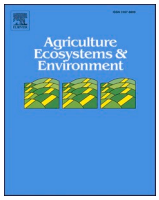
Agriculture, Ecosystems and Environment 414 (2027) 110730



Contents lists available at ScienceDirect

Agriculture, Ecosystems and Environment

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





Positive effects of regenerative organic agriculture on soil biology

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ARTICLE INFO

Keywords:
Regenerative organic agriculture
Soil biodiversity
Earthworms
Soil arthropods

ABSTRACT

Despite growing interest in regenerative organic agriculture (ROA), no well-replicated field experiment has simultaneously quantified the effects of multiple ROA practices on soil microbial, earthworm, and arthropod communities. The combined net effect of ROA practices (crop rotations, cover cropping, organic fertilization, minimum soil disturbance etc.) on key soil biological indicators has rarely been quantified. Here we use a plot-scale randomized block-design experiment to assess how multiple ROA practices have influenced the (1) composition of the soil microbial community, (2) abundance of earthworms, and (3) abundance and diversity of the soil arthropod community. We found that ROA had overall positive effects on soil biology. Phospholipid Fatty Acid (PLFA) and Neutral Lipid Fatty Acid (NFLA) analyses show how, over only two years since the introduction of ROA practices, the soil microbial community shifted towards an increasing abundance of fungal markers and in particular a significant increase of the 16:1ω5 biomarker for arbuscular mycorrhizal (AM) fungi. On the other side, soil bacterial groups that are generally considered to be more copiotrophic (which grow and reproduce rapidly in nutrient-rich environments), including Actinobacteria, increased in relative abundance in the conventional soils receiving synthetic nutrient fertilization. We also observed a significant increase in the number of earthworms in the ROA-managed soil. Number of earthworms in regenerative organic soils varied between 103 and 249 m^{-2} whereas in the conventional soils, the number of earthworms varied between 0 and 4 m^{-2}. We found that the total number of soil arthropods significantly increased in the regeneratively organic managed soils. The number of euedaphic arthropods adapted to life in the soil was higher in the regenerative organic soils as well as the number of epiedaphic generalist organisms, not strictly tied to the soil environment. The regenerative organic soils showed greater species richness, a more even distribution of species, and a more complex community structure than conventional soils, which receive synthetic fertilizer, pesticide and herbicide inputs, and are subject to greater soil disturbance. The increasing abundance of AM fungi and earthworms and the greater arthropod diversity observed in our regenerative organic soils strongly suggest that ROA may contribute to soil biological conditions that are associated with long-term sustainability and resilience. Many farmers and policymakers are reluctant to adopt regenerative organic practices partly because perceived benefits may take decades to materialize. Our findings within 2–3 years provides encouraging evidence that soil health benefits can accrue relatively quickly, potentially lowering perceived barriers to adoption.

1. Introduction

Regenerative Organic Agriculture (ROA) embraces a series of practices (e.g. minimum soil disturbance, crop rotations, cover crops, organic fertilization etc.), which contribute to ameliorate soil health in the long-term (Khangura et al., 2023; Regeneration International, 2017; Rodale Institute, 2014). There is an increasing number of studies showing the potential benefits of regenerative organic agronomic practices on soil health and quality across different agroecosystems from arable croplands to vineyards and grazing systems (Díaz de Otálora et al., 2021; Villat and Nicholas, 2023). ROA contributes to the accumulation of soil organic carbon (SOC) (Gattinger et al., 2012) facilitated by the adoption of practices such as reduced tillage, cover cropping, and ley-arable rotations (Jordon et al., 2022). Recent scientific evidence also suggests that ROA has a positive impact on soil biology by promoting greater soil microbial activity (Yang et al., 2024), microbial diversity and abundance (Prescott et al., 2021) and higher earthworm diversity (Llanos et al., 2025).

Despite ROA can have significant positive effects on soil biology, evidence from well-replicated field studies, which specifically addressed

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https://doi.org/10.1016/j.agee.2026.110730
Received 13 February 2026; Received in revised form 19 August 2026; Accepted 28 August 2026
Available online 8 September 2026
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how multiple ROA practices interact through time to affect soil microbial community composition is still scarce. In particular, we are still missing data from the simultaneous measurement of multiple biological indicators from plot-scale field experiments, which specifically compared ROA and conventional agriculture. The primary objective of this study was to quantify the combined net effects of multiple ROA practices on three key soil biological indicators: (1) microbial community composition, (2) earthworm abundance, and (3) arthropod community diversity and abundance. This set of indicators is useful because includes different trophic levels and functional roles—microbes as decomposers and symbionts, earthworms as ecosystem engineers, and arthropods as indicators of food web complexity—providing a holistic assessment of soil biological responses to management.

Recent evidence suggests that low-intensity soil management (i.e. longer crop rotations, organic fertilization, increased plant species diversity) contributes to increase the abundance of arbuscular mycorrhizal fungi (AM fungi) and fungal-to-bacterial ratios in both croplands and grasslands (Sünnemann et al., 2021). A field study which addressed changes in soil microbial community composition across nine years since land conversion from conventional to regenerative organic, found that the biomass of AM fungi increased in most croplands, together with fungal saprotroph, whereas putative fungal pathogens showed the opposite response (Lekberg et al., 2024). Similarly, a review paper on the effects of chemical-based *vs.* organic farming shows how the application of synthetic fertilizers and pesticides has a negative impact on the diversity of the soil microbial community and on the earthworm community (Datta et al., 2016).

Earthworms play a key role in enhancing soil health and soil ecosystem functioning (Liu et al., 2019). Findings from four organic farms located in the Pampas region of Argentina show how the highest abundance, biomass and species richness of earthworms were observed in natural grasslands, followed by large-scale organic farming whereas the lowest earthworm abundance was found in conventional agricultural systems (Rodríguez et al., 2025). Similarly, Hole et al. (2005) in their review study observed a general trend of higher earthworm abundance and biomass under organic management in comparison to conventional farming. These results, coupled with changes in the soil microbial community, suggest that a more comprehensive characterization of soil biology is needed to better understand potential soil ecosystem-level impacts of ROA *vs.* conventional agriculture. A comprehensive assessment of potential soil biology responses to management should also include changes in the diversity and abundance of the soil arthropod community. For example, the QBS-ar (Soil Biological Quality-arthropod) index has been shown effective in assessing differences in the biodiversity of the soil microarthropod community in human-managed soils (Menta et al., 2018).

A recent study carried out across 78 farms in Hungary found a negative correlation between QBS-ar and soil bulk density, suggesting a negative effect of soil compaction on soil microarthropod community (Tóth et al., 2025). A research study comparing organically and conventionally managed vineyards found that soils from organic systems exhibited higher QBS-ar values compared to the conventional systems (Ghiglieno et al., 2021).

Regenerative organic practices have the potential to reduce soil bulk density and increase soil moisture thanks to the use of cover crops, organic amendments, crop rotations, among others. Therefore, they can create better environmental conditions for different communities of key soil organisms including soil microbes, microarthropods and earthworms. No studies so far have simultaneously addressed how these soil communities have been impacted by multiple soil management practices (regenerative organic vs. conventional) using replicated field experiments, which lasted for at least few years. This provides a cleaner test of the effects of eliminating synthetic agrochemicals alongside other regenerative practices.

Here we used a replicated field experiment where we compared three different agroecosystems (cereal, tomato and a mixed crop system), which have been managed using ROA and conventional agricultural practices for 3 years. Our ROA treatment strictly excluded all synthetic fertilizers, pesticides, and herbicides. While the bundled nature of ROA practices prevents attribution to individual components, it also represents a strength: farmers implement multiple practices simultaneously. Our study therefore tests the real-world effectiveness of ROA as a holistic system, not the isolated effect of any single practice. We expect that potential ROA-induced changes in soil health will start with changes in soil biology, which will be detected in the 3-year timeframe of our field experiment. We measured changes in the soil (1) microbial, (2) microarthropod, and (3) earthworm communities. Our working hypotheses are that soils 'conditioned' by ROA management practices, which included crop rotations, cover cropping, animal manure amendments, minimum soil disturbance and no use of synthetic chemicals (synthetic fertilizers, pesticides and herbicides), will show: H1: Greater microbial biomass and abundance of AM fungi, H2: Greater earthworm abundance, H3: Greater arthropod diversity, when compared to conventional soils.

