigeic springtails (Collembola), and rove beetles (Coleoptera, Staphylinidae), which were particularly abundant and well represented. In addition, particular attention was given to carabids (Coleoptera: Carabidae), spiders (Arachnida: Araneae), and ants (Hymenoptera: Formicidae), which were isolated and identified mostly to the species level (and, in some cases, to the genus level) using dichotomous keys and online taxonomic resources. Specifically, ants were identified using Blatrix et al. (2013), spiders using online taxonomic resources (Nentwig et al., 2025), and carabids using the Xper3 online key (Heintz, 2015) as well as Hurka (1996), Jeannel (1941), and Roger et al. (2014).

The sum of the individuals captured in the 5 traps of the same plot was used to estimate abundance-activity. The total number of species obtained in the 5 traps of the same plot was used to estimate species richness and Shannon index.

Traits related to dispersal capacity and resource acquisition were selected for spiders and carabids. For carabids, body length, diet, and wing development were considered, while for spiders, body length and hunting strategy were included. Trait information for each species was primarily obtained from the BETSI database (Biological & Ecological Functional Traits of Soil Invertebrates) (Hedde et al., 2012). For species not recorded in BETSI, additional data were sourced from the literature. The morphological identification data for this study have been deposited in Zenodo (10.5281/zenodo.16576909).

2.4. Statistical treatment

Richness and Shannon's diversity indices were calculated for the studied taxa using both molecular and morphological approaches, with abundance values additionally considered in the morphological analysis. Then, correlations were calculated between biodiversity metrics derived from different primer sets, as well as between molecular and morphological methods, to assess the consistency and comparability of soil biodiversity estimates across methodological approaches. For each case study, the mean, standard deviation, and sample size for both treatment and control groups were extracted for various structural and functional metrics of microorganisms, microfauna, microarthropods, and macrofauna. Hedges' g was calculated as the standardised mean difference for each individual study using the *escalc(.)* function in the *metafor* package for R (Viechtbauer, 2010). A random-effects model was subsequently employed to synthesise effect sizes for each metric, yielding the pooled Hedges' g and its corresponding 95% confidence interval using the *rma(.)* function with the Restricted Maximum Likelihood (REML) method. The results were visualised using forest plots generated with the *ggplot2* package (Wickham et al., 2007). Finally, a circular network plot was generated using the R package *circlize* (Gu et al., 2014) to visualise positive and negative associations between agricultural management practices and soil biodiversity metrics. For each taxon, the mean Hedges' g value was calculated for all biodiversity metrics, with the sign inverted for metrics representing clearly undesirable traits (e.g., prokaryotic human pathogens and fungal plant pathogens). Edges were coloured green for Hedges' $g > 0.5$ and red for Hedges' $g < -0.5$, while intermediate values were left uncoloured.

3. Results

3.1. Molecular vs. morphological approaches for metazoan diversity assessment

Two primer sets, COI and 18S rRNA, were used to target metazoan diversity. Average metazoan richness was markedly higher with COI (mean = 304) than with 18S rRNA (mean = 108), though the two primers yielded positively correlated metrics (Supplementary Fig. 1). However, 18S rRNA generally provided higher alpha diversity at lower taxonomic levels; for instance, average Collembola richness was 0.95 with COI versus 6.65 with 18S rRNA.

Metabarcoding did not match the resolution of morphological identification for most faunal taxa, and correlations varied by group. For example, no significant correlation was observed between Collembola richness and Shannon indices from metabarcoding versus morphological methods (Fig. 3). Similarly, metabarcoding detected only five carabid (Carabidae, 18S rRNA only), six spider (Araneae, 18S rRNA only), and four ant (Formicidae, COI only) ASVs across all samples, and correlation with morphological data was poor (Supplementary Fig. 2). Nonetheless, metabarcoding enabled the detection of faunal groups not targeted by the sampling strategy. Earthworms, amplified with a specific primer pair, showed limited and scattered detection, while nematodes were well represented by both COI and 18S rRNA, though their results would require validation through morphological identification.

Consequently, when morphological identification data were available, they were prioritised over metabarcoding data for subsequent analyses. Additionally, COI and 18S rRNA results were compared, and the primer yielding more complete data was selected for each taxonomic group.

3.2. Soil biodiversity responses to agricultural practices

In the context of standardised mean differences, such as Hedges' g or Cohen's d, a value around 0.5 is commonly interpreted as a moderate effect size, with thresholds of 0.2, 0.5, and 0.8 conventionally corresponding to small, medium, and large effects, respectively (Cohen, 1988). When assessing the effects of all agricultural practices on soil biodiversity, i.e. comparing conventional and alternative management systems, only the total abundance of macrofauna captured with pitfall traps showed a statistically significant increase under alternative management (Supplementary Fig. 3e). Additionally, both the richness of endogeic Collembola and the abundance of epigeic Collembola exhibited positive Hedges' g values exceeding 0.5 (Supplementary Fig. 3c and e).

