

Ecological facilitation: Terrestrial isopods indirectly modulate collembolan behaviour in biochar-amended soils

Lorenzo Federico*, Asia Rosatelli, Niccolò Lamanna, Veronica Termopoli, Heiko Lange, Andrea Franzetti, Isabella Gandolfi, Sara Villa

DISAT, Department of Earth and Environmental Sciences, University of Milano-Bicocca, Piazza della Scienza 20126, Milan, Italy

* Corresponding author. E-mail: lorenzo.federico@unimib.it (L. Federico)

Received April 30, 2026; Revised July 16, 2026; Accepted August 11, 2026

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ABSTRACT

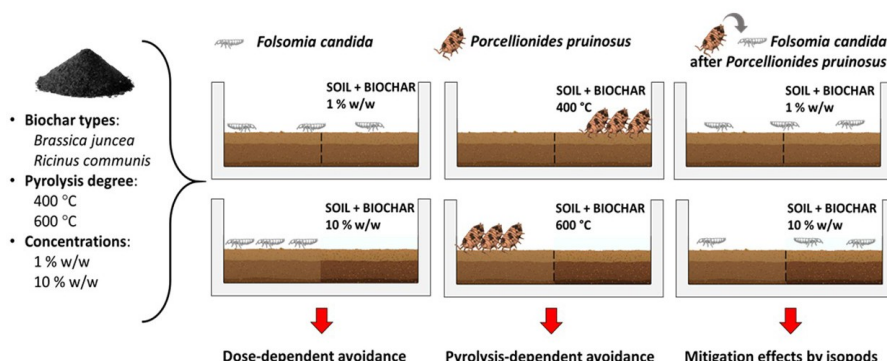
• Collembolans showed dose-dependent avoidance in response to biochars.

• Terrestrial isopods' behaviour was affected both by dose and pyrolysis degrees of biochars.

• Terrestrial isopods' presence reduced collembolan avoidance of biochar-treated soils.

Biochar is applied to soils to enhance fertility and sequester carbon, but its impacts on soil fauna remain debated. This work assessed the behavioural responses of collembolans (*Folsomia candida*) and woodlice (*Porcellionides pruinosus*) to soils amended with different biochar derived from *Ricinus communis* and *Brassica juncea* feedstocks, both pyrolyzed at 400 °C and 600 °C, and tested at 1% and 10% (w/w). Results revealed that *F. candida* avoided *R. communis* biochar at high concentrations, suggesting a stronger role of dosage than pyrolysis temperature. In contrast, *P. pruinosus* was attracted to *B. juncea* biochar at 400 °C but avoided the sample pyrolyzed at 600 °C, while gregariousness was affected at the highest concentration, highlighting the influence of dose and pyrolysis. Furthermore, we examined whether woodlice presence alters collembolans responses through ecological facilitation—non-trophic interactions that benefit at least one species. Remarkably, collembolans no longer avoided soils previously inhabited by woodlice. Multivariate analysis revealed that responses of soil arthropods to biochar were primarily determined by the type of feedstock, while ecological facilitation was mainly driven by soil conductivity, suggesting that woodlice actively modulate soil properties likely by bioturbation, or faecal deposition. In an ecological perspective, facilitation by woodlice may buffer stress and enhance habitat suitability for other soil fauna, while their high sensitivity positions them as key early indicators of biochar-induced changes in soil ecosystem functioning. It is recommended that ecological sustainable management be adopted, as high soil biodiversity can prevent soil degradation and facilitate natural processes that can mitigate the effects of biochar.

Keywords biochar, ecological facilitation, soil fauna, behavioural ecotoxicology, species interactions



1 Introduction

The utilisation of biochar has been identified as a promising method for the removal of atmospheric carbon and the amelioration of carbon storage in forests and soils, as outlined in the Carbon Removals and Carbon Farming (CRCF) Regulation (EU/2024/3012). Biochar is produced through the pyrolysis of biomass, e.g. crop residues, green waste, wood chippings, or chicken manure, and can be

amended in soils to improve soil fertility, facilitate soil water management, sequester CO₂ and manage organic waste (Li et al., 2011). In general, the application of biochar improves the structures (texture, pH, porosity and aeration) and processes (water retention capacity, conductivity, biogeochemical cycling) of soil ecosystems (Glaser et al., 2002; Verheijen et al., 2010; Lehmann and Joseph, 2015), maintaining the fertility and integrity of soils. Furthermore, biochar is effective in removing contaminants from interstitial water and absorbs heavy metals up to six times more effectively than activated carbon (Cao et al., 2009).

However, it is crucial to ensure that biochar application does not negatively affect soil biodiversity or ecological functions, to fully realize these benefits. In this regard, behavioural bioassays represent a pivotal screening tool in the identification and prioritisation of soils that are susceptible to depopulation and diminished soil fauna biodiversity (Federico et al., 2026a, 2026b). Indeed, previous studies have shown that biochar negatively affected growth, survival, and behaviour in both earthworms (Liesch et al., 2010; Li et al., 2011; Tammeorg et al., 2014) and collembolans (Marks et al., 2014; Prodana et al., 2019; Bastos et al., 2022). In contrast, woodlice seem to prefer soils amended with biochar (Madžarić et al., 2018; Bastos et al., 2022). To date, most of these studies have focused on direct effects of biochar on single model species, with limited attention to recruitment and facilitation phenomena or probiosis which are non-trophic interactions that benefit at least one species without harming others (Stachowicz, 2001). Facilitation can occur through ecological niche regulation by pioneer species, which facilitate the colonisation of other edaphic organisms in areas that would otherwise be unsuitable for them.

Considering these processes is essential for a more realistic assessment of biochar impact on soil habitat function, as it allows the inclusion of species that occupy different spatial ecological niches, not yet considered in ecotoxicological assays, to be incorporated beyond trophic level differentiation. In this way, it is possible to balance ecological complexity with the need to reduce the number of model organisms, according to the Directive 2010/63/EU of the European Parliament, which mandates the application of the 3Rs (Replacement, Reduction, Refinement) for animal use in scientific research. For instance, collembolans inhabit a variety of vertical soil layers, ranging from the litter layer to deeper soil horizons, reducing interspecific competition through spatial segregation. In contrast, woodlice act as primary decomposers of litter, occupying an epiedaphic spatial niche. Ecologically, they could be among the first organisms affected by the presence of soil improvers such as biochar, which could influence the responses of other edaphic organisms.