To our knowledge, this is the first well-replicated, plot-scale randomized block experiment to simultaneously examining microbial, earthworm, and arthropod responses during the early transition from conventional to ROA management.

2. Materials & methods

We conducted our study at the European Regenerative Organic Center (EROC) established in Parma, Italy (44.849394 N, 10.279305 E) in March 2022. Experiment 1 (Fig. 1) within EROC is laid out as a full randomized block design with four replicates, which includes two experimental treatments. The initial characteristics of the soil (March 2022) are shown in Table 1. Experiment 1 includes 56 experimental plots, arranged in 8 rows containing 7 plots each (Fig. 1). To avoid cross contamination from fertilizers drifts or water runoffs, the 4 blocks (2 rows of plots) were separated by grassland strips 8 m wide. Likewise, rows of plots were separated by 8 m wide grassland strips and by 3 m wide grassland strips between two adjacent plots in the same row (Fig. 1). Grassland strips in the corridors among plots consist of perennial ryegrass lawns. The size of each experimental plot is 36 m^2 (6 m x 6 m). The first experimental treatment is associated with the type of agroecosystem (conventional *vs.* ROA) where we compare (a) Conventional cereal (CC) *vs.* Regenerative organic cereal system (RC), (b) Conventional tomato (CT) *vs.* Regenerative organic tomato-based system (RT) and Conventional Mixed (CMS) *vs.* Regenerative organic mixed system (RMS); RMS includes a tomato-wheat-sunflower rotation managed either in conventional or regenerative organic. The second treatment is associated with the number of crops (i.e. plant species diversity) grown in a strip-cropping system, which is compared to monoculture. Here the comparison is between higher diverse Regenerative Vegetable strip-cropping systems (RV) *vs.* each of the Regenerative organic monocultures (RMon), which are currently growing in the strip-cropping system and change according to the specific crop rotations. A synthetic description of soil management differences between ROA and conventional agriculture is shown in Table 2, while a detailed description of the crop rotations, cover crops, fertilizers type and amount used in the experiment is provided in Annex 1. Note that this study mainly focuses on the potential effects of the first treatment (ROA *vs.* conventional) on soil biology and not the plant species diversity treatment. This is because we could not identify yet any potential species-diversity induced effect on soil biology (see below Data Analysis). Soil samples were collected following this timeline: in 2022 (time zero, soil characterization including PLFA soil sampling before the experimental treatments started), in 2023 (PLFA sampling), 2024 (PLFA, NLFA, earthworm sampling) and 2025 (arthropod sampling). Thus, soil was collected after 1, 2 and 3 growing seasons for different analyses.



Fig. 1. Layout of Experiment 1 established within the European Regenerative Organic Center (EROC) in Parma, Italy. We focus on the Regenerative Organic vs. Conventional agricultural treatment of three agroecosystems (a) Conventional cereal (CC) vs. Regenerative organic cereal system (RC), (b) Conventional tomato (CT) vs. Regenerative organic tomato (RT) and Conventional Mixed (CMS) vs. Regenerative organic mixed system (RMS). Each experimental plot is replicated 4 times in a randomized block design. The remaining experimental treatments, which include the regenerative organic monocultures (RMon 1–6) and their associated strip-cropping systems (RV1-2) were not included in the current study. This is because the majority of these crops are rotated every year and the more dynamic management has not shown yet clear soil biology responses (see also Data Analysis). The only exception is a monoculture of *Achillea millefolium*, which was used for comparison in the arthropod study because the plot with this perennial plant was not disturbed over the 3 years of the experiment.

Table 1
Initial soil characteristics at time zero before the beginning of the experimental trial in 2022.

Soil depth layer	Bulk density	pH	Soil Organic Matter	Sand	Silt	Clay
[cm]	[g cm^{-3}]		[%]	[%]	[%]	[%]
0–15	1.04	8.41	3.48	4.2	69.4	26.4
15–30	1.13	8.24	3.15	5.5	69.9	24.6

Table 2
Comparison of soil management practices between Regenerative Organic and Conventional Agriculture.

Management system	Depth	Equipment	Seedbed preparation	Passages per year
Conventional	30 cm	Mouldboard plow	Rotary harrow	1 or 2 ploughing events + 2 passages of rotary harrow each time
Regenerative Organic	15 cm	Minimum tillage cultivator	Minimum tillage cultivator	2 for every crop

2.1. PLFA and NLFA analyses

Each of the 56 experimental plots was sampled at 5 random sites within each plot, 0–20 cm soil depth, and a composite soil sample was freeze-dried and analyzed for Phospholipid Fatty Acid Analyses. PLFAs were measured in 2022 (time zero before we started the plot experiment) and again in 2023 and 2024. We also performed Neutral Lipid Fatty Acid analysis (NLFAs) in 2024. In 2022 a total of 16 composite samples (4 replicates per block) were collected not in the same location of the plots, but in the center of each experimental block before the creation of the individual plots. Microbial lipids (from 3 analytical replicates) were extracted from 2 g of each freeze-dried soil with a solvent system that included methanol, chloroform and a phosphate buffer. Total extracted lipids collected in the organic phase were fractionated into neutral, glyco- and polar lipids with chloroform, acetone and methanol using silicic acid chromatography. By using a GC-FID (Agilent Technologies 6890 N) equipped with an HP-5 (Agilent Technologies) fused silica capillary column (30 m length, 0.32 mm ID, 0.25 m film), fatty acid methyl esters were analyzed (see Pawlet et al., 2013). We used 15:00, 15:0i, 15:0ai, 16:0i, 17:0i, a17:0, 18:0 (10Me), Me 17:0 isomer, Me 17:0 isomer2 and 17:0 (12Me) PFLAs as indicative of Gram + bacteria whereas 16:1ω7c, 17:1ω7, 18:1ω7 t, cyc17:0 isomer PFLAs were indicative of Gram- bacteria. We used the fatty acid 16:1ω5 (using both NLFAs and PLFAs extraction process of 16:1ω5) as a biomarker for arbuscular mycorrhizal (AM) fungi in soils. We used 18:2ω6,9, 16:1ω5 as, 18:1ω9c as a biomarker for total fungal and 18:0 (10Me), Me 17:0 isomer, Me 17:0 isomer2 for Actinobacteria (Frostegård et al., 1993; Lewe et al., 2021; Pawlett et al., 2009; Willers et al., 2015). NLFAs were also extracted in 2024 together with PLFAs. Total lipids were extracted, and subsequent fractionation was performed by separating lipids based on their polarity, followed then by derivatization. The resulting fatty acid methyl esters were measured via gas chromatography. Ethanol (2%) was added to chloroform to allow the complete recovery of TAGs in the neutral lipid fraction (see Gorka et al., 2023). As additional parameters, the ratio between Gram + and Gram- bacteria, the ratio between total fungal and total bacterial PLFAs, and the net change of AM

fungi and actinobacteria (difference between 2024 and 2022) were calculated. PLFA and NLFA analyses provide information on broad microbial guilds (e.g., Gram-positive vs. Gram-negative bacteria, AM fungi) but lack the taxonomic resolution of amplicon sequencing (e.g., 16S rRNA or ITS). Future studies using metagenomic or metabarcoding approaches could reveal whether specific microbial taxa respond differentially to ROA management.

