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Development of a new biosensor based on behavioural responses of *Porcellionides pruinosus* (Ord. Isopoda) as a screening tool for detecting the quality of soil ecosystems

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Abstract

The loss of soil ecosystem functions and services represents a major threat to environmental sustainability and human well-being, particularly in the context of increasing anthropogenic pressures and large-scale soil degradation. Traditional approaches to soil quality assessment, largely based on physical and chemical parameters, are often insufficient to capture the biological complexity and ecological functioning of soils. Consequently, there is an urgent need for innovative, ecologically relevant, rapid, and cost-effective indicators capable of detecting early signs of soil degradation and supporting risk assessment and management strategies.

This PhD thesis addresses these challenges by developing and validating a novel behavioural ecotoxicological endpoint based on the combined assessment of avoidance and population disaggregation responses in gregarious soil invertebrates, with a particular focus on the terrestrial isopod *Porcellionides pruinosus*. By integrating behavioural ecology into soil ecotoxicology, the proposed approach aims to capture not only individual stress responses but also alterations in population structure and adaptive capacity. The thesis explores the sensitivity and versatility of this endpoint under a wide range of conditions, including exposure to physical and chemical contaminants, abiotic and biotic stressors, real urban soils, and soils amended with biochar.

Experimental results demonstrate that disaggregation responses occur at different levels of soil contamination than avoidance, revealing sublethal effects that may compromise population cohesion and soil habitat function even when organisms are able to migrate, reinforcing the need to use the two endpoints jointly. We demonstrated that disaggregation effects may affect even by abioic and biotic factors, suggesting the versatility of the bioassay even for other soil stressors. Furthermore, specific physicochemical properties of contaminants, such as Henry law constant of chemicals, were identified as a potential key descriptor of behavioural disruption, suggesting a potential role of volatility in the insurgence of social alterations. Behavioural outcomes were also shown a great versatility as rapid screening test for assessing reductions in invertebrate biodiversity in real soils, highlighting the ecological relevance of the proposed endpoint. Overall, this thesis demonstrates that behavioural bioassays integrating avoidance and disaggregation responses provide a sensitive and realistic early-warning system for assessing soil health, supporting their application in soil monitoring, biodiversity conservation, and sustainable land management.

Chapter 1

Introduction: Soil Health and Novel Monitoring Strategies

1.1 The Legacy of Soil Health

In “*The Language of the Goddess”* (1989), Marija Gimbutas postulates that in the Neolithic cultures of Old Europe (ca. 7000–3500 BCE), the *soil* held a central symbolic and cultic function, conceptualized as a sacred element intrinsically linked to the archetypal figure of the Great Goddess. It was not merely perceived as an agricultural resource or ecological substrate, but as an ontologically living space (panentheistic), within which the fundamental processes of birth, death, and regeneration were inscribed. This agro-symbolic dimension of soil, understood as a *locus sacer*, emerges systematically through the iconographic and typological analysis of over 2,000 artefacts, some previously known and others uncovered by Gimbutas herself during excavations in the Danube basin and northern Greece (Gimbutas, 2001). These findings reveal a deep integration between material infrastructures and mytho-religious systems, focused on the cult of Mother Earth as a generative, cosmological, and social principle. A comparable structural correlation between soil, fertility, and sacrality is found in Erich Neumann’s theoretical elaboration in “*The Great Mother”* (1981), where soil is thematized as an expression of the maternal archetype in its dual valence: on one hand, as a fertile and nurturing womb; on the other, as a devouring and chaotic abyss. For Neumann, soil is not merely a mythological metaphor, but a profound psychic configuration that reflects the dialectical tension between containment and separation, generation and dissolution. Despite being distinct archaeological and psychological frameworks, both perspectives underscore the semantic centrality of soil health within symbolic systems of early societies: not merely an agricultural matrix, but an ontological and ecological entity, a vital ecosystem fundamental to collective life that demonstrates the interdependence between humans and soil.