When analysing management types (i.e., organic amendments, cropping pattern modifications, bioinoculations) separately, compost addition showed statistically significant positive effects on the abundance of epigeic Collembola and total macrofauna abundance (Fig. 4e), and large effect sizes (>0.8) for prokaryotic richness, chemoheterotrophs, and spider richness (Fig. 4a,b,e). Conversely, compost addition had a large negative effect on the Shannon index of Crassiclitellata (Fig. 4e). Overall, compost addition produced effect sizes > 0.5 in 13 of 57 metrics (9 positive, 4 negative), while mulching produced such effects in 32 metrics (22 positive, 10 negative), although none were statistically significant (Fig. 4). Several metrics showed large positive or negative trends under mulching, including increased values for prokaryotic human pathogens, fungal saprotrophs, phototrophic protists, and richness and Shannon of Collembola, carabids, and ants. Reduced values included prokaryotic Shannon, nitrogen-fixers, parasitic protists, and abundance of Collembola (Fig. 4).

In relation to cropping patterns, the only statistically significant and negative effects were observed for nematode richness and Shannon diversity (18S rRNA) under cover crops (Fig. 5a). Additional metrics under cover crops showing large negative effect sizes included those related to carabids, spiders, and coleoptera abundance, and Crassiclitellata

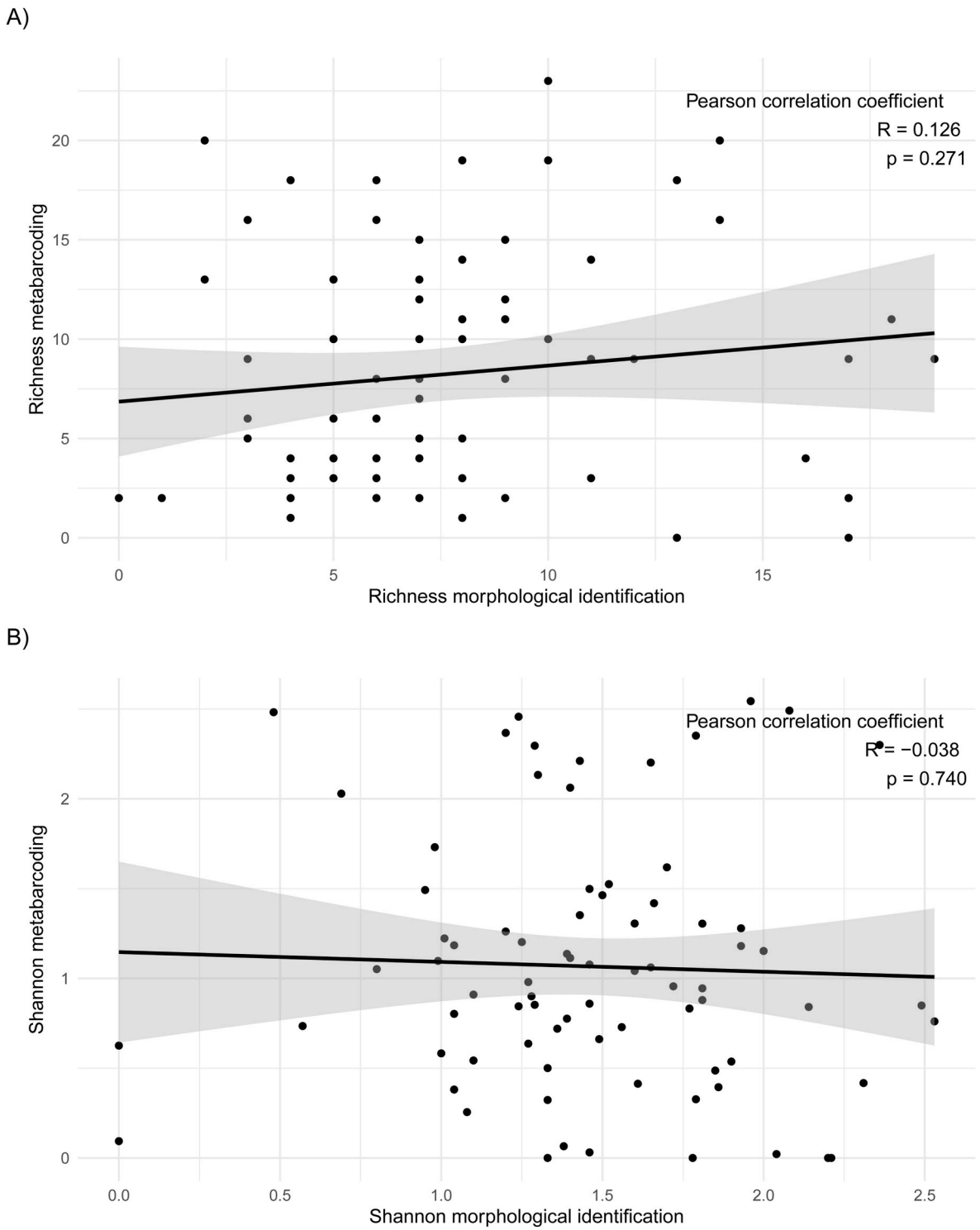

Fig. 3. Pearson correlations between (A) richness (R = 0.126, p = 0.271) and (B) Shannon (R = −0.038, p = 0.740) diversity metrics of Collembola obtained through 18S rRNA metabarcoding and morphological identification (n = 78).