Based on this ecological framework, the present study aimed to investigate the effects of biochar on soil fauna through a series of behavioural assays. Specifically, the objectives were to:

- 1) Assess the direct behavioural responses of model soil invertebrates to different parental feedstocks, concentrations, and pyrolysis degree of biochar;
- 2) Evaluate potential facilitation mechanisms mediated by biochar-amended soils, with a focus on non-trophic interactions;
- 3) Explore how biochar influences recruitment dynamics and interspecific interactions within soil communities, going

beyond species-specific toxicity to capture broader ecological impacts.

2 Materials and methods

2.1 Model organisms

Individuals of *Folsomia candida* (Willem, 1902) and *Porcellionides pruinosus* (Brandt, 1833) were bred in the Laboratory of Ecotoxicology of the Department of Earth and Environmental Sciences at the University of Milano-Bicocca, Milan (Italy). Collembola were cultured in glass Petri-dishes ($\varnothing = 8.5$ cm) on a moist substrate of Calcium Sulphate Hemihydrate, also known as plaster of Paris ($\text{CaSO}_4 \cdot 1/2 \text{H}_2\text{O}$), and activated charcoal (8:1). The collembolans were maintained at temperature of 20 ± 2 °C, at 16:8 h light:dark photoperiod regime and fed weekly with dried baker's yeast (*Saccharomyces cerevisiae*). Collembolan eggs were periodically separated from the culture to obtain synchronized individuals for tests. Woodlice were cultured at 21 °C at 16:8 h (light-dark) photoperiod in a *terrarium* and were fed *ad libitum* with alder leaves (*Alnus glutinosa*). The cultures were sprayed with ultra-pure water, and food was provided twice a week.

2.2 Biochar production, extraction and chemical analysis

Four different types of biochar obtained from waste biomasses *Brassica juncea* and *Ricinus communis* pyrolyzed at two different temperatures (400 °C and 600 °C) were tested. Pressed cake pellets of *B. juncea* were by-products of the processing of the original feedstock, while *R. communis* stems, branches and leaves were collected from sites subjected to phytoremediation treatments. The two parental feedstocks were desiccated at 80 °C for 48 h and then pyrolysed for 1 h in a horizontal split tube furnace (Nabertherm®) at the chosen temperatures (400 °C and 600 °C), applying a thermal gradient of 300 °C h⁻¹. Before pyrolysis, the furnace was flushed with ultra-high purity nitrogen at a rate of 100 cm³ min⁻¹ for 20 min to ensure anoxic conditions, which was maintained throughout the entire pyrolysis process and during the cooling stage (Onay, 2007). Biochar was then collected and stored in hermetic containers at room temperature. Biochar samples were subjected to Soxhlet extraction using toluene. Unlike the EBC protocol, which specifies a 6-hour extraction with 2.5 g of biochar using 100 mL of toluene (EBC v10.5), we used the extended 36-hour version proposed and optimized by Hilber et al. (2012) and confirmed by Mayer et al. (2016) and Madej et al. (2016), to enhance the recovery of a broad range of extractable organic compounds from the biochar matrix. Initially, the biochar samples were dried in a ventilated oven at 80 °C for 24 h, then grounded and finally sieved to

obtain particles smaller than 1 mm and a homogeneous material. Then, 2.5 g of biochar were weighed and carefully placed inside the cellulose thimble. Soxhlet extraction was performed with 100 mL of toluene (w:v 1:40) for 36 h (at a siphoning rate of 6 cycles h^{-1}) under reflux conditions at a constant temperature equal to the boiling point of toluene between 110 and 115 °C. Once collected and cooled to room temperature, the extracted samples were transferred to a rotary evaporator to concentrate them and then transferred to 1 mL vials for qualitative analysis by GC-MS. The chromatographic separations were performed using a Shimadzu GCMS QP2020NX (Shimadzu Corporation, Kyoto, Japan) equipped with Shimadzu autosampler AOC20i. An SH-Rxi-5ms fused silica capillary column (stationary phase (5%-phenyl)-methylpolysiloxane, 30 m $\times$ 0.25 mm i.d., 0.25 μm , Shimadzu Corporation, Kyoto, Japan) was used as stationary phase, and He (UHP purity grade) as carrier gas. The GC system was operated in linear velocity mode at 39.5 cm s^{-1} , with the injector and MS interface temperatures maintained at 280 °C. The injection volume was 1 μL , the injection port operated in splitless mode. The temperature program was set as follows: the initial temperature of 60 °C was held for 3 min, then increased at a rate of 10 °C min^{-1} to 280 °C, which was maintained for 33 min. The MS operated in electron ionization mode (EI) at 70 eV, acquiring in full-scan mode in the m/z range of 35–600. LabSolutions–GCMS Version 4.54 software (Shimadzu Corporation) was used as system control, instrument management and data acquisition. Compounds were identified using NIST MS Search, version 2.4 (2020).

2.3 Behavioural tests

Individuals of *F. candida* were used for performing the avoidance bioassays (ISO, 2020), while individuals of *P. pruinosus* were used for both investigation of the avoidance and disaggregation effect (Federico et al., 2024). These endpoints were chosen as sensitive indicators of soil habitat quality, as avoidance or reduced aggregation behaviour reflect declining habitat suitability and the loss of suitable microhabitats (Federico et al., 2026b).