2.2. Earthworm survey

We conducted a survey on the abundance of earthworms in each of the 56 experimental plots. Earthworm sampling was conducted at two different time-points: April 2024 and September 2024. Sampling was performed using chemical expulsion with allyl isothiocyanate (AITC), without hand-sorting, following the protocol of Pelosi et al., (2009). Briefly, a metal frame measuring 0.4 × 0.4 m and 15 cm in height was pushed 5 cm into the soil in each plot of Experiment 1 to retain the solution on the sampling site (three times in the case of RV plots, once for each rotation). A solution was prepared by mixing 2 mL of pure AITC [Sigma-Aldrich, CAS number 57–06–7] and 40 mL of pure isopropanol in 20 L of deionized water, resulting in a final concentration of 0.1 g L^{-1} . The solution was applied twice at 15-minute intervals. Emerging earthworms were collected for 30 min after the first application and counted. Hand-sorting was not performed, in accordance with the conclusions of Pelosi et al. (2009), and due to the small size of the experimental plots to avoid disturbing future sampling. We understand that this is a limitation of our study, the absence of hand sorting might have resulted in a underestimation of the number of earthworms. We recognize the limitation of the methodology we have selected, despite our result are consistent with another published study (Hawes et al., 2025, see results below).

2.3. Arthropod diversity

For this activity, we focused on the same agronomic treatments Conventional Cereal (CC), Regenerative Cereal (RC), Conventional Tomato (CT), Regenerative Tomato (RT), Conventional Mixed System (CMS), Regenerative Mixed System (RMS), and we also included as comparison with these cropping systems a less-disturbed soil as the Regenerative Monoculture treatment including the perennial plant yarrow (*Achillea millefolium*). On July 6th, 2025, for each replicate plot, three soil samples (10 × 10 × 10 cm clods) were randomly collected for a total of 84 samples, which were transported to the laboratory immediately and placed in Berlese flasks (Supplementary Fig. 1) for arthropod extraction. In brief, fresh soil was put in a plastic tube (10cm dimeter x 13cm height), over a commercial white funnel cover with a mesh of 2mm of size. Soil was put under a light bulb (12cm of distance from the samples, 60 W of power) over a period of 7 days and collected by 50 mL plastic tube with 40 mL of a hydroalcoholic solution (70% Ethanol – 30% Distilled Water). Soil subsamples were also collected to measure gravimetric moisture (H') to verify that the values fell within the optimal range for the application of the Soil Biological Quality Index (BQI) (15% < H' < 45%). BQI is calculated as the sum of the EMI (Eco-Morphological Index) scores linked to the type of arthropods occurring in our samples. EMI is a qualitative index that reflects the degree of morphological adaptation to life in the soil and we adopted the scoring between 1 and 20 to identify different functional groups: (a) Eudaphic (EMI = 15–20) completely adapted to life in the soil (absence of eyes and pigmentation, reduced appendages, etc.), (b) Hemiedaphic (EMI 5 to <15), partially adapted to the soil environment, (c) Epiedaphic (EMI < 5) general organisms, not strictly associated with the soil environment. The EMI score is assigned to the single form present in the soil sample, even if detected in only one subsample. The QBS-ar (now referred to as BQI) index is a valuable tool because it incorporates morphological adaptations to soil life (EMI scores), not just taxonomic presence or abundance. This functional approach provides a more ecologically meaningful assessment of soil arthropod community quality than simple richness or abundance metrics alone.

In addition to calculating the BQI, a taxonomic classification of arthropods was conducted at least down to the family level, and where possible, down to the species level. An alpha and beta diversity analysis was also performed, with particular attention to functional groups (decomposers, predators, detritivores, etc.) to better understand the ecological contribution of arthropod communities in different systems. For the alpha diversity, the following list of indicators and indices was calculated:

1) Richness, calculated as total number of taxa in each system
2) Shannon Index (H), calculated as: $H = -\sum_{i=1}^{S}(p_i x \ln(p_i))$, where p_i is the proportion of the individuals of each taxa and S is the total number of taxa
3) Simpson (D), calculated as: $D = \sum\left(\frac{n_i}{N}\right)^2$, where n_i is the number of individuals of the taxa and N is the total number of individuals

Arthropods were sampled once in July 2025. Seasonal variation in arthropod activity and emergence patterns means that our diversity estimates may not capture the full community present across the year and a multi-year sampling would provide a more complete assessment.

2.4. Data analysis

2.4.1. Analysis of variance

One-way Analysis of Variance (ANOVA) (1) was used to assess potential effects of changes in soil management (regenerative organic *vs.* conventional) and changes in species diversity (regenerative monoculture *vs.* regenerative strip-cropping) on every individual biomarker of 2024 PLFAs and NLFAs data (response variables). Block was added to the model as fixed factor.

$$Y_{ij} = \mu + Treatment_i + Block_j + \varepsilon_{ij} \quad (1)$$

ANOVA assumptions were tested through Shapiro-Wilk (for Normality) and Levene's test (for Homogeneity). Log transformations or square root transformations were performed to satisfy assumptions of normality and heteroskedasticity when needed. The transformation log (y + 1) was applied to earthworm data before ANOVA was performed to address zero inflation and heteroscedasticity. The function *emmeans* was used to estimate the marginal means and Tukey HSD post hoc tests were used to assess significant differences between treatments when the F-tests indicated statistically significant effects. All test results were considered statistically significant at $p \leq 0.05$. Dunn-Sidak correction was applied for multiple comparison test. With four replicates per treatment, our study is adequately powered to detect large effects (e.g., 50–100 × differences in earthworm abundance) but may lack power to detect more modest biological responses or interactions between management and crop type. Post-hoc power analysis suggests that detecting a 20% change in bacterial PLFAs would require approximately 8–10 replicates per treatment. The second experimental treatment, which includes monoculture and strip-cropping plots managed with the same regenerative organic agriculture approach, did not result in statistically significant differences due to the short duration of the experimental trials. This experimental trial has not run enough through time to allow meaningful ecological and biogeochemical interactions to become statistically detectable. This is mainly because the strip-cropping system includes crops, which are rotated in the field every year, and will require longer time to quantify potential species-diversity induced effects on soil biology. For this reason, in this study, we focused on the two contrasting experimental treatments (ROA *vs.* Conventional). Our study design included four replicates for each agricultural treatment (ROA *vs.* Conventional), and composite soil samples were collated in each experimental plot (i.e. 5 soil samples to give a composite sample for the PLFA analyses). Data on earthworm abundance did not conform with normal

distribution of data (e.g., Shapiro-Wilk test) and thus a non-parametric test (e.g., Wilcoxon/Kruskal Wallis rank sum) was used as sensitivity analysis**.** The soil Biological Quality Index (BQI), the various biodiversity indices and earthworm abundance were analyzed with one-way ANOVA to separate the effect of the different agro-ecosystems. The ANOVA was performed with the R 4.2.0 (R Core Team, 2020).

2.4.2. *Multivariate analysis*

Patterns in microbial community composition were explored using Principal Component Analysis (PCA) based exclusively on PLFAs profiles. The analysis was performed using the *vegan* package (v2.6) (Oksanen et al., 2013) through the *rda* function on centred data. To address the unbalanced temporal structure of the dataset, the PCA was calculated using samples collected in 2023 and 2024, when the experimental design was fully balanced across treatments. Samples from 2022 (baseline, T0), for which fewer replicates were available, were subsequently projected onto the ordination space as supplementary observations using the *predict()* function, allowing comparison of baseline conditions with later years without influencing the ordination axes Some PLFA biomarkers were displayed as vectors to aid interpretation of the contribution of individual biomarkers to the ordination axes. Group-level patterns were visualized as covariance ellipses. All PCA plots were produced using *ggplot2* (v4.0.2)(Wickham et al., 2021). Using these methods, we assessed the effects of ROA on soil microbial communities, earthworm abundance, and arthropod diversity.