Shannon diversity (Fig. 5e). Conversely, 21 metrics showed large positive effects, including prokaryotic richness and Shannon diversity, several microbial traits (e.g., chemoheterotrophs, saprotrophs, pathogens), collembola richness and abundance, epigeic fauna, and macrofaunal metrics. This resulted in 37 out of 57 metrics with effect sizes > 0.5 (21 positive, 16 negative) (Fig. 5). For intercropping, 18 of 43 metrics surpassed this threshold (11 positive, 7 negative), with large positive effects on spider and ant diversity, as well as the abundance of coleoptera (especially rove beetles) and small spiders (0–5 mm body length). A large negative effect was observed on prokaryotic richness, smallest carabid beetles (0–5 mm), and spiders with intermediate body length (5–10 mm) (Fig. 5). Crop rotation showed strong positive effects on several soil biodiversity metrics, including those for protists and nematodes, as well as on the abundance of Acari (particularly Oribatida), carabids (notably smaller species), and Coleoptera. In contrast, negative effects were observed on fungal diversity and certain fungal traits, specific protist groups, larger-bodied Collembola, and carabid diversity. Overall, 33 out of 57 diversity metrics showed effect sizes

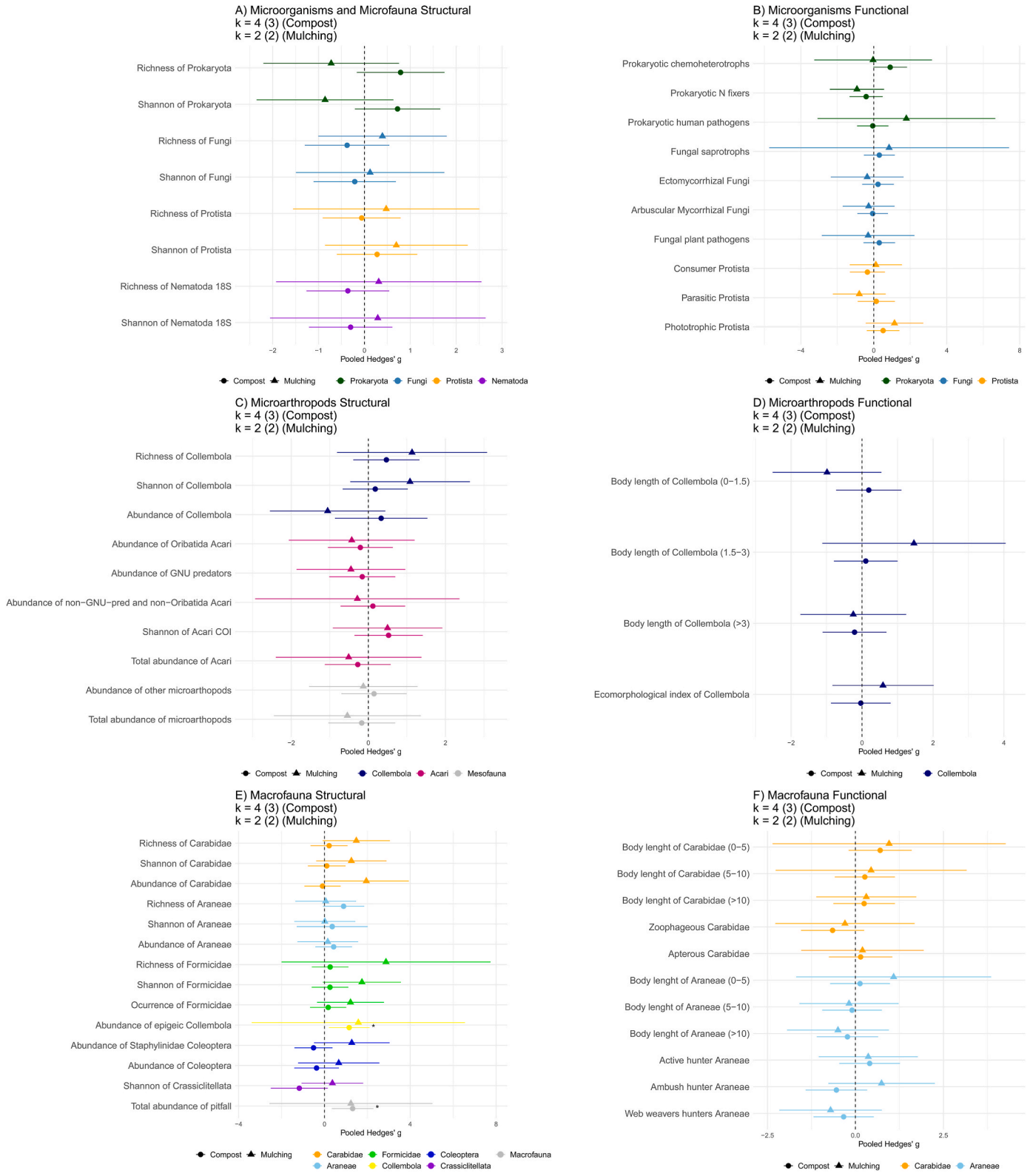

Fig. 4. Pooled effect sizes for (a) microorganisms and microfauna structural; (b) microorganisms functional; (c) microarthropods structural; (d) microarthropods functional; (e) macrofauna structural; and (f) macrofauna functional metrics for organic amendment practices. Annotations on the top of the plot refer to the number of comparisons in each metric/group of metrics (k) and, in parentheses, the number of studies they are taken from. Asterisks indicate when effect sizes are significantly different from zero.