Collembolan adults (9–12 days after hatching) and woodlice adults (wet weight of 14–30 mg without sex distinction) were used, respectively. Glass boxes (9.5 cm $\times$ 7.3 cm $\times$ 5 cm) were used as test arena. LUFA 2.2 Soil Speyer was used for the ecotoxicological bioassays. The properties included pH = 5.5 ± 0.2 (0.01 M CaCl_2), WHC = 44.5 ± 3.0 (g/100 g), C = 1.77 ± 0.2 (%), N = 0.17 ± 0.02 (%), texture = 7.3 ± 1.2 (%) clay; 13.8 ± 2.7 (%) silt and 78.9 ± 3.5 (%) sand. Treated soils were prepared by amending LUFA 2.2 with 1% and 10% (w/w) biochar, representing realistic and worst-case application scenarios, corresponding to approximately 22 and 225 t ha^{-1} , respectively (Conti

et al., 2018). Biochar-amended soils were allowed to stabilize in the dark for at least 96 h before testing. Responses of the test species were then compared to those in unamended LUFA 2.2 soil to evaluate attraction, avoidance, or indifference, following the procedure of ISO guidelines (ISO, 2020). These responses were assessed by placing an aliquot of 50 g d.w. of untreated soil (control) on one side of the container and 50 g d.w. of biochar-amended soil on the other. Dual controls (i.e. glass boxes with only LUFA 2.2 soil) were also performed for each bioassay to verify the homogeneous distribution of organisms and validate the tests. Soil moisture in both the bioassays was maintained at WHC of 45 %. Both control and treated soils were slightly flattened to eliminate clumps in the woodlice bioassays. This technique is necessary to reduce the thigmotaxis of woodlice and induce them to aggregate only with conspecifics (Federico et al., 2026a). For each experiment, ten individuals of the representative species were added separately in each test and five replicates for each condition were performed for statistical reasons. The tests were carried out in thermostatic chambers at 20 ± 2 °C, photoperiod 16:8 h (light:dark). After 48 h, arenas of collembolans were separated by a plastic divisor and the number of individuals per each side was counted following the ISO (2020), while the arenas of woodlice were gently positioned beneath a camera (Trust Teza 4K Ultra HD webcam, 3840 $\times$ 2160 pixels) inside the thermostatic chamber, maintaining a fixed distance of 15 cm from the focal point, and high-resolution colour images were recorded for subsequent analysis by ImageJ software.

To assess the effect of potential ecological facilitation, at the end of the woodlice bioassays, individuals were removed and additional ten collembolans were added in the glass boxes for another period of 48 h under the same environmental conditions, at the end of which the distribution of the population was analysed following the method reported in ISO (2020). At the end of all behavioural tests, pH and electrical conductivity were measured by pH meter (WTW-pH 330i/set meter) and conductometer (WTW 3110/set meter) in a 1:5 soil:distilled water ratio as outlined in ISO 10390 (ISO, 2021) and ISO 11265 (ISO, 2025), respectively.

Avoidance behaviour, assessed both in *F. candida* and *P. pruinosus* bioassays, was expressed as Avoidance (A%):

$$A(\%) = \frac{C - T}{N} \times 100 \quad (1)$$

where C is the number of individuals moved to unamended soils, T is the number of individuals which moved towards the biochar-amended soils, and N is the total number of alive individuals recovered at the end of the experiment. Sample soils with less than 20% of individuals were considered as having “limited habitat functions” (ISO, 2020).

The disaggregation effects, assessed only for *P. pruinosus*, were measured by the disaggregation index (DI):

$$DI(\%) = \frac{s + 2d}{n} \quad (2)$$

where s is the number of groups with 1 individual, d is the number of groups with 2 individuals, and n is the number of alive individuals at the end of the experiment. A group represents all the individuals who touch each other with at least one part of the body. The index varies between 0 and 1, which represent the maximum degree of aggregation and disaggregation, respectively, while 0.5 was fixed as the threshold level above which disaggregation affects 50% of the population. Further details referred to the indices can be found in [Federico et al. \(2024\)](#).

2.4 Statistical analysis

Behavioural data were analysed using generalized linear models (GLMs) assuming a Gaussian error distribution with an identity link function. Prior to analysis, potential outliers were excluded (Grubb's test). Model assumptions were evaluated by inspecting residual diagnostics, including tests for normality (Shapiro–Wilk) and homogeneity of variances (Levene's test). When deviations from these assumptions were detected, heteroscedasticity-consistent (Wald robust test) standard errors were applied. Post hoc pairwise comparisons among treatments were conducted using estimated marginal means (emmeans) with Tukey's adjustment for multiple testing. Model significance was assessed using F-statistics and p -values, while goodness-of-fit was quantified using R^2 . To assess whether the presence of woodlice influenced collembolan recruitment in biochar-amended soils, avoidance responses were analysed using a Bayesian linear model with treatment condition (pre- vs. post-isopod exposure) specified as a fixed effect. The model was fitted in rstanarm using Markov chain Monte Carlo (MCMC) sampling with Hamiltonian Monte Carlo as implemented in Stan. Four MCMC chains were run for 4000 iterations each, including 2000 warm-up iterations per chain. Convergence was evaluated using the potential scale reduction factor (R). Treatment effects were estimated from the posterior distribution of the regression coefficient. Evidence for a directional effect was quantified as the posterior probability of reduced avoidance in the post-isopod condition. Differences in soil pH and electrical conductivity in collembolans bioassay before and after woodlice presence were assessed using linear models with controls and treatments as a fixed factor. Responses included initial (pH_i , EC_i) and final (pH_f , EC_f) measurements, as well as temporal changes ($\Delta pH = pH_f - pH_i$; $\Delta EC = EC_f - EC_i$). Model assumptions were evaluated through residual diagnostics, including Shapiro–Wilk tests for normality and Levene's tests for homogeneity of variance. Post-hoc pairwise comparisons were conducted using Tukey HSD tests for ΔpH and ΔEC . Statistical significance was set at $\alpha = 0.05$. Behavioural responses of soil arthropods

were analysed using a multivariate framework integrating collembolan baseline avoidance (*F. candida*, no facilitation) and woodlice responses (*P. pruinosus* avoidance and DI). Data were standardised using z-score transformation. Distance-based redundancy analysis (dbRDA) was implemented via capscale in the vegan package, with biochar type (Control, *B. juncea*, *R. communis*), concentration (0, 1, 10% w/w), and pyrolysis temperature (400, 600 °C) included as explanatory variables. Statistical significance of the model and individual terms was evaluated using permutation tests (999 permutations). Permutational multivariate analysis of variance (PERMANOVA; adonis2) was applied using the same model structure and distance metric. Homogeneity of multivariate dispersion among groups was tested using beta dispersion, followed by permutation tests (999 permutations), to ensure that observed differences were not driven by heterogeneity in dispersion. Redundancy analysis (RDA) was used to relate avoidance response variables of collembolans before and after woodlice facilitation. Predictor variables included biochar type, pyrolysis intensity, and concentration, as well as soil pH and conductivity measured both prior to and following colonization. PERMANOVA based on Bray–Curtis dissimilarities were applied to test for overall treatment effects. Homogeneity of multivariate dispersion among treatment groups was assessed using beta dispersion analysis to verify that observed PERMANOVA effects were not driven by differences in within-group variability. All multivariate analyses were conducted using distance-based and constrained ordination frameworks to jointly assess treatment effects and their contribution to total explained variance. Significance was evaluated using 999 permutations. All statistical analyses were conducted in R version 4.2.1 ([R Core Team, 2024](#)).