Indeed, soils are among the most species rich ecosystems, and their functions contribute to biomass production, the maintenance of biogeochemical cycles, water storage and purification, organic carbon storage, and the supply of medicines, particularly antibiotics (Blum, 2005; Pulleman et al., 2012; Brevik et al., 2018). Due to the integrity of their ecological functions, healthy soils provide essential ecosystem services that are useful for achieving various Sustainable Development Goals (SDGs) (Sachs, 2012), including zero hunger, clean water and sanitation, life on land, flood control, and biodiversity conservation (FAO and ITPS, 2015). At the same time, it is well known that soils are becoming increasingly vulnerable due to various environmental and/or anthropogenic pressures. The main drivers include land use change, deforestation, agricultural intensification, urbanization, pollution, acidification, salinization, erosion, and climate change, which often act synergistically to reduce soil diversity and function, leading to desertification (FAO, 2020). Since soil formation or recovery rates are too slow to cope with current rates of soil loss and degradation, strategies are urgently needed to ensure that this non-renewable natural resource is preserved for future generations (Orgiazzi et al., 2016; Kraamwinkel et al., 2021; Panagos et al., 2022). There is an urgent need for integrative and mechanistic research to assess the cumulative and interactive effects of global change drivers on soil ecosystems (Borrelli et al., 2018).

The urgency of monitoring and safeguarding soil health is justified historically, as well as ecologically, by the deleterious effects that erosion has had on societies. Several ancient civilizations experienced irreversible decline due to soil degradation driven by unsustainable anthropogenic practices. In Mesopotamia, the Sumerian civilization was undermined by salinization of irrigated lands and upland erosion, processes identified by Perlin (1991) and Montgomery (2007) as central to its collapse. The Epic of Gilgamesh (ca. 2700 BCE) preserves a mythopoetic account of this ecological unravelling: the hero's deforestation of the great cedar forests is followed by a divine curse upon the land, which "turned white", a likely reference to salt-encrusted soils, signalling the onset of agricultural decline and the subsequent demographic shift northward to Babylon and Assur. A parallel trajectory is evident in the decline of the Maya civilization, where intense deforestation of the plains and slopes of Central America caused severe erosion and sedimentation of the black fertile soils (*Eklu'um*), contributing to the socio-ecological collapse of the region (Beach et al., 2006). Similarly, the ancient Greeks, as observed by Plato himself, documented soil erosion and the consequent decline in agricultural productivity (Panagos et al., 2024). Soil degradation was closely linked to the population growth of early sedentary civilizations, and unsustainable agricultural practices often led to soil degradation, compromising food security, political stability, and social resilience. While in the past such collapses were localized, today's global population of 7.9 billion people, which is projected to reach 9.8 billion by 2050, combined with a highly interconnected global economy, makes soil degradation a serious threat (Kopittke et al., 2017). For this reason, soil degradation should be considered as the tenth planetary boundaries framework (Kraamwinkel et al., 2021). In the European Union alone, annual costs linked to soil degradation exceed €50 billion (Broothaerts et al., 2024), and such degradation has been associated with mass migration and armed conflict (IPBES, 2018). Alarmingly, the Intergovernmental Technical Panel on Soils (FAO and ITPS, 2015) estimates that up to 90% of global soils may be degraded by 2050.

1.2 A Soil Deal for Europe

Over 60% of soils in the European Union (EU) are unhealthy (Veerman, 2023), and crop productivity levels and other soil functions may already be compromised. To prevent irreversible damage to soil health, the EU has implemented a series of policies to protect soils and agroecosystems over the past two decades, such as the Thematic Strategy for Soil Protection (EC, 2006) and the greening of the Common Agricultural Policy (CAP) (EC, 2013). The Commission Communication "Thematic Strategy for Soil Protection" represents the first comprehensive document aimed at establishing a European policy for soil protection. It identifies the main threats to soil, such as erosion, loss of organic matter, contamination, compaction, salinization, sealing, and loss of biodiversity, and emphasizes the need for a common regulatory framework to ensure the long-term sustainability of the ecological function of soil in the Union. Regulation (EU) No. 1307/2013 establishes rules for direct payments to farmers under the CAP, introducing agricultural practices that are beneficial to the climate and the environment in a greening strategy approach. These measures include the obligation to allocate at least 5% of agricultural land to Ecological Focus Areas (EFAs), crop diversification, and the maintenance of permanent grassland, with the aim of reducing the environmental impact of intensive agriculture and preserving soil biodiversity.