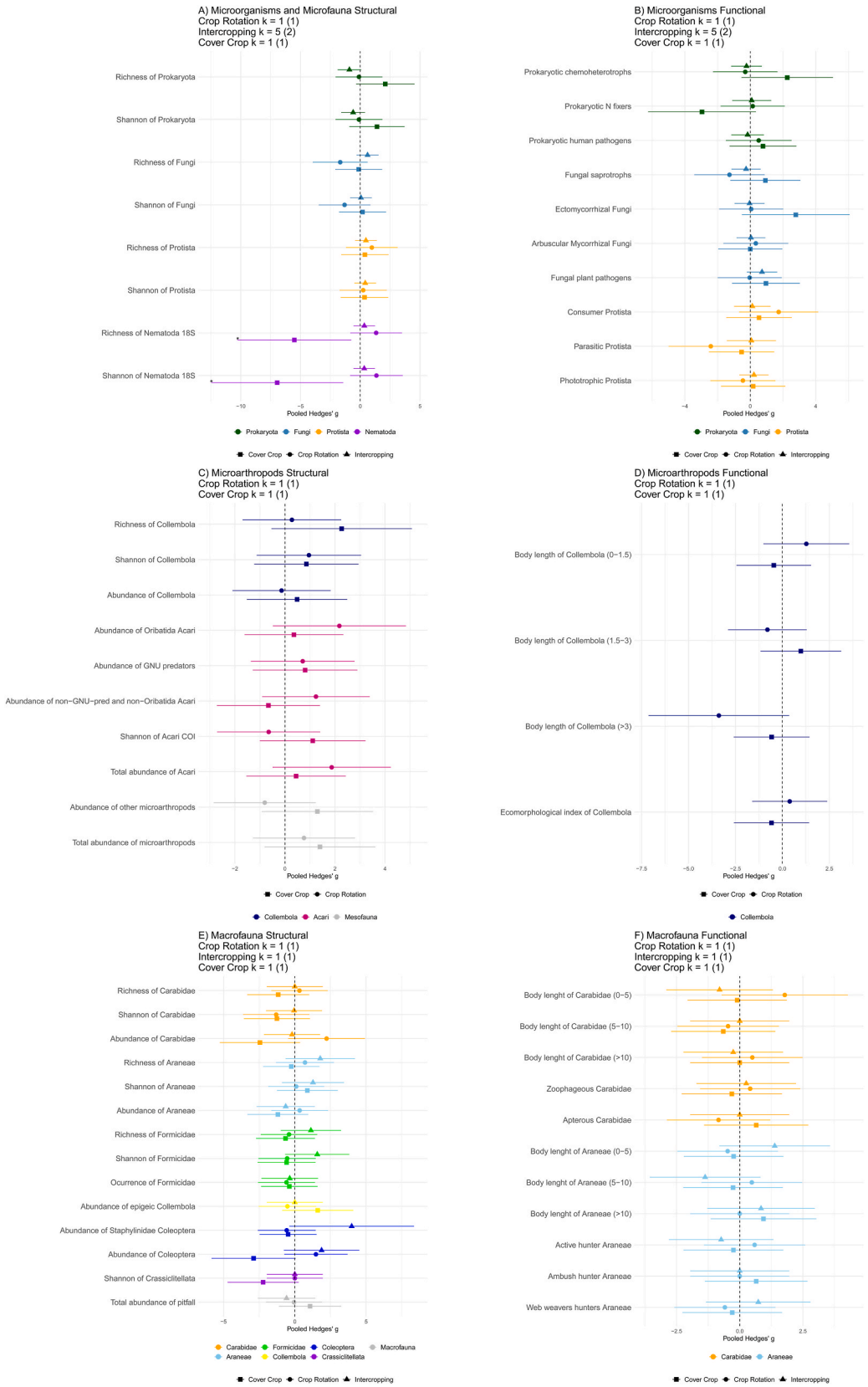

Fig. 5. Pooled effect sizes for (a) microorganisms and microfauna structural; (b) microorganisms functional; (c) microarthropods structural; (d) microarthropods functional; (e) macrofauna structural; and (f) macrofauna functional metrics for cropping pattern practices. Annotations on the top of the plot refer to the number of comparisons in each metric/group of metrics (k) and, in parentheses, the number of studies they are taken from. Asterisks indicate when effect sizes are significantly different from zero.

greater than 0.5 under crop rotation (18 positive, 15 negative) (Fig. 5).

Regarding bioinoculations, the only metric showing a statistically significant and positive effect from inoculated samples compared to controls was the Shannon diversity of Acari detected using COI primers (Fig. 6c). In contrast, both the abundance of oribatid Acari and that of non-GNU predator and non-oribatid Acari exhibited large negative effect sizes (Fig. 6c). Overall, only 6 of the 57 evaluated metrics displayed Hedges' *g* values exceeding 0.5 (two positive and four negative) (Fig. 6).

Fig. 7 summarises the overall associations between management practices and all the soil taxa. Some practices, such as bioinoculation, showed no consistent effects on soil biodiversity. Compost amendments were primarily associated with negative responses in Crassiclitellata, possibly because compost addition often reduces earthworm diversity by favouring species that are better adapted to thrive under such conditions. In contrast, mulching and intercropping were positively associated with some groups, such as carabids (only for mulching), ants, and coleoptera. Crop rotation and cover crops displayed both positive and negative associations, depending on the taxon. Similarly, some taxa, such as protists and spiders, showed no consistent response to management, while others exhibited exclusively negative (e.g., Crassiclitellata), exclusively positive (e.g., prokaryotes, collembola, acari), or mixed associations (e.g., fungi, nematodes, carabids, ants).

4. Discussion

4.1. Molecular vs. morphological approaches for metazoan diversity assessment

The simultaneous deployment of multitargeted metabarcoding and morphological identification in this study was a deliberate methodological choice grounded in the recognised complementarity of these approaches. By applying both approaches to the same samples across 11 case studies, we were able to cross-validate biodiversity estimates, identify the specific contexts in which molecular methods perform adequately, and determine where morphological data should be prioritised. This has direct implications for the interpretation of our results: when available, morphological data were given preference, ensuring that management-driven biodiversity responses were interpreted on the