3 Results

3.1 Behavioural test results

All behavioural tests performed with individuals of *Folsomia candida* and *Porcellionides pruinosus* showed stochastic population distribution, aggregation (in woodlice bioassays) and no mortality in controls, validating the behavioural tests.

In collembolans, GLMs revealed a significant overall effect in *R. communis* bioassays ([Fig. 1A](#); $F_{4,55} = 4.02$, $p = 0.0063$, $R^2 = 0.226$). Post hoc pairwise comparisons showed that 10% w/w biochar treatments differed significantly from the control, with a stronger effect at 600 °C ($t = -3.24$, $p = 0.017$) and at 400 °C ($t = -2.75$, $p = 0.045$). This pattern suggests that avoidance responses were more influenced by application rate than by pyrolysis temperature. Model diagnostics indicated no evidence of heteroscedasticity (Levene's test: $F_{4,55} = 1.57$, $p = 0.196$), although residuals

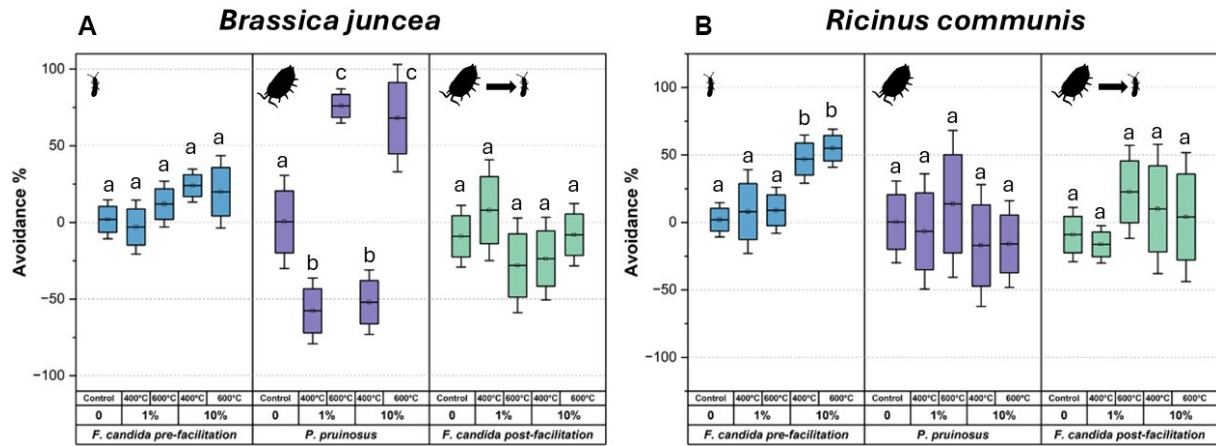


Fig. 1 Avoidance test results for *Folsomia candida* pre- and post-facilitation effects and *Porcellionides pruinosus* exposed to soils amended with *Brassica juncea* and *Ricinus communis* biochar pyrolyzed at 400 and 600 °C at concentrations of 1% and 10% w/w. Dashed lines indicate those values above or below which 50% of the population avoids or is attracted to soils with biochar. Values are expressed as mean ($\pm$ standard error). Different letters indicate significant differences among treatments (post hoc test, $p < 0.05$).

showed a slight deviation from normality (Shapiro–Wilk $W = 0.96$, $p = 0.027$). Nevertheless, results remained robust after heteroscedasticity-consistent (Wald test) corrections ($F_4 = 5.44$, $p = 0.0009$), confirming a significant effect of *R. communis* biochar on collembolan behavioural responses. In contrast, biochar derived from *B. juncea* had no detectable effect on avoidance behaviour (Fig. 1B; $F_{4,55} = 1.08$, $p = 0.37$, $R^2 = 0.073$), highlighting feedstock-dependent differences in collembolan avoidance patterns. In woodlice, GLMs revealed a strong overall effect in *B. juncea* bioassays (Fig. 1A; $F_{4,32} = 6.13$, $p = 0.00088$, $R^2 = 0.434$). Post hoc pairwise comparisons identified significant differences between pyrolysis temperatures at both 1% w/w (400 vs. 600 °C: $t = 3.20$, $p = 0.024$) and 10% w/w (400 vs. 600 °C: $t = 3.64$, $p = 0.008$). Additional differences were observed between 1% (400 °C) and 10% (600 °C) ($t = 3.34$, $p = 0.017$), and between 10% (400 °C) and 1% (600 °C) ($t = 3.54$, $p = 0.010$). Together, these patterns indicate that avoidance responses were more strongly driven by pyrolysis temperature than by application rate. Model diagnostics indicated a slight deviation from normality in residuals (Shapiro–Wilk $W = 0.94$, $p = 0.034$) and heteroscedasticity (Levene's test: $F_{4,32} = 7.14$, $p = 0.0003$). However, results remained highly significant after heteroscedasticity-consistent (Wald-type) corrections ($F_4 = 36.43$, $p < 0.0001$), supporting the robustness of the model. In contrast, biochar derived from *R. communis* had no detectable effect on avoidance behaviour (Fig. 1B; $F_{4,33} = 0.15$, $p = 0.96$, $R^2 = 0.018$), indicating that behavioural responses in woodlice depend on biochar feedstock type.

The reintroduction of collembolans into the woodlice bioassays resulted in no significant avoidance after 48 h for either *B. juncea* ($F_{4,22} = 0.54$, $p = 0.71$) or *R. communis* ($F_{4,27} = 0.48$, $p = 0.75$), indicating that woodlice may modulate

soil microhabitats through faecal pellet deposition and bioturbation, creating conditions that help collembolans maintain homeostasis. Bayesian modelling indicated a consistent reduction in collembolan avoidance behaviour following exposure to woodlice. The posterior mean effect of treatment was negative (-20.9), with a 95% credible interval ranging from -34.5 to -7.5 , indicating a robust directional shift towards reduced avoidance. The posterior probability that avoidance was lower in the post-treatment condition was high ($p = 0.974$), providing strong probabilistic evidence for a reduction in avoidance behaviour following woodlice presence, consistent with ecological facilitation. This signal occurred against a backdrop of substantial behavioural variability ($\sigma = 48.5$), suggesting that while the direction of the effect is stable, its magnitude is modest relative to overall system noise. Model diagnostics supported reliable posterior estimation and good convergence. All parameters showed potential scale reduction factors close to 1 ($R^* = 1.0$), with large effective sample sizes (n_{eff} : 3600–7900) and low Monte Carlo standard errors. Posterior predictive checks further supported model adequacy (mean_PPD = 13.3), consistent with the observed data structure.