Presented in 2019 and repealed in 2020, the EU adopted a sustainable growth strategy known as the European Green Deal (EC, 2019), with the aim of transforming the EU economy into a climate-neutral, resource-efficient, and socially equitable model by 2050 (Čavoški, 2020), placing soil protection at the top of the political agenda (Panagos et al., 2024). In line with the objectives of the Green Deal, the European Commission (EC) has adopted the EU Soil Biodiversity Strategy 2030 (EC, 2020), the Zero Pollution Action Plan (EC, 2021a), the EU Strategy on Adaptation to Climate Change (EC, 2021b), and the ninth Horizon Europe program (EC, 2021c), followed by the Nature Restoration Law (EC, 2024) and the proposed Soil Monitoring Act (EC, 2025). The EU Biodiversity Strategy for 2030 sets binding conservation targets: at least 30% of the Union's terrestrial area and 30% of its marine areas must be legally protected by 2030, with at least 10% subject to strict protection. The strategy also requires a 50% reduction in the use and risk of chemical pesticides and the restoration of at least 20% of degraded terrestrial and marine areas by 2030, promoting ecological resilience and soil functionality. In the EU Action Plan "Towards Zero Pollution for Air, Water and Soil," the EC sets complementary quantitative targets, including a 50% reduction in nutrient losses (nitrogen and phosphorus) and a 50% reduction in overall pesticide use by 2030. The plan recognizes the close connection between soil health, water quality, and air quality, and introduces integrated monitoring tools to mitigate diffuse pollution from agricultural and industrial activities. The EU Strategy on Adaptation to Climate Change focuses instead on increasing the resilience of European territory to climate impacts, providing for the strengthening of green and blue infrastructure and improving the water and carbon retention capacity of soils. In September 2021, Horizon Europe was also launched as the EU's ninth framework program for research and innovation, with the aim of addressing some of Europe's most pressing and global challenges. Among these challenges, "A Soil Deal for Europe" is one of five flagship missions (alongside Adaptation to climate change, Cancer, Climate-neutral and smart cities, and Restore our Ocean and Waters), which aims to establish 100 living labs and lighthouses to lead the transition towards healthy soils by 2030, combining research, innovation, and active citizen engagement to build a more sustainable and resilient future.

The first binding legislative act for EU member states is Regulation (EU) 2024/1991 on nature restoration, dedicated to large-scale ecological restoration. It requires Member States to take measures to restore at least 20% of the EU's terrestrial and marine areas by 2030 and all degraded ecosystems by 2050, with specific targets for soil organic matter recovery and ecological connectivity. Complementary to the Nature Restoration Law, the proposed Soil Monitoring Act also represents a fundamental step towards the protection and sustainable management of soils in the European Union. The main objective is to ensure healthy soils by 2050, in line with the “Zero Pollution” strategy. In the legislative proposal, the concept of soil health is also defined for the first time as "*the physical, chemical, and biological condition of the soil that determines its ability to function as a vital living system and to provide ecosystem services*." The legislation requires Member States to adopt common methodologies for monitoring and assessing soil health, based on physical, chemical, and biological descriptors. A soil status classification system (good, moderate, critical) is also established to guide targeted interventions. The directive introduces principles for mitigating land consumption, combating soil sealing and topsoil removal. It also requires the management of contaminated sites through public registers and corrective measures. Finally, the legislation promotes the strengthening of technical and laboratory capacities to ensure reliable and consistent data at European level. This integrated approach aims to elevate soil to a fundamental natural resource, on a par with water and air, contributing to the continent's sustainable development goals and environmental resilience. To achieve these objectives, the proposed law requires the development of rapid and environmentally relevant tools that can be used both prognostically and diagnostically to identify, monitor, and preserve soil health.

1.3 Soil Ecotoxicology – the need of practical and relevant tools

Ecotoxicology is an applied ecological science that aims to assess the risk posed by chemical or physical contaminants introduced into the environment at specific ecological levels. The first pioneering studies in soil ecotoxicology date back to the 1960s (Volpe, 1964; Edwards, 1969) and documented the negative effects of pesticides on soil invertebrates, such as earthworms and collembolans (Ghabbour and Imman, 1967; Scopes and Liechtenstein, 1967). These studies stimulated the development of official guidelines for chemical testing, supporting risk assessment procedures and pesticide registration adopted in most Western countries. The first standardized toxicity test for soil invertebrates was the acute (survival) earthworm test (OECD, 1984).