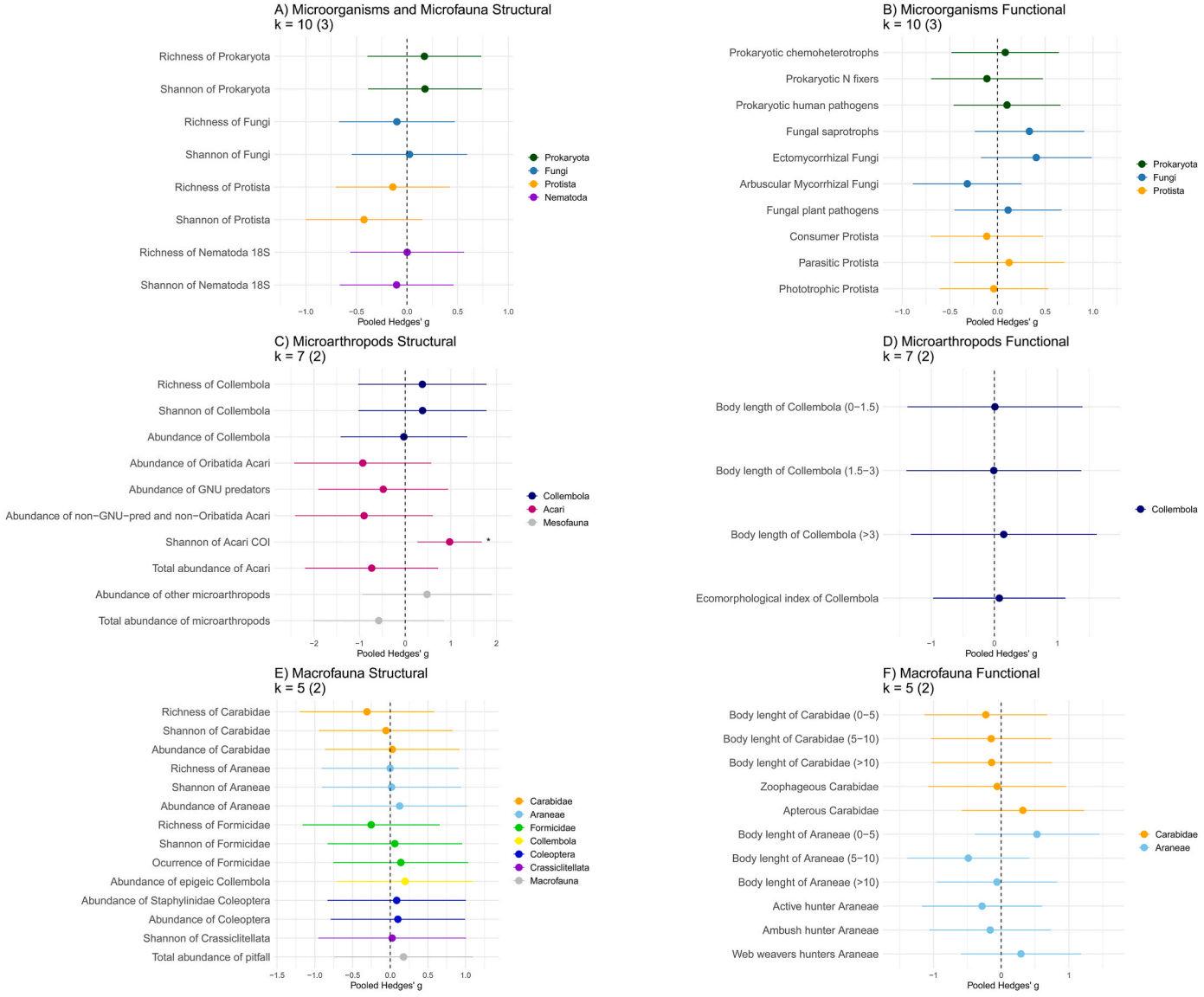

Fig. 6. Pooled effect sizes for (a) microorganisms and microfauna structural; (b) microorganisms functional; (c) microarthropods structural; (d) microarthropods functional; (e) macrofauna structural; and (f) macrofauna functional metrics for bioinoculation practices. Annotations on the top of the plot refer to the number of comparisons in each metric/group of metrics (k) and, in parentheses, the number of studies they are taken from. Asterisks indicate when effect sizes are significantly different from zero.