Woodlice further exhibited a dose-dependent disaggregation response across all biochar treatments (Fig. 2). GLMs detected significant overall effects for both biochar treatments of *B. juncea* ($F_{4,32} = 4.48$, $p = 0.0055$) and *R. communis* ($F_{4,29} = 3.66$, $p = 0.0157$). Post hoc pairwise comparisons indicated that 10% w/w biochar treatments differed significantly from controls for *B. juncea* (400 °C: $t = 3.66$, $p = 0.0075$; 600 °C: $t = 3.09$, $p = 0.031$) and for *R. communis* at 10% w/w (400 °C: $t = -3.01$, $p = 0.039$), whereas the 10% w/w treatment at 600 °C was not significant ($p > 0.05$). Model diagnostics supported the validity of both models, with normally distributed residuals (Shapiro–Wilk: $W = 0.96$,

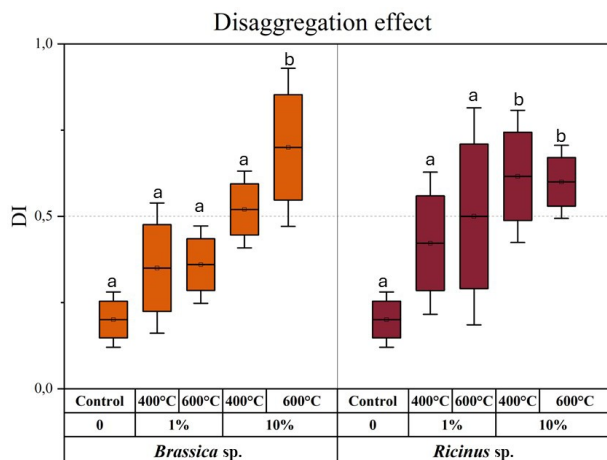


Fig. 2 Scatter plot referred to the disaggregation index (DI) results for *Porcellionides pruinosus* exposed to soils amended with *Brassica juncea* and *Ricinus communis* biochar pyrolyzed at 400 and 600 °C at concentrations of 1 and 10% w/w. Dashed lines indicate those values above or below which 50% of the population is fragmented in groups. Different letters indicate significant differences among treatments (post hoc test, $p < 0.05$).

$p = 0.15$ for *B. juncea*; $W = 0.97$, $p = 0.42$ for *R. communis*) and no evidence of heteroscedasticity (Levene's test: $F_{4,32} = 0.28$, $p = 0.89$; $F_{4,29} = 1.38$, $p = 0.27$, respectively), confirming a good overall model fit.

3.2 Changes of pH and conductivity in collembolan bioassays before and after woodlice introduction

Compared to the control, pre-colonization soil pH (pH_{pre}) increased under *B. juncea* ($\beta = 1.58$, $t = 6.07$, $p = 0.0001$) and *R. communis* ($\beta = 2.88$, $t = 11.06$, $p < 0.0001$). A similar pattern was observed for post-colonization soil pH (pH_{post}), with significant increases under *B. juncea* ($\beta = 1.77$, $t = 7.36$, $p < 0.0001$) and *R. communis* ($\beta = 2.48$, $t = 10.31$, $p < 0.0001$), indicating soil alkalisation following biochar

application. Both concentration and pyrolysis degree were significant positive predictors of soil pH_{pre} (concentration: $\beta = 0.15$, $t = 8.10$, $p < 0.0001$; pyrolysis: $\beta = 0.0028$, $t = 7.29$, $p < 0.0001$), and soil pH_{post} (concentration: $\beta = 0.082$, $t = 5.91$, $p < 0.0001$; pyrolysis: $\beta = 0.003$, $t = 11.1$, $p < 0.0001$). Pre-colonization soil conductivity (EC_{pre}) was unchanged under *B. juncea* ($\beta = 188$, $t = 0.66$, $p = 0.73$) but strongly increased under *R. communis* ($\beta = 2130$, $t = 7.51$, $p < 0.0001$). Post-colonization soil EC (EC_{post}) showed the same pattern, with no effect of *B. juncea* ($\beta = 187$, $t = 0.49$, $p = 0.83$) and a significant increase under *R. communis* ($\beta = 1311$, $t = 3.45$, $p = 0.0091$), indicating soil salinization following treatment. Concentration was a strong positive predictor of both soil EC_{pre} ($\beta = 203$, $t = 8.50$, $p < 0.0001$) and soil EC_{post} ($\beta = 110$, $t = 3.48$, $p = 0.0045$), whereas pyrolysis showed no detectable effect on soil EC ($p > 0.05$), suggesting that ion release and soil accumulation are mainly dose-dependent processes. Temporal changes in soil pH (ΔpH) did not differ between *B. juncea* and the control ($\beta = 0.185$, $t = 1.43$, $p = 0.3$), whereas *R. communis* significantly reduced soil ΔpH ($\beta = -0.407$, $t = -3.14$, $p = 0.016$), indicating dampened pH dynamics over time. Similarly, soil ΔEC was unaffected by *B. juncea* ($\beta = -1.1$, $t = -0.006$, $p = 0.99$) but strongly reduced by *R. communis* ($\beta = -819.4$, $t = -4.67$, $p = 0.0011$). Tukey post-hoc tests confirmed these patterns, with significant differences between *R. communis* and both other treatments for soil ΔpH ($p = 0.00032$ vs. *B. juncea*; $p = 0.022$ vs. control) and soil ΔEC ($p = 0.00027$ vs. *B. juncea*; $p = 0.0015$ vs. control), while no differences were detected between control and *B. juncea*. Model diagnostics indicated adequate adherence to assumptions, with normally distributed residuals for soil ΔpH ($W = 0.94$, $p = 0.43$) and soil ΔEC ($W = 0.95$, $p = 0.51$), and homogeneity of variance for soil ΔpH ($F_{2,12} = 2.55$, $p = 0.12$), although soil ΔEC showed moderate heteroscedasticity ($F_{2,12} = 8.28$, $p = 0.0055$), without affecting overall inference. In general, the

Table 1 summarizes the level of soil pH and EC measured at the end of the collembolan bioassay before and 48 h after the introduction of woodlice for both biochar types.