3. Results

3.1. *Regenerative organic agriculture effects on soil microbial community*

Baseline (2022) measurements showed no significant differences between plots subsequently assigned to conventional vs. ROA treatments for any measured indicator (data not shown, all $P > 0.05$), supporting that observed differences at years 2–3 are attributable to management rather than pre-existing gradients. We found that total fungal PLFAs (mol%), as measured in 2024 (2 years after the implementation of ROA) were significantly higher in ROA-managed soils compared to conventionally managed soils ($F = 6.55$, $P = 0.002$). No significant differences in total bacterial PLFAs (mol%) were instead found between ROA and conventional agriculture. Principal Component Analysis (PCA) shows how the soil microbial community has changed from 2022 (before the start of the experimental treatments) to 2024 following the implementation of conventional and regenerative organic practices (Fig. 2). The PCA axes 1 and 2 explain 43.7% and 21.2% of the variability associated with changes in the soil microbial community, which starting from 2022 show a divergent trend between conventional or regenerative organic treatments (Table S2). Soil microbial communities of conventional and regenerative organic soils tend to separate with increasing abundances of Actinobacteria and Gram + guilds in conventional soils on one side and total fungi, AM fungi and Gram- bacteria increasing in regenerative organic soils on the other side (Fig. 2).

The 16:1ω5 biomarker for arbuscular mycorrhizal (AM) fungi estimated either through the NLFA ($F [3,5] = 28.00$, $P = 0.0001$) or PLFA ($F [3,5] = 12.07$, $P = 0.00008$) extraction process, significantly increased in the ROA-managed soils (Fig. 3, Annex 1). A methodological strength of our study is the concurrent use of both PLFA and NLFA extraction to quantify the AM fungal biomarker 16:1ω5. NLFA primarily reflects storage lipids (neutral lipids) and is considered a more sensitive indicator of AM fungal biomass than PLFA, which measures membrane lipids. The consistent direction of response across both methods (Fig. 3) provides strong evidence that ROA management increases AM fungal abundance.

Total fungal PLFAs increased in the ROA-managed plots ($F [3,5] = 6.55$, $P = 0.002$) compared to conventional plots, whereas total bacterial PLFAs did not change significantly between regenerative organic soils and conventional-managed soils ($F [3,5] = 2.32$, $P = 0.09$) (Annex 1). We found that the fungal:bacterial ratio increased in ROA-managed

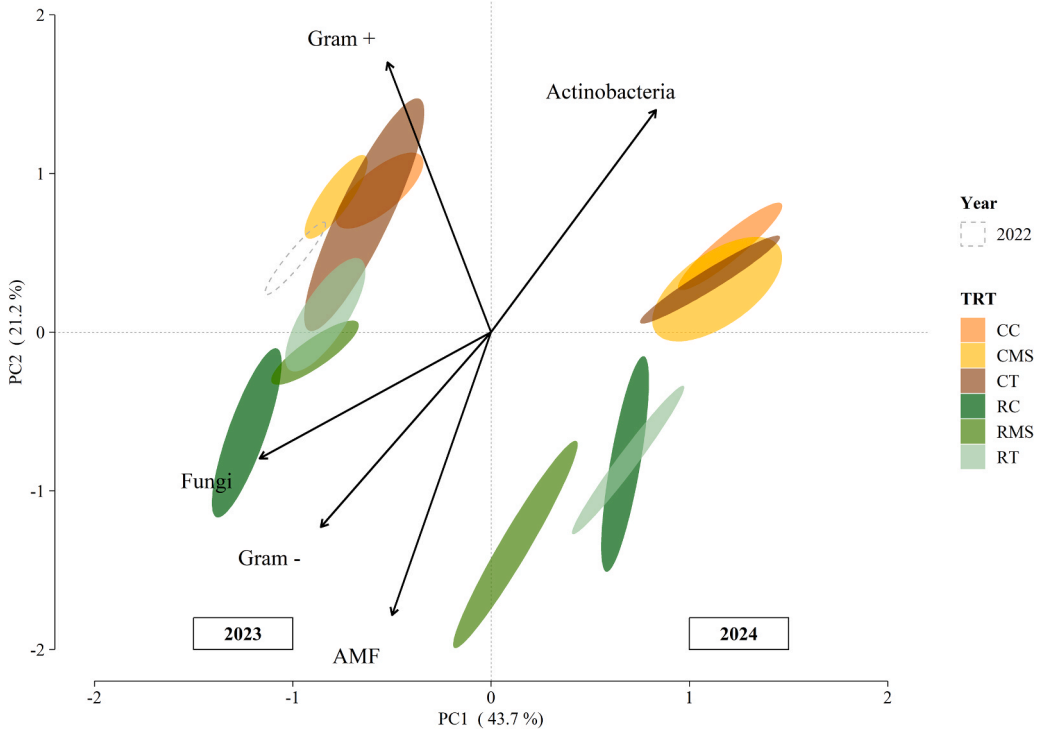


Fig. 2. Results from Principal Component Analysis (PCA), which show how the soil microbial community has changed from 2022 (small, dashed line polygon) to 2024 following the implementation of conventional and regenerative organic practices. Note the increasing divergence of microbial guilds between conventional (orange-red-brown colors) and regenerative organic (green colors) treatments. There is an increasing abundance of Actinobacteria and Gram + guilds in conventional soils on one side and total fungi, AM fungi and Gram- bacteria increasing in regenerative organic soils on the other side.

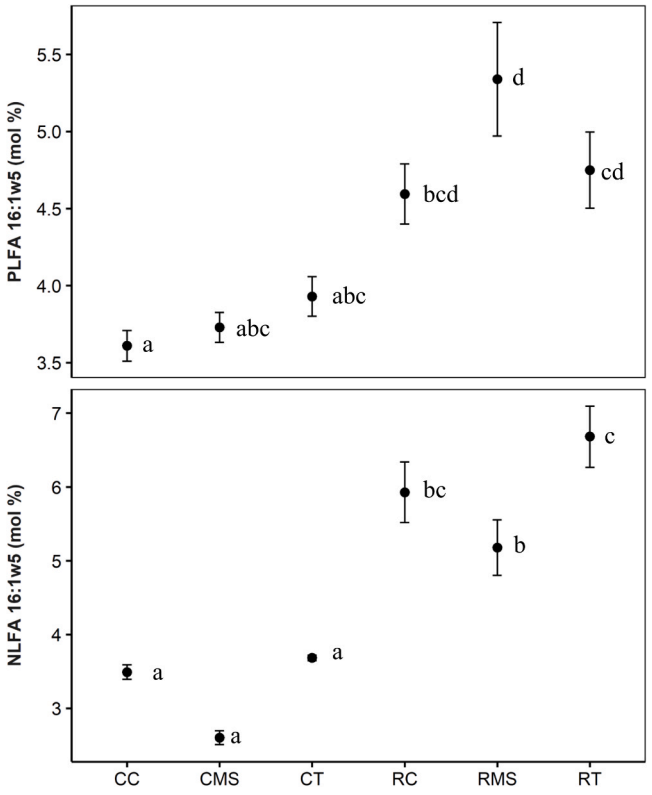


Fig. 3. Abundance of the arbuscular mycorrhizal (AM) fungal biomarker 16:1ω5 in conventional and regenerative organic soils. (A) PLFA-extracted 16:1ω5 (mol% of total PLFAs), (B) NLFA-extracted 16:1ω5 (mol% of total NLFAs). Treatments: CC = Conventional Cereal, CT = Conventional Tomato, CMS = Conventional Mixed System, RC = Regenerative Cereal, RT = Regenerative Tomato, RMS = Regenerative Mixed System. Bars represent mean ± standard error (n = 4 plots per treatment). Significant differences among individual treatment comparisons are shown by letters above bars (Tukey HSD, α=0.05).