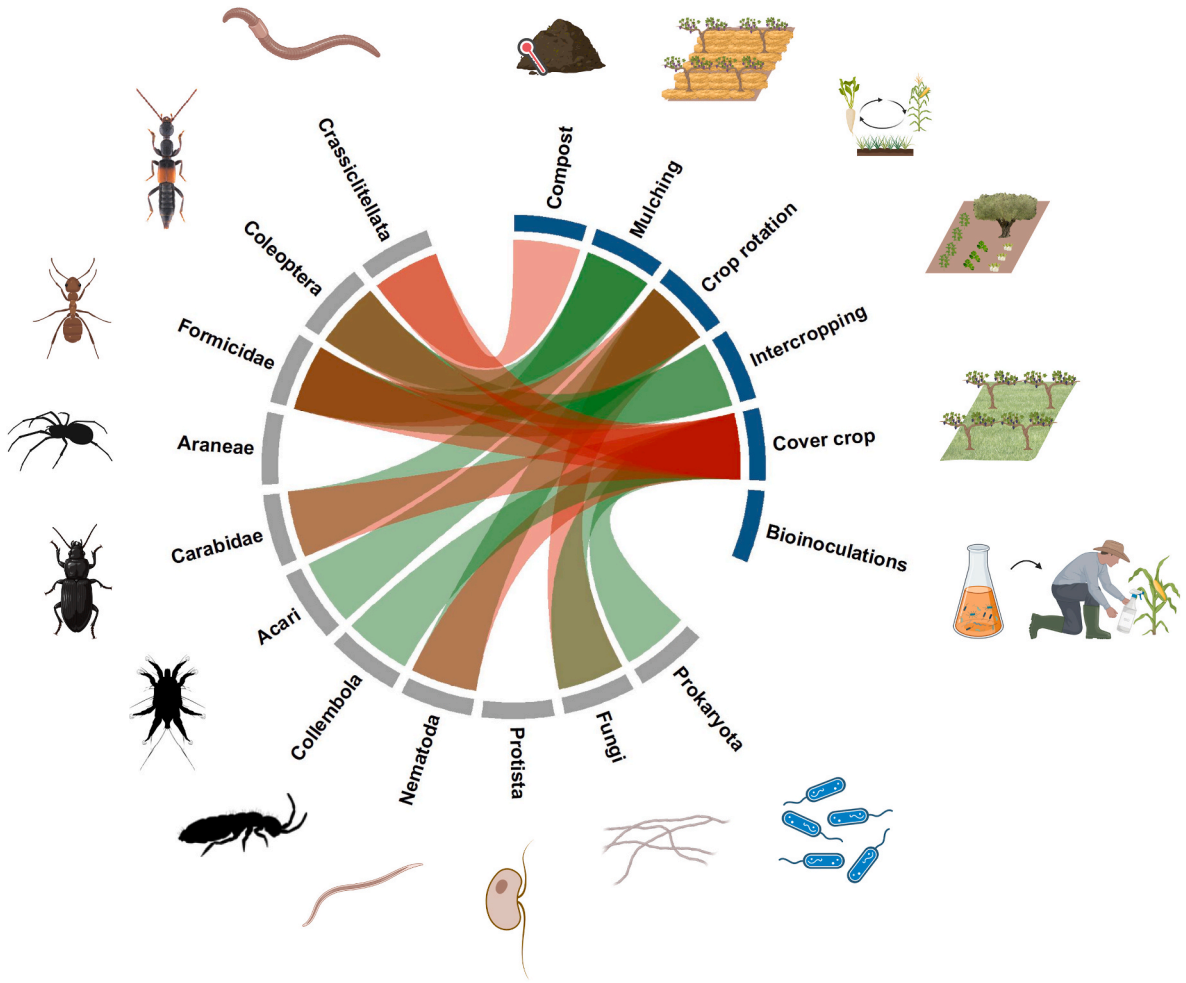

Fig. 7. Circular diagram illustrating associations between soil taxa and management practices. Green links indicate moderate positive associations (mean Hedges' $g > 0.5$), while red links indicate moderate negative associations (mean Hedges' $g < -0.5$) based on the average effect size of the diversity metrics calculated for each taxon. Created with Biorender.

basis of the most reliable evidence available.

COI metabarcoding yielded more sequences than 18S rRNA, yet 18S rRNA generally allowed finer taxonomic resolution. It has previously been described that universal primers such as those targeting COI, exhibit heterogeneous amplification efficiencies across taxa due to sequence mismatches and GC-content variation, potentially biasing detection and underrepresenting less compatible taxa (Ficetola and Taberlet, 2023). Therefore, despite a positive correlation between the diversity metrics from both primers, primer choice remains a critical limitation for inter-study comparability.

Oliverio et al. (2018) showed that COI-metabarcoding-based methods can properly reflect arthropod diversity, but our data reveal a poor correspondence between molecular and morphological datasets. It is important to note that the direct observation methods employed in this study (the Macfadyen-type high thermal gradient extractor and pitfall traps) depend on the active movement of individuals and therefore capture only living organisms. In contrast, direct DNA extraction from soil detects genetic material from both living and dead or inactive fauna, which may partially explain the differences observed between the two approaches, as suggested by Donhauser et al. (2023). Nevertheless, the results obtained underscore the need for improved, standardised, and validated molecular protocols. Taxon-specific primers (Calderón-Sanou et al., 2022) or metagenomic approaches (Schmidt et al., 2022), along with more complete reference databases (Keck et al., 2022), may enhance accuracy. Furthermore, the low and inconsistent detection of certain taxa may stem from an insufficient amount of soil sample: we used 0.75 g soil per sample, compared to 0.60 g in the LUCAS protocol (Köninger et al., 2023), whereas studies using ≥10 g show better macrofauna recovery (Lilja et al., 2023). Future studies targeting fauna via eDNA should consider substantially larger soil inputs or complementary bulk tissue approaches (Oliverio et al., 2018) to improve detection reliability.