Treatment	Dose (% w/w)	Pyrolysis (°C)	Soil pH_{pre}	Soil EC_{pre} ($\mu S cm^{-1}$)	Soil pH_{post}	Soil EC_{post} ($\mu S cm^{-1}$)
Control	—	—	6.08 (± 0.12) a	212.6 (± 9.1) a	6.14 (± 0.03) a	226.1 (± 3.6) a
<i>B. juncea</i>	1	400	6.61 (± 0.04) b	208.0 (± 2.1) a	6.96 (± 0.06) b	226.3 (± 3.6) a
		600	6.75 (± 0.14) b	200.7 (± 3.9) a	6.85 (± 0.04) b	248.0 (± 11.8) a
	10	400	6.96 (± 0.03) b	371.3 (± 31.1) b	7.04 (± 0.03) b	304.8 (± 3.8) b
		600	6.74 (± 0.02) b	333.3 (± 59.1) b	6.96 (± 0.03) b	291.4 (± 1.6) b
<i>R. communis</i>	1	400	7.64 (± 0.12) c	255.7 (± 13.0) a	7.77 (± 0.12) c	255.8 (± 19.9) a
		600	7.69 (± 0.21) c	545.7 (± 100.0) b	7.93 (± 0.03) c	580.0 (± 19.7) c
	10	400	8.53 (± 0.04) d	1888 (± 123.6) c	8.11 (± 0.06) d	1006.6 (± 5.9) d
		600	9.41 (± 0.07) e	2797 (± 261.3) d	9.01 (± 0.04) e	2077 (± 512) d

Soil conductivity (EC, expressed as $\mu S cm^{-1}$) and soil pH measured at the end of the collembolan bioassay before the introduction (pre-ecological facilitation) and 48 h after the introduction of woodlice (post-ecological facilitation) for *B. juncea* and *R. communis* biochar. Different letters indicate significant differences among treatments (post hoc test, $p < 0.05$).

model outcomes indicates that woodlice activities modulated soil alkalisation and specifically soil salinization, suggesting a regulation niches.

3.3 Multivariate analysis

Multivariate analysis integrating collembolan and woodlice behavioural responses revealed a significant but moderate shift in response to biochar treatments. Distance-based redundancy analysis (dbRDA, Fig. 3A) showed that experimental factors explained a limited proportion of total variance (constrained inertia = 0.322; $R^2 = 0.107$), indicating a weak but consistent community restructuring. Despite low explanatory power, the permutational multivariate analysis of variance (PERMANOVA) detected a significant overall effect of biochar properties on community composition ($F_{4,95} = 2.85$, $p = 0.003$). Partitioning of the response identified biochar type as the main driver ($F_{2,95} = 3.21$, $p \leq 0.01$), primarily along CAP1 ($\lambda = 0.183$), which captured more variance than CAP2 ($\lambda = 0.132$) and separated control from amended soils. Concentration had a weaker but significant effect ($F_{1,95} = 2.1$, $p = 0.021$), while pyrolysis temperature showed only a minor influence ($F_{1,95} = 1.89$, $p = 0.047$). Beta dispersion showed no differences among groups ($F_{2,97} = 0.40$, $p = 0.67$; permutation $p = 0.68$), confirming that PERMANOVA reflects true centroid shifts rather than dispersion effects. Overall, soil arthropod responses to biochar were statistically robust but moderate, with effects driven mainly by feedstock type > concentration > pyrolysis degree.

Redundancy analysis (RDA, Fig. 3B) integrating collembolan avoidance pre- and post-facilitation of woodlice showed that experimental factors explained approximately 47% of total variance (constrained inertia = 0.937; $R^2 = 0.469$). PERMANOVA results detected a marginally

significant overall effect of biochar properties on community composition ($F_{8,18} = 1.98$, $p = 0.037$), suggesting a real shift in multivariate centroids rather than stochastic variation. Partitioning of the response identified pyrolysis intensity as a significant driver ($F_{1,18} = 3.54$, $p = 0.044$), primarily associated with shifts along RDA1 ($\lambda_1 = 0.591$), which explained most of the constrained variance (63.1%) compared to RDA2 ($\lambda_2 = 0.346$). Soil EC_{pre} also exerted a significant effect ($F_{1,18} = 4.06$, $p = 0.026$), indicating that soil salinization strongly influences *F. candida* behavioural responses. No significant effects were detected for biochar type ($F_{2,18} = 2.32$, $p = 0.063$), and concentration ($F_{1,18} = 1.30$, $p = 0.27$). Beta dispersion analysis showed no significant differences among treatment groups ($F_{2,24} = 1.12$, $p = 0.34$; permutation $p = 0.36$), confirming that PERMANOVA results reflect true centroid shifts rather than dispersion artefacts. Overall, collembolan responses to biochar are statistically significant but moderate in magnitude, with effects primarily driven by soil EC > pyrolysis degree > feedstock and concentrations of biochar.

4 Discussion

This study clearly demonstrates that the addition of biochar derived from *Brassica juncea* and *Ricinus communis* significantly altered soil physicochemical properties, which in turn influenced the behavioural responses of two key soil arthropods, *Folsomia candida* and *Porcellionides pruinosus*. These effects were strongly dependent on biochar type, application rate, and pyrolysis temperature, highlighting the importance of these factors in shaping ecological outcomes in soil ecosystems.