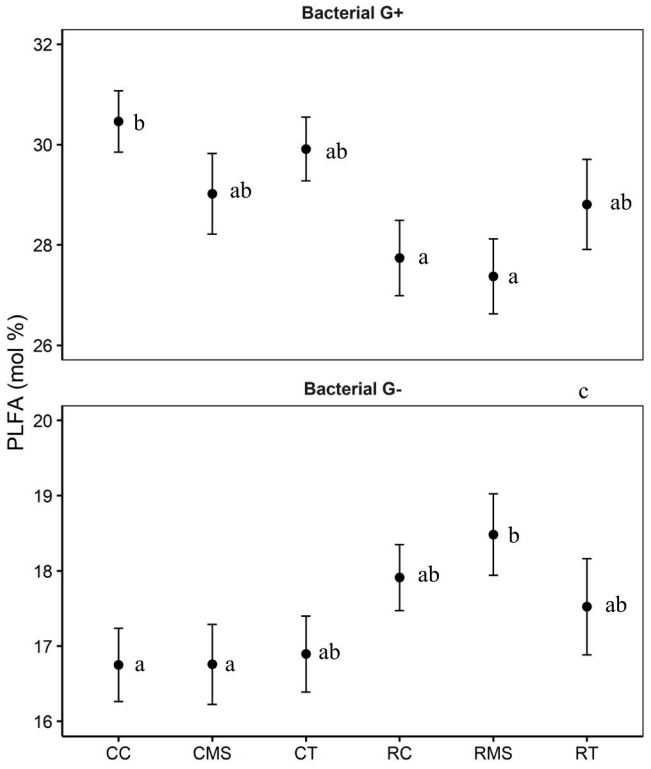


Fig. 4. Results from PLFA analyses, which show a higher in the abundance of Gram- bacteria (G-) compared to Gram + bacteria (G+) in regenerative organic soils compared to conventional soils. Treatments: CC = Conventional Cereal, CT = Conventional Tomato, CMS = Conventional Mixed System, RC = Regenerative Cereal, RT = Regenerative Tomato, RMS = Regenerative Mixed System. Bars represent mean ± standard error (n = 4 plots per treatment). Significant differences among individual treatment comparisons are shown by letters above bars (Tukey HSD, α=0.05).

soils (F [3,5] = 5.54, P = 0.001). The Gram + Gram- bacteria ratio decreased significantly in ROA soils when compared to conventional soils (F [3,5] = 5.29, P = 0.001), possibly due to an increase in Gram- bacteria in regenerative organic soils compared to conventional (Fig. 4, Annex 1). On the other hand, PLFA biomarkers for Actinobacteria increased in the conventional agriculture plots (F [3,5] = 7.98, P = 0.001; Fig. 5). We also assessed net changes in AM fungi and Actinobacteria abundance between 2022 and 2024 and found a significant positive effect of ROA practices on net changes in AM fungi abundance (F [3,5] = 12.07, P = 0.001) between 2022 and 2024 and a negative ROA effect on net changes in Actinobacteria abundance (F [3,5] =7.98, P = 0.001) (Fig. 6, Annex 1). We found also a significant positive effect of ROA on the fungal biomarker 18:2ω6,9 (F [3,5] = 10.81, P = 0.0001; Fig. 7).

3.2. Regenerative organic agriculture effects on earthworm abundance

We performed two earthworm count surveys in April and September 2024 and we found on average, a significantly higher number of earthworms in the regenerative organic soils (F [3,5] = 12.1, P < 0.0001; Fig. 8). Kruskal Wallis test ($\chi^2 = 38.5$, p < 0.0001). Number of earthworms per square meter in regenerative organic soils was on average 249 ± 44.9 m^{-2} [95% CI: 192, 306], 108 ± 35 m^{-2} [95% CI: 52, 165], 103 ± 39.4 m^{-2} [95% CI: 52, 165], in the RMS, RT and RC plots, respectively. The number of earthworms was 0 m^{-2} [95% CI: −57, + 57], 3.9 ± 3.1 m^{-2} [95% CI: −53, 61], and 1.6 ± 1.5 m^{-2} [95% CI: −55, 58], m^{-2} in the CC, CMS and CT plots, respectively. Our use of a standardized, published protocol for earthworm sampling (AITC chemical expulsion) ensures reproducibility and allows direct comparison with other studies using the same method. While hand-sorting was not performed (see limitations in the Discussion), the AITC method is non-destructive to plot integrity, enabling repeated sampling over time—a critical advantage for longitudinal studies.

3.3. Regenerative organic agriculture effects on arthropod diversity

We found that the total number of arthropods was significantly higher in the ROA-managed plots compared to conventionally managed soils (F [3,6] = 11.1, P < 0.0001; Table 3). Our Soil Biological Quality Index (BQI) shows how the number of euedaphic arthropods, which are completely adapted to life in the soil (i.e. absence of eyes and pigmentation, reduced appendages, etc.) was higher in the regenerative organic soils (F [3,6] = 5.2, P = 0.03). Similarly, the number of epiedaphic generalist organisms, not strictly tied to the soil, was greater in the regenerative organic plots (F [3,6] = 21, P < 0.0001). The number of hemiedaphic arthropods, partially adapted to the soil environment, was not significantly different between regenerative organic and conventional plots (F [3,6] = 1.2, P = 0.27). The Shannon index showed significant differences (F [3,6] = 9.5; p < 0.001) with the highest taxa diversity in the RC system and the lowest richness in CT. The Simpson index also showed significant differences (F [3,6] = 4.3; p < 0.01) with the highest value in RC and the lowest in CT and RMon3 (Table S1).

4. Discussion

Overall, our study demonstrates how regenerative organic

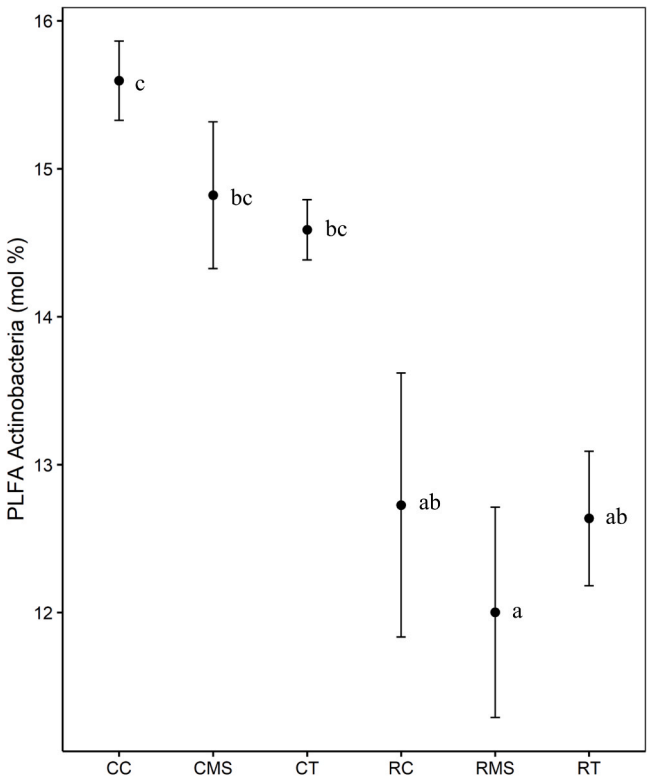


Fig. 5. Results from PLFA analyses, which show higher abundance (mol%) of Actinobacteria in conventional soils compared to regenerative organic soils. Treatments: CC = Conventional Cereal, CT = Conventional Tomato, CMS = Conventional Mixed System, RC = Regenerative Cereal, RT = Regenerative Tomato, RMS = Regenerative Mixed System. Bars represent mean ± standard error (n = 4 plots per treatment). Significant differences among individual treatment comparisons are shown by letters above bars (Tukey HSD, α=0.05).