Molecular methods dominate large-scale surveys due to efficiency, but they lack the resolution of morphological identification, which remains laborious and expertise-intensive (Köninger et al., 2025). AI-assisted optical imaging and bioacoustic monitoring may provide a scalable alternative to traditional morphological approaches (Dombos et al., 2017; Robinson et al., 2024). While these technologies generate large volumes of high-dimensional data that require robust analytical pipelines capable of addressing the complexity and heterogeneity of ecological datasets, recent advances are beginning to resolve key methodological limitations (Belaud et al., 2025). Finally, we agree with Köninger et al. (2025) that integrative approaches are indispensable, as molecular approaches reveal cryptic and microscopic taxa while morphological approaches supply key trait and abundance data.

4.2. Soil biodiversity responses to agricultural practices

When the effects of all practices were examined collectively, across 23 treatment–control pairs from 11 case studies in six countries and three crop types, only three metrics (i.e., richness of Collembola, abundance of epigeic Collembola, and total macrofauna abundance)

showed moderate positive responses. This supports the notion that larger-bodied soil fauna may be more responsive to management changes, as suggested by Tsiafouli et al. (2015), due to greater mobility and/or traits that increase their sensitivity to habitat alteration. Effects at the macrofaunal level could subsequently cascade down to lower levels of the trophic chain (top-down effects). However, such conclusions should be drawn with caution, given the limited knowledge currently available on soil trophic relationships (brown food webs) (Delgado-Baquerizo et al., 2025; Potapov, 2022). Other factors expected to play important roles include the vertical strata (epigeic versus endogeic), the taxon's mobility and dispersal capacity, and its trophic group.