The observed increases in soil pH and electrical

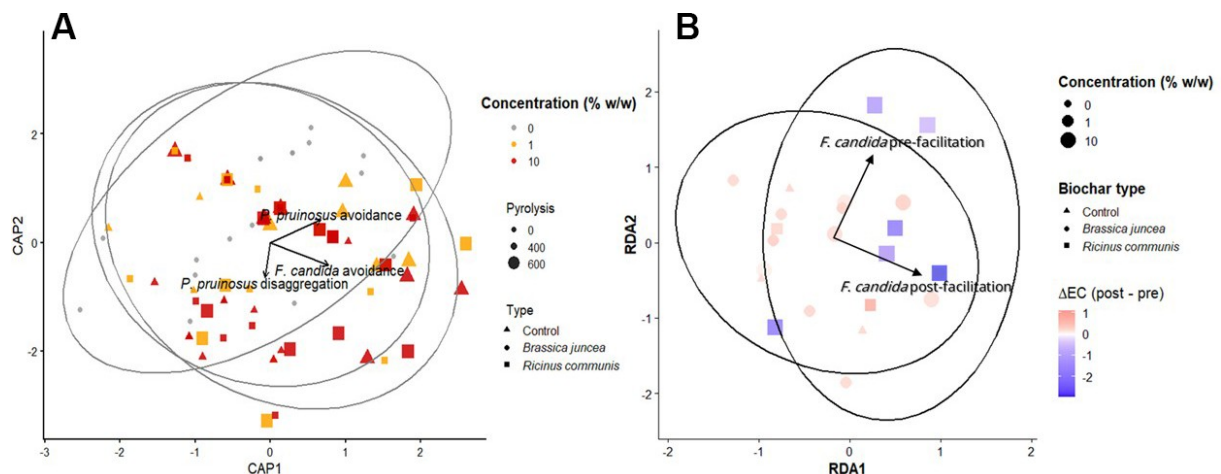


Fig. 3 dbRDA (A) ordination of behavioural responses of *F. candida* and *P. pruinosus* to biochar treatments, and RDA (B) ordination of behavioural responses of *F. candida* pre- and post-facilitation phenomenon. Points are coloured by concentration (0, 1%, 10% w/w), shaped by type (control, *B. juncea*, *R. communis*), and sized by pyrolysis temperature (400, 600 °C). Colour gradient in RDA represents delta values of soil EC pre- and post-facilitation. Ellipses represent 95% confidence intervals. Data were z-score normalized.

conductivity at the initial sampling confirmed the well-documented capacity of biochar to modify soil properties (Lehmann et al., 2011). Notably, *R. communis* biochar exerted a more pronounced effect in soil physiochemical properties than *B. juncea*, especially at higher concentrations, likely reflecting differences in feedstock composition and ash content.

F. candida exhibited a clear avoidance response to soils amended with biochar of *R. communis* at 10% (w/w) compared to soils amended at 1% (w/w). The responses at 10% (w/w) concentrations are consistent with findings from other studies in the literature, where application rates above 10% have been shown to be unsuitable to *F. candida* (Conti et al., 2018). Usually, such concentration reflects only worst-case scenario conditions, as field applications of biochar are not expected to exceed 5%. However, there is no regulation at the EU level that imposes an explicit 5% (w/w) limit on the application of biochar in soil, while such limit is derived from established agronomic practices and technical guidelines (Lehmann and Joseph, 2015), rather than from EU legal obligations. In conclusion, this study showed that the effects of biochar on collembolans depended entirely by the application dose, rather than by the degree of pyrolysis, and adverse effects were consistent with previous studies showing that extreme shifts in soil pH and EC can negatively affect collembolan activity and distribution (Hopkin, 1997; Bastos et al., 2022).

Compared to collembolans, *P. pruinosus* demonstrated attraction to soils amended with *B. juncea* biochar pyrolyzed at 400 °C, while those treated with the same biochar pyrolyzed at 600 °C induced avoidance responses and altered aggregation behaviour. This indicates that pyrolysis temperature critically influences biochar quality and toxicity, in line with literature research (Godlewska et al., 2021). GC–MS analysis showed that *B. juncea* biochar pyrolyzed at 400 °C contained alkanes together with free fatty acids, and esterified fatty acids. In contrast, free fatty acids were not detected in the *B. juncea* pyrolyzed at 600 °C, which was dominated by alkanes, and contained minor amounts of esterified fatty acids. Furthermore, in *B. juncea* biochar pyrolyzed at 400 °C traces of steroid derivatives were additionally detected. Figure S1 (Supplementary information) shows the overlay of the GC–MS chromatograms obtained for the four biochar extracts, in which the main compositional differences are highlighted. The observed *P. pruinosus* attraction at 400 °C in the *Brassica juncea* assay may be attributed to enhanced moisture retention, which is compatible with the presence of free acids, rendering the environment more hydrophilic; moreover, biochar itself can serve as a food source for isopods, comparable to natural detrital substrates like alder leaves (Madžarić et al., 2018). The neutral behavioural response of *P. pruinosus* to *R. communis* biochar suggests a degree of tolerance or adaptation to

altered soil conditions.

The disaggregation of social behaviour observed across the concentration range may be influenced by the granularity of the biochar itself, as coarser particles may enhance thigmotactic behaviour by providing shelter or reducing evapotranspiration. However, in the case of the *B. juncea* 600 °C treatment, most terrestrial isopods were not found in the amended soil but still showed a disaggregation index greater than 50%. This suggests that the observed disaggregation reflects an effect beyond simple thigmotaxis. Biochar-induced changes in soil chemical properties, including increases in salinity and alkalinity, may contribute to the behavioural responses observed in terrestrial isopods. In this context, Federico et al. (2026a) showed that salinity can modify aggregation patterns in *P. pruinosus*, affecting gregariousness through a non-linear response. These findings suggest that changes in soil ionic conditions may influence social aggregation dynamics, but their effects are likely context-dependent and cannot be attributed solely to variations in EC or pH. This is analogous to observations in salt-enriched soils, where, at comparable EC levels, terrestrial isopods show a preference for NaCl-amended soils (0.46–1.87 dS m⁻¹) but not for KCl-amended soils with similar EC (0.74–1.67 dS m⁻¹) (Škarková et al., 2016).