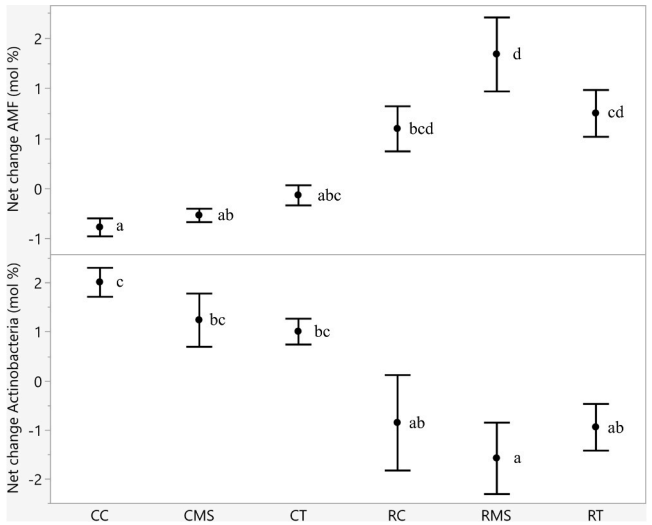


Fig. 6. Results from PLFA analyses, which show net changes (2022–2024) in the abundance (mol%) of AMF and Actinobacteria groups in regenerative organic soils compared to conventional soils. Treatments: CC = Conventional Cereal, CT = Conventional Tomato, CMS = Conventional Mixed System, RC = Regenerative Cereal, RT = Regenerative Tomato, RMS = Regenerative Mixed System. Bars represent mean ± standard error (n = 4 plots per treatment). Significant differences among individual treatment comparisons are shown by letters above bars (Tukey HSD, α=0.05).

agriculture has positive effects on soil biology. Our findings show how

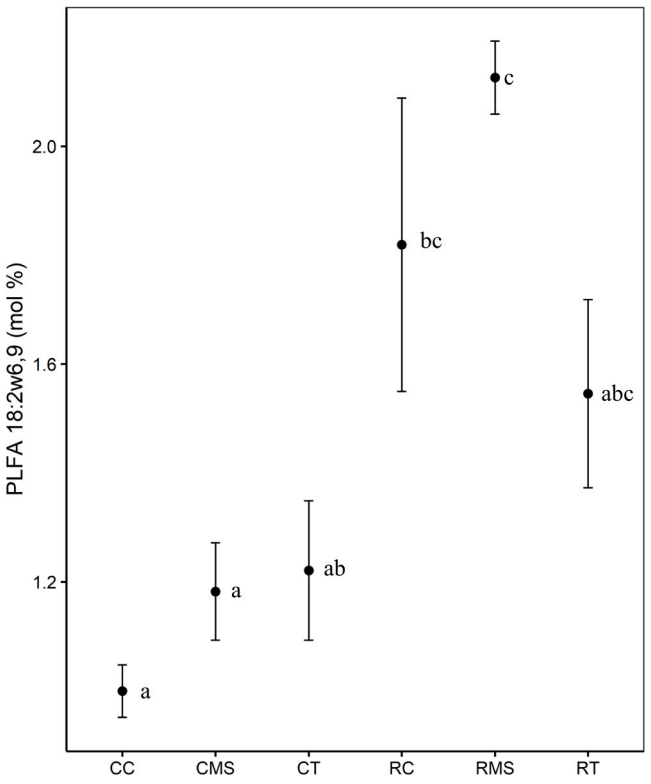


Fig. 7. Results from PLFA analyses, which show significant positive effects of ROA on the fungal biomarker 18:2ω6,9. Treatments: CC = Conventional Cereal, CT = Conventional Tomato, CMS = Conventional Mixed System, RC = Regenerative Cereal, RT = Regenerative Tomato, RMS = Regenerative Mixed System. Bars represent mean ± standard error (n = 4 plots per treatment). Significant differences among individual treatment comparisons are shown by letters above bars (Tukey HSD, α=0.05).

several soil biological indicators can shift detectably within 2–3 years following the implementation of combined ROA practices, though longer-term studies are needed to assess the stability of these responses. We observed shifts in the soil microbial community towards an increasing abundance of PLFA and NFLA fungal markers in particular a significant increase in the 16:1ω5 biomarker for AM fungi. We also observed a significant increase in the number of earthworms and greater arthropod diversity including the number of microarthropods adapted to life in the soil ecosystem of the regeneratively organic managed experimental plots. By including three distinct agroecosystems—a cereal grain system, a vegetable (tomato) system, and a mixed rotation system—our study demonstrates that ROA benefits are not limited to a single crop type. The consistency of responses across systems (e.g., increased AM fungi in RC, RT, and RMS; increased earthworms in all three) suggests that positive ROA effects on soil biology may be broadly generalizable across Mediterranean arable systems.

4.1. *Changes in the soil microbial community*

We found that total fungal (but not bacterial) PLFAs (mol%) were significantly higher in ROA-managed soils compared to conventionally managed soils, suggesting a potential increase in microbial fungal biomass following regenerative organic practices. This expectation is confirmed by the results of a recent study, which found a 38–178% increase in overall microbial biomass across crops and years during the agroecological transition between conventional and regenerative organic management (Lekberg et al., 2024). Moreover, experimental evidence shows that soil microbial biomass increased following the inclusion of cover cropping and living mulch into a conventional potato

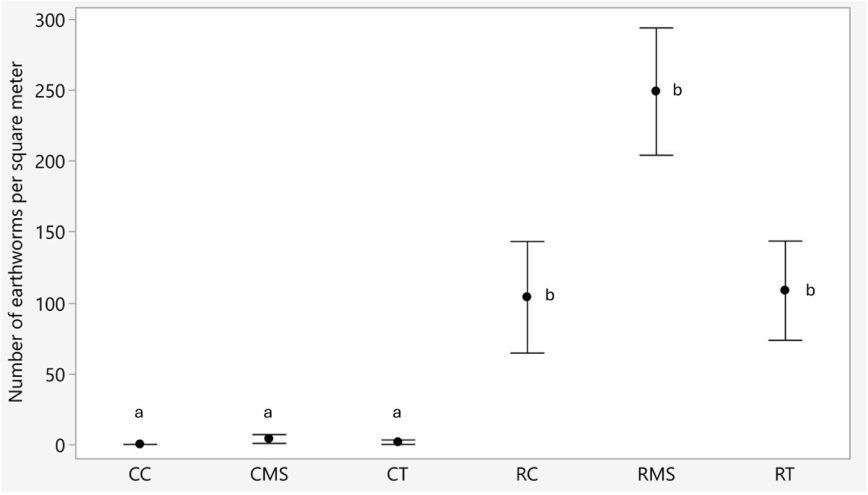


Fig. 8. Results from earthworm counts, which show significantly higher number of earthworms in the regenerative organic soils. Treatments: CC = Conventional Cereal, CT = Conventional Tomato, CMS = Conventional Mixed System, RC = Regenerative Cereal, RT = Regenerative Tomato, RMS = Regenerative Mixed System. Bars represent mean ± standard error (n = 4 plots per treatment). Significant differences among individual treatment comparisons are shown by letters above bars (Tukey HSD, α=0.05).

Table 3
Results from the Soil Biological Quality Index (BQI) analysis including the average number of different arthropod functional guilds in each soil sample per square meter of soil. Total number of arthropods was significantly higher in the Regenerative Monoculture and in the ROA-managed plots compared to conventionally managed soils. Eudaphic are arthropods completely adapted to life in the soil (absence of eyes and pigmentation, reduced appendages, etc.), Hemiedaphic are partially adapted to the soil environment, Epigeic are general organisms, not strictly associated with the soil environment. Significant differences among individual treatment comparisons are shown by letters (Tukey HSD, α=0.05).