To enable clearer comparisons among similar interventions, management practices were grouped accordingly. Compost addition again positively influenced epigeic Collembola and total macrofauna abundance, along with increased prokaryotic richness and chemoheterotrophs, likely due to enhanced organic matter availability (Epelde et al., 2025; Giffard et al., 2022). Beyond stimulating decomposers, this increase in basal resources may also enlarge the prey pool available to predatory organisms, potentially reinforcing trophic interactions (Thomson and Hoffmann, 2007). Mulching, however, elicited broader and mostly positive responses across soil taxa compared to compost. The continuous surface habitat and moisture retention provided by mulching may create stable microclimatic conditions that better support diverse soil organisms. In contrast, organic amendments are often incorporated into the soil, causing short-term disturbance and more transient effects on soil communities.

Cropping patterns elicited the most widespread responses. Cover crops were associated with reductions in nematode richness and diversity, raising the question of whether specific functional groups were disproportionately affected (Zhang et al., 2024). In fact, for spiders, carabids, and Collembola, the effects also varied within each taxon depending on body size. However, both cover cropping and crop rotation consistently showed strong and generally positive effects on overall soil biodiversity. These outcomes may suggest that cropping patterns enhance biodiversity primarily through increased spatiotemporal heterogeneity in root structure and exudation profiles, but impacts remain group- and trait-specific.

In our study, bioinoculation had clearly the most limited impact on soil biodiversity. This can be taken as a positive outcome, as bioinoculation has been considered a deliberate introduction of non-native organisms that should have specific effects on particular functions but negligible overall effects on resident communities (Mawarda et al., 2020). Li et al. (2024) provide a meta-analysis of 335 studies, revealing a positive effect of microbial inoculants on soil microbial biomass. Although microbial inoculants did not alter microbial diversity, they induced major changes in the structure and bacterial composition of soil microbial communities, reducing the complexity of bacterial networks and increasing network stability.

Overall, these findings are consistent with Cozim-Melges et al. (2024), who concluded that no single management practice enhanced all taxonomic groups and that biodiversity responses depend on both the taxonomic group and the intervention type. In our case, we reached this conclusion through a study that analysed a relatively large and diverse set of case studies and control pairs using consistent methodologies and both multitargeted metabarcoding and morphological identification approaches. A limitation of our study remains the short duration of most field experiments, with sampling conducted between three months and four years after management implementation, which may underestimate long-term effects. Furthermore, the limited number of studies per management type constrains the generalizability of our findings. Finally, variability in edaphoclimatic conditions and crop systems likely also contributed to the observed heterogeneity in outcomes. This is supported by Fernández-Huarte et al. (2023), who, analysing nine long-term UK field trials, found that site-specific factors often exerted effects equal to or greater than those of the management interventions themselves.

4.3. Conclusions

These findings underscore the need for integrative, standardised approaches that combine molecular and morphological methods to accurately characterise soil biodiversity in agroecosystems. Based on our comparative analyses, we specifically recommend: (i) standardisation of primer choice across studies, as COI and 18S rRNA yielded markedly different diversity estimates and taxonomic resolution; (ii) an increase in soil input mass beyond the 0.75 g used here, given that this total input likely underrepresents larger and less abundant taxa; and (iii) systematic integration of morphological identification alongside metabarcoding, since the former provides abundance and trait data for soil fauna while the latter enables detection of microscopic taxa.

Within this specific set of 11 case studies spanning six Mediterranean countries and three cropping systems, sustainable management practices generated variable responses in both structural and functional soil biodiversity indicators, with positive responses being observed more frequently than negative ones for several taxa and management interventions. However, most individual effects were not statistically significant, and responses varied considerably among taxa, practices, and study contexts. Consequently, these results should be interpreted as indicative trends within the studied systems rather than as broadly generalisable conclusions for Mediterranean agriculture. Given the context-dependent nature of soil biodiversity responses, practice-specific assessments tailored to local edaphoclimatic conditions and cropping systems are strongly recommended.

Funding sources

This research has been financially supported by the grant ReCROP funded by (i) MICIU/AEI/10.13039/501100011033 (PCI2021-121935 and PCI2021-121981) and the European Union Next Generation EU/PRTR, (ii) Fundação para a Ciência e Tecnologia, (iii) the Moroccan Ministry of Higher Education, Scientific Research and Innovation (MESRSI)/DRSI under the framework of the Program for Research and Innovation solutions in the Mediterranean region (PRIMA/0002/2020). NEIKER acknowledges funding from the European Union's Horizon Europe research and innovation programme under grant agreement No. 101086179 (AI4SoilHealth). The UCP team also acknowledges support from the CBQF research centre through the FCT project UIDB/50016/2020.

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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.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.soilbio.2026.110284.

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