The significant differences in the pre–post changes (Δ) of soil pH and observed after 48 hours in isopod-colonized soils suggests that biotic activity modifies soil chemical conditions, likely through faecal pellet deposition, bioturbation, or microbial input. This is supported by the shift in *F. candida* avoidance patterns upon reintroduction to soils previously inhabited by terrestrial isopods. Such results point to biochar transformation or detoxification processes mediated by isopods and associated microorganisms, ultimately influencing collembolan habitat preferences and indicating complex trophic and microbial interactions (Scheu, 2002; Frouz, 2018). The role of woodlice as facilitating species emerged in laboratory studies by Bastow (2011), in which the abundance of bacterivorous nematodes was influenced by the facilitating effect of litter degradation by *P. scaber* population. In other studies, the synchronous presence of isopods and collembolans accelerates and controls nutrient supply and mobilization in urban soils (Pieper and Weigmann, 2008). Some myrmecophilous woodlice species, such as *Porcellionides myrmecophilus* and *Porcellio scaber*, can live as facultative guests in ant nests, including those of *Lasius fuliginosus*, where they contribute to nest hygiene by feeding on faecal pellets and fungal spores (Parmentier et al., 2025). The multivariate analysis helped to reveal and quantify the magnitude of facilitation effect exerted by woodlice on collembolans. The observed facilitation, particularly for *R. communis* biochar at 10% and 600 °C, confirmed that terrestrial isopod activity can indirectly enhance collembolan colonization in biochar-amended soils.

This underscores the complexity of ecological responses where abiotic amendments and biotic interactions collectively shape soil biodiversity and functions.

The results of this study suggest that the effects of biochar on soil ecosystems cannot be reduced to its physicochemical properties alone, but instead emerge from the interaction between material characteristics, production and application practices, and the activities of soil biological communities themselves. In particular, the observation that woodlice activity modifies soil properties and attenuates collembolan avoidance responses provides experimental evidence of the role of biotic interactions in modulating biochar effects, suggesting that soil fauna represent an active component involved in determining soil suitability. This finding expands the current paradigm of ecological assessment of biochar, traditionally based on chemical and physical parameters, by indicating that the functioning of soil ecosystems also depends on interaction networks among organisms belonging to different trophic levels.

In this context, the interpretation of biological responses requires an ecological perspective that considers the different niches occupied by epiedaphic and euedaphic fauna. Since biochar is incorporated into the soil at depths ranging from 5 cm to 30 cm (Verheijen et al., 2010), its direct effects are likely to primarily affect euedaphic communities, which are more exposed to the amendment. However, the results obtained suggest that these effects may be modulated by the activity of epiedaphic fauna, which, through bioturbation and faecal pellet production, modifies substrate quality, resource availability, and soil physicochemical properties. Consequently, the ecological effects of biochar should also be evaluated by considering functional interactions with litter-associated compartments. Neglecting this dimension could indeed lead to an underestimation of effects on epiedaphic communities and to potential changes in litter decomposition processes and organic matter recycling, compromising an overall assessment of soil functioning.

From an applied perspective, this study suggests that the design of biochar should integrate ecological compatibility criteria alongside agronomic performance and carbon sequestration capacity. The inclusion of behavioural endpoints, given their versatility, rapid implementation, and their ability to be used as tools for investigating interactions among soil fauna organisms within assessment protocols, could enable the identification of materials capable of improving soil quality without compromising the ecological processes that sustain soil fertility and resilience. This perspective is consistent with the establishment of modern ecological risk assessment paradigms, in which numerous regulatory agencies and international organizations recognize behavioural tests as high-predictive-value tools to be integrated, together with traditional endpoints, into environmental risk assessment decision-making processes (Ford

et al., 2026).

In summary, our findings highlight that biochar effects on soil fauna are strongly modulated by feedstock origin, pyrolysis conditions, and amendment rates. Behavioural responses of soil mesofauna are governed by intricate interactions between altered soil physicochemical properties and facilitation activity of soil fauna. These insights emphasize the necessity of integrating biochar characteristics and soil biota dynamics when designing sustainable biochar applications in agroecosystems and soil management strategies.

5 Conclusion

This study examined the effects of two types of biochar derived from *Brassica juncea* and *Ricinus communis* biomass on the behavioural responses of *Folsomia candida* and *Porcellionides pruinosus*. The results demonstrated that the effects of biochar on soil fauna were modulated by the type of feedstock, pyrolysis temperature, and application rate. The responses of *F. candida* confirmed a sensitivity influenced by the application dose, at least for one of the two tested feedstocks. The outcomes of *P. pruinosus* revealed an influence of both pyrolysis and dose conditions, and this information should be taken in consideration in management strategies. In light of the varied responses exhibited by organisms, it is advised that the impact of diverse biochar conditions on behavioural responses at the population level be examined. This approach will facilitate the identification of combinations that have the potential to induce depopulation and thereby contribute to the reduction of soil fauna biodiversity. Supporting soil biodiversity is essential, given the crucial role that woodlice play in ecological facilitation processes. Their ecological relevance was further supported by the observation that the presence of *P. pruinosus* modulated the behavioural responses of *F. candida*, thus suggesting a mitigation response likely mediated by bioturbation and the deposition of faecal pellets. It is therefore recommended that ecological sustainable management be adopted, as high soil biodiversity can prevent soil erosion and facilitate natural processes that can mitigate the effects of biochar itself.

Electronic supplementary material

Supplementary material is available in the online version of this article at <https://doi.org/10.1007/s42832-026-0513-5> and is accessible for authorized users.

Acknowledgements

This work was supported by the MUSA—Multilayered Urban

Sustainability Action—project (contract number ECS 000037) and funded by the European Union—NextGenerationEU, under the National Recovery and Resilience Plan (NRRP) Mission 4 Component 2 Investment Line 1.5: Strengthening of research structures and the creation of R&D “innovation ecosystems”, set up by “territorial leaders in R&D”. We thank Dr. Beatrice Pistore and Dr. Emanuele Defendi for their contribution in laboratory analysis. Open access funding provided by Università degli Studi di Milano-Bicocca within the CRUI-CARE Agreement.

Author contributions

Lorenzo Federico: Conceptualization, Methodology, Resources, Data curation, Formal analysis, Investigation, Validation, Writing – original draft. Asia Rosatelli: Conceptualization, Investigation, Writing – reviewer & editing. Niccolò Lamanna: Investigation, Writing – reviewer & editing. Heiko Lange: Resources, Writing – reviewer & editing. Termopoli Veronica: Resources, Data curation, Writing – reviewer & editing. Isabella Gandolfi: Conceptualization, Writing – reviewer & editing. Andrea Franzetti: Supervision, Writing – reviewer & editing. Sara Villa: Conceptualization, Project administration, Resources, Data curation, Supervision, Validation, Writing – reviewer & editing.

Conflicts of interest

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

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