Treatment	Code	BQI index	Number Euedaphic (n)	Number Emiedaphic (n)	Number Epigeic (n)	Number Euedaphic (n m^{-2})	Number Emiedaphic (n m^{-2})	Number Epigeic (n m^{-2})
Conventional Cereal	CC	49.0 ± 15.2c	1.5 ± 0.6 b	1.5 ± 0.6 bc	3.0 ± 2.0 bc	708 ± 189 bc	850 ± 199 bc	542 ± 330 bc
Conventional Mixed System	CMS	45.8 ± 3.8c	1.0 ± 0.1 b	1.5 ± 0.6 bc	4.8 ± 0.5 ab	317 ± 155 ab	175 ± 83 ab	483 ± 235 ab
Conventional Tomato	CT	36.2 ± 5.2c	1.0 ± 0.1 b	1.3 ± 0.5c	1.8 ± 0.5c	433 ± 66c	225 ± 126c	150 ± 64c
Regenerative Cereal	RC	77.5 ± 8.4 a	1.8 ± 0.5 b	2.8 ± 0.5 a	7.3 ± 0.5 a	1566 ± 82 a	1350 ± 561 a	1300 ± 86 a
Regenerative Monocolture 3 (*A. millefolium*)	RMon3	83.0 ± 2.9 a	2.8 ± 0.5 a	2.3 ± 0.5 abc	4.5 ± 1.7 b	1950 ± 459 b	208 ± 88 b	1925 ± 453 b
Regenerative Mixed System	RMS	68.5 ± 8.7 ab	1.8 ± 0.5 b	2.5 ± 0.6 ab	4.5 ± 0.6 b	675 ± 206 b	1375 ± 652 b	933 ± 547 b
Regenerative Tomato	RT	52.2 ± 4.9 bc	1.0 ± 0.1 b	2.8 ± 0.5 a	2.8 ± 0.5 bc	542 ± 260 bc	1075 ± 250 bc	358 ± 134 bc
F-value		19.15	10.46	5.75	10.76	10.14	9.45	15.49
P-value		< 0.001	< 0.001	0.001	< 0.001	< 0.001	< 0.001	< 0.001
Sig.		***	***	**	***	***	***	***

Sig. Codes: '***' = <0.001, '**' = <0.01, '*'= <0.05. Different letters correspond to a significant difference among the treatments according to the Tukey's test (α=0.05)

cropping system (Henzel et al., 2025).

Our findings suggest that AM fungi positively responded to the implementation of regenerative organic practices. Similar results were found in recent studies, Lekberg et al., (2024); Martínez-García et al., (2018), which show an increase in the biomass of arbuscular mycorrhizal fungi after the transition to regenerative organic agriculture. Also, evidence from a recent glasshouse study associated with the cultivation of the cereal *Sorghum bicolor* demonstrates how organic cereal systems were associated with a larger proportion of AM fungal taxa (Heuck et al., 2025). Our results show that AM fungi decreased in conventionally managed soils suggesting that conventional agricultural practices might negatively influence AM fungi while promoting the abundance of other microbial guilds. For example, we found that soil bacterial groups that are generally considered to be more copiotrophic, including Actinobacteria increased in relative abundance in our conventional soils, perhaps because these soils are managed receiving synthetic nutrient additions (Leff et al., 2015). A meta-analysis study using datasets produced from different regions worldwide show how greater synthetic nitrogen (N) fertilization favored the growth of Actinobacteria (Dai et al., 2018). Synthetic N inputs tend to promote copiotrophic bacterial growth, including some Alproteobacteria and Actinobacteria, due to fast growth of these bacteria and their lower C use efficiency (Fierer et al., 2012; Ling et al., 2017).

Actinobacteria are also Gram-positive bacteria, and we observed a general increase of G + bacteria in the conventional soils whereas Gram-negative bacteria increased more in the regenerative organic soils. Previous evidence suggests that G + bacteria use more SOM-derived carbon sources than Gram- bacteria, which use more plant biomass-derived C sources (Kramer and Gleixner, 2008). Similarly, Fanin et al. (2019), found that G- bacteria were associated with plant-derived

simple C compounds (alkyls) whereas G + bacteria were more strongly associated with SOM-derived complex C forms (carbonyls). Thus, increases in Gram- bacteria in ROA soils possibly associated with greater inputs of labile plant-derived carbon (e.g., from root exudates and/or cover crop residues), could enhance short-term C cycling with potentially more microbial-derived C accumulating in soils. Conversely, the increase in Gram + bacteria in conventional soils may reflect greater reliance on more recalcitrant soil organic matter, with possible negative implications for long-term carbon storage. While synthetic N fertilization is a plausible explanation for increased Actinobacteria in conventional soils (Dai et al., 2018), we cannot rule out contributions from other management differences, including tillage intensity or pesticide applications.

We suggest that the presence of crop roots in ROA soils (e.g., from cover crops during winter) may have contributed to greater AM fungi and Gram-negative bacteria abundance through higher inputs of plant-derived carbon (see also Lange et al., 2024), though root biomass was not directly measured in this study. Shifts in the soil microbial community towards lower G + : G- ratios and higher AM abundance could have significant effects on soil organic C accumulation either through the turnover and processing of labile plant material by G- bacteria (Fornara et al., 2011) or through increased positive AM-associated effects on organic C inputs in soil organo-mineral aggregates (Horsch et al., 2023) or both. The lack of change in total bacterial PLFA abundance despite shifts in community composition suggests that ROA management may alter bacterial community structure without necessarily changing total biomass, a finding that is different from what we observed in total fungal PLFAs and merits further investigation with complementary methods such as 16S rRNA sequencing.

4.2. Changes in earthworm abundance

We found a higher number of earthworms per square meter in the regenerative organic soils when compared to conventionally managed soils. Our estimate is that average earthworm number varied between 100 and 250 individuals m^{-2} compared to 0–4 earthworms m^{-2} in the conventionally managed soils. Our survey was conducted only 2 years since soils started to be managed in a regenerative organic way, and this short period of time might not have captured the potential of these soils to further increase earthworm abundance. For example, an earthworm survey from a field experiment that lasted > 10 years shows a higher number of earthworms whereby the average number of earthworms across years and crop rotations was 225 earthworm m^{-2} in conventionally managed soils and 358 earthworm m^{-2} in regenerative soils (Hawes et al., 2025). However, direct comparisons across studies should be made cautiously given differences in extraction methods; our exclusive use of chemical expulsion may underestimate earthworm abundance relative to studies that also used hand-sorting (Pelosi et al., 2009).

A recent study which assessed earthworm abundance in conventionally managed arable fields and in strips of grass-clover ley introduced in the same agricultural fields found a $> 55\%$ increase in earthworm numbers into the third year of the leys (Llanos et al., 2025). Similarly, a study carried out in regenerative agroecosystems in the UK also integrating livestock grazing show that the total number of earthworms, including epigeic and juvenile functional groups was significantly higher under zero tillage associated with mob-grazing (Trickett and Warner, 2022).

Among regenerative practices, the application of farm manure could have had an important positive effect on earthworm abundances in our study. A previous study carried out across organic arable farming systems under Mediterranean climate shows how the density and biomass of earthworms increased following farmyard manure applications (Baldivieso-Freitas et al., 2018). However, because ROA practices were implemented as a bundle, we cannot isolate the effect of manure application from other concurrent practices (cover cropping, reduced tillage, crop rotation). More studies should address potential changes in the abundance of different earthworm functional groups to better understand how regenerative organic practices might influence soil ecosystem functioning.

4.3. Changes in the arthropod community

Findings from our Soil Biological Quality Index (BQI) analyses show a significant increase in the total number of arthropods in the ROA-managed plots compared to conventionally managed soils. The number of euedaphic arthropods adapted to life in the soil was higher in the regenerative organic soils as well as the number of epiedaphic generalist organisms, not strictly tied to the soil.