Soil ecotoxicology thus emerged and became established at the regulatory level. In the 1990s, through the European SECOFASE project (Development, Improvement and Standardization of Test Systems for Assessing Sublethal Effects of Chemicals on Fauna in Soil Ecosystems, 1993–1996), supported by the dissemination of the Handbook of Soil Invertebrate Toxicity Tests by Professors Løkke and van Gestel (1998), the possibility of developing toxicity tests for a wide range of soil invertebrates was explored. These included enchytraeids, nematodes, staphylinid beetles, mites, centipedes, millipedes, and isopods. The project laid the groundwork for the development of new guidelines, some of which are still undergoing standardization, and for the introduction of sublethal endpoints, which had previously been subordinate to acute lethal tests. Today, soil ecotoxicology encompasses studies ranging from single chemical compounds to mixture models, physical contaminants at both micro- and nanoscale dimensions, and the combined effects of contaminants and environmental stressors, in line with the paradigm calling for “more ecology in ecotoxicology” (van Straalen, 2003). Despite the substantial advances achieved in soil ecotoxicology, the core framework of ecotoxicological risk assessment has remained essentially unchanged, with two main approaches being distinguished: prognosis and diagnosis (van Gestel, 2012). The prognostic approach aims to estimate the potential effects of new or existing chemical substances in order to regulate their use or prevent their introduction onto the market. This estimation is carried out using laboratory toxicity tests, from which ecotoxicological endpoints—such as the effective concentration affecting 50% of the population (EC50) or the no observed effect concentration (NOEC)—are derived to establish environmental safety levels. The diagnostic approach, by contrast, is aimed at assessing the ecological risk posed by chemical substances at contaminated sites. In this case, toxicity tests are integrated with chemical analyses and field-based ecological observations following a TRIAD approach (Jensen and Mesman, 2006), which is useful for setting management priorities for contaminated sites.

Taken together, the two ecotoxicological approaches pursue a common goal: the protection of biological communities, in terms of both structure and function, from the effects of chemical and physical pollution through environmental risk assessment (ERA). As outlined above, conventional ERA approaches primarily rely on endpoints related to mortality, growth, and reproduction; however, these endpoints are often not sufficiently informative with regard to the potential effects that contaminants may exert on organisms. Indeed, sublethal concentrations can still induce deleterious effects on individuals that may substantially impair soil ecological functions. For example, highly neurotoxic chemicals with specific modes of action (MoAs) can affect population-relevant endpoints by reducing individual mobility, with direct consequences for foraging and mating behaviour (Legradi et al., 2018). For these MoAs, no consolidated ERA approach currently exists, despite their recent introduction into the regulatory debate under REACH through the European Chemicals Agency (ECHA, 2024) report on Key Areas of Regulatory Challenge. Such sublethal endpoints are classified as semi-conventional (defined by standardised guidelines for a limited number of species) or non-conventional (not defined by any standardised guidelines), in order to distinguish them from conventional standardised measures used in chemical risk assessment (Hilgendorf et al., 2025). Within this conceptual framework, semi-conventional and non-conventional endpoints can be incorporated into ERA through a MoA-based approach following an Adverse Outcome Pathway (AOP) structure up to the population level for biochemically active substances, including genotoxic, teratogenic, neurotoxic, immunotoxic, and endocrine-disrupting compounds (Hilgendorf et al., 2025). As most of these endpoints act at the molecular or biochemical level, measurements at sub-individual biological levels, such as biomarkers, are commonly used under the assumption that they are predictive of ecologically meaningful effects. However, this assumption is not always validated. Over the past 40 years, biomarkers have been widely applied in ecotoxicology and ecological risk assessment as alternative early warning signals to detect measurable effects on individual performance (Forbes et al., 2006). Biomarkers have proven valuable in elucidating chemical modes of action, providing mechanistic insight into ecotoxicological effects on soil organisms across different levels of biological organisation. Nevertheless, they have largely failed to demonstrate their actual relevance in terms of potential impacts on population dynamics or on community structure and function (Vighi et al., 2006).

In this context, the most informative ecotoxicological endpoints are those that allow the prediction of relevant effects at the population, community, and ecosystem levels (Figure 1). Alterations in foraging behaviour, destabilisation of trophic networks, avoidance responses, and habitat selection are among the main non-conventional endpoints with direct effects on population performance or community stability.