The regenerative organic systems (RC, RT, RMS) showed greater species richness, a more even distribution of species, and a more complex community structure than conventional systems (CC, CT, CMS). Species richness was similar between the three regenerative organic systems and the regenerative organic monoculture of *Achillea millefolium*, which we used as comparison. The soil beneath the *A. millefolium* system had not been disturbed by management for 3 years and this together with significant aboveground biomass production by yarrow (which provides shelter), could explain the high arthropod richness.

A recent study carried out at the International Rice Research Institute in India focusing on rice—wheat-mung bean systems, shows how the arthropod community increased following the transition to organic farming (Mishra et al., 2024). Similarly, a study carried out in the US Midwest region of cereal production systems shows how regenerative practices fostered greater soil arthropod diversity (Prairie et al., 2026). Findings from a study in South Africa show how no-till coupled with diversified crop rotations and cover crop mixtures under conservation and integrated agricultural management contributed to enhance the diversity of earthworms, collembola, beetles, and spiders (Mamabolo et al., 2024).

In our study, in terms of individual arthropod abundance, the undisturbed regenerative organic monoculture of *Achillea millefolium* (RMon3) showed the highest density of euedaphic (1950 ± 459 n m^{-2}) and epigeic (1925 ± 453 n m^{-2}) organisms, while RC hosted the most abundant hemiedaphic communities (1350 ± 561 n m^{-2}). The conventionally managed systems (CT and CMS) recorded the lowest abundances in all groups, highlighting poorer soil biology. Interestingly, despite the regenerative organic monoculture (RMon3) being associated with the highest numbers of individuals, it also showed lower values of the Simpson's Diversity index suggesting greater dominance of a few taxa. This pattern—high abundance but low evenness—suggests that a single perennial plant species can support large populations of a subset of arthropod taxa but does not promote the broader taxonomic diversity observed in rotationally managed ROA systems (e.g., RC). This highlights a potential trade-off between supporting high arthropod biomass vs. high biodiversity.

This was not the case, for example, for the regenerative organic cereal system (RC), which beyond showing high individual arthropod abundance also shows highest value of Simpson's Richness index suggesting a more diversified arthropod community.

4.4. Changes in soil biology and ecosystem functioning

Our results suggest that regenerative organic systems are associated with greater soil health and may also have greater ecological resilience, and a higher potential for maintaining ecosystem services related to soil fauna, such as organic matter decomposition, soil structure formation, and biological pathogen control. In terms of potential relationships between arthropod diversity and soil ecosystem functioning, our expectation is that increased numbers of microarthropods (and earthworms) in regenerative organic soils should be related, for example, to faster rates of leaf litter decomposition, as has been found in arable agroecosystems in Scotland (Hawes et al., 2025). Likewise, the observed

increases in AM fungi abundance in ROA soils have potential functional implications for nutrient acquisition (particularly phosphorus), soil aggregation, and plant stress tolerance. Similarly, the 50- to 100-fold greater earthworm abundance in ROA soils likely enhances macropore formation, water infiltration, and organic matter incorporation into deeper soil horizons. If we assume that ROA promotes more complex soil food webs and potentially greater rates of litter decomposition, nutrient cycling and carbon accumulation, quantifying these functional outcomes—rather than merely the biological indicators—will represent an important next step.

4.5. Limitations and strengths of our study

There are limitations to our study including its duration, which assessed changes in key soil biological indicators in the first 2–3 years since establishing the randomized block design field experiment. Longer-term responses may differ in magnitude or direction. Our earthworm sampling used chemical expulsion without hand-sorting, which may underestimate abundance, particularly for deep-burrowing species. Also, a multi-year earthworm sampling would provide a more complete assessment of potential seasonal variations in arthropod activity and emergence. The study was conducted at a single site in northern Italy; results may not generalize to different soils, climates, or cropping systems. Our study, includes however, important strengths such as a randomized block design field experiment with 4-fold replication, baseline (pre-treatment) sampling in 2022, the simultaneous measurement of three biological indicator groups under multiple ROA practices and across three cropping systems (cereal, tomato, mixed), standardized, published protocols for all measurements and the evidence of short-term response detection (2–3 years). Most regenerative agriculture studies have compared established organic vs. conventional farms, making it difficult to separate management effects from pre-existing differences. Our time-zero data confirm that treatment plots were statistically indistinguishable at the start of the experiment, strengthening causal inferences about management effects. We believe that our baseline sampling strengthens management-related inference, replication reduces sensitivity to plot-level variability, and simultaneous measurement of three biological groups under combined ROA practices provides a solid assessment of potential changes in soil biology.

5. Conclusions

This study contributes to a growing literature assessing the impacts of multiple regenerative practices on soil biological indicators and is among the few to simultaneously quantify microbial, earthworm, and arthropod responses in a single replicated experiment. We show that within 3 years of regenerative organic soil management (1) the soil microbial community has changed towards higher abundance of AM fungi, (2) the number of earthworms has significantly increased, (3) arthropod diversity has also increased in the regenerative organic soil ecosystems. The next step is to assess how these changes in soil biology are related to changes in soil ecosystem functioning including soil nutrient cycling, soil C accumulation etc. Our evidence from a well-replicated full-randomized block designed experiment is that soil biology can respond quickly to the application of multiple regenerative organic practices on agricultural soils previously managed following conventional agricultural practices. Mediterranean agricultural systems face challenges including seasonal drought, high temperatures, and low soil organic matter. Demonstrating positive soil biological responses to ROA in this challenging environment also suggests that the net effect of these combined ROA practices should be tested in more temperate or higher-rainfall regions. Future research should focus on (1) linking these biological changes to ecosystem functions such as nutrient cycling, carbon sequestration, and crop productivity; (2) disentangling the relative contributions of individual ROA practices; and (3) assessing the long-term stability of these biological responses beyond 3 years.

CRediT authorship contribution statement

Dario Fornara: Writing – review & editing, Writing – original draft, Methodology, Data curation, Conceptualization. **Enrico Martani:** Writing – review & editing, Writing – original draft, Validation, Methodology, Investigation.

Declaration of Generative AI and AI-Assisted technologies in the manuscript preparation process

Authors declare that no Generative AI and AI-assisted technologies were used during the writing process of this paper.

Funding

Dario Fornara reports financial support and article publishing charges were provided by Davines SpA.

Enrico Martani reports financial support and article publishing charges were provided by Davines SpA.

Dario Fornara reports a relationship with Davines SpA that includes: employment.

Enrico Martani reports a relationship with Davines SpA that includes: employment.

The research activities of the European Regenerative Organic Center (EROC) on Regenerative Organic Agriculture are separated from the commercial activities of the Davines Group. The Davines Group source biomass from Regenerative Organic Certified farms which are not linked to EROC research activities.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Dario Fornara reports financial support and article publishing charges were provided by Davines SpA. Enrico Martani reports financial support and article publishing charges were provided by Davines SpA. Dario Fornara reports a relationship with Davines SpA that includes: employment. Enrico Martani reports a relationship with Davines SpA that includes: employment. The research activities of the European Regenerative Organic Center (EROC) on Regenerative Organic Agriculture are separated from the commercial activities of the Davines Group. The Davines Group source biomass from Regenerative Organic Certified farms which are not linked to EROC research activities If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

We thank Dr Mark Pawlett at Cranfield University in England for guidance and for carrying out the PLFAs and NLFAs analyses in his lab at the Centre for Soil, Agrifood and Biosciences. Thanks to Simona Mazza, Marco Vettese and Fabio Torchio for their invaluable support in field work. This study could not have been done without the financial support of the Davines Group, which has promoted the creation and operational implementation of the European Regenerative Organic Center (EROC) in Parma, Italy.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.agee.2026.110730.

Data availability

Original dataset and R code are available in Annex 1 (Data) and

Zenodo (R code) at this link: 10.5281/zenodo.21276218

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