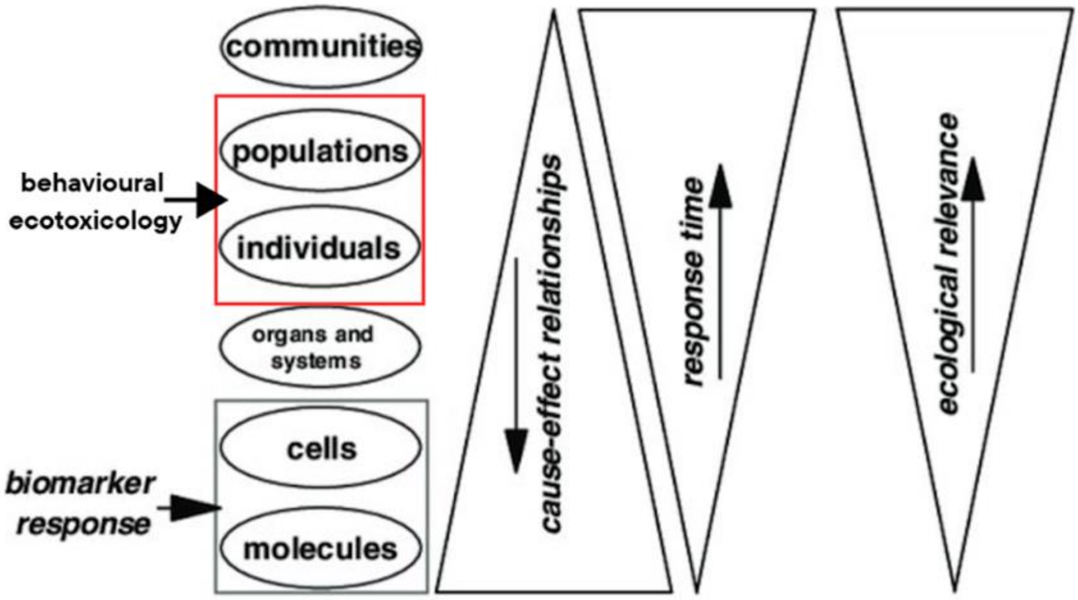

Figure 1. Relationships between responses at different hierarchical levels, their ecological relevance, and challenges in interpretation, with the specific roles and relevance positions of biomarker and behavioural ecotoxicology (modified from Vighi et al., 2006).

However, such endpoints are often excluded from ERA due to concerns regarding their reliability and, in particular, their high sensitivity, often considered overly conservative, which may hinder the market introduction of new chemical substances (Ågerstrand et al., 2020). Consequently, they are consistently assigned lower weight in regulatory decision-making compared to standard studies, for example when submitted as supporting studies within registration dossiers. Despite this, these endpoints provide strong ecological relevance. In light of this scenario, the most promising and ecologically relevant early warning indicators may be those associated with behavioral responses.

1.4 Behavioural bioassays as early warning tools

In recent years, there has been a notable increase in the interest surrounding the investigation of the impact of stressors and sublethal concentrations of contaminants on behaviour as early and sensitive indicators of soil stress (Coyle et al., 2017; Ågerstrand et al., 2020). Among these, behavioural responses related to the structure and dynamics of soil populations represent ecologically relevant warning signals for detecting soil with habitat fragmentation. Therefore, understanding the ethological traits associated with the migration or communication of edaphic populations and the effects of soil stressors on their ecological niche contributes to enhancing ecological realism in the ecotoxicological risk assessment framework (van Straalen, 2003). Among the main behavioural responses, alterations in communication, mobility, feeding, physical fitness, and social behavior of soil fauna are the most studied indicators (De Vries et al., 2012; Boiteau and MacKinley, 2013; Morgado et al., 2015; McAfee et al., 2022; Trigos-Peral et al., 2024), as such alterations can affect population dynamics, reproduction, distribution, and survival. At the same time, these responses are highly species-specific, depending on the tolerance and adaptability of the species, and the choice of appropriate model organisms must be justified in terms of ecological relevance and functional role. Despite their importance, behavioural bioassays remain underutilized in regulatory contexts, making difficult to obtain standardisable and widely comparable data (Ford et al., 2021). A survey conducted by Ford et al. (2025) clearly shows that most academic and government sectors consider behavioural testing to be essential and should be included in risk decisions. However, such inclusion would likely increase costs for regulatory authorities, but more importantly, it could have a direct economic impact on chemical companies, making these sectors less likely to endorse this approach, as a higher degree of sensitivity in ecotoxicological testing could highlight additional risks and lead to stricter regulatory restrictions (Schäffer et al., 2023). Despite the critical issues, some standardized behavioural tests already exist.

Among the relevant, rapid, and cost-effective ecotoxicological bioassays, the avoidance behaviour test is considered a sensitive screening tool with improved ecological realism for evaluating the “limited habitat function” of the soil ecosystem (ISO, 202oa, 2020b), using soil organisms’ ability to choose or avoid harmful substances within the soil. This test provides both prognostic and diagnostic information about soil depopulation, allowing the assessment of the suitability of chemicals and amendment, such as compost and biochar. However, although easy to perform and highly versatile, the avoidance behaviour of soil invertebrates varies between species and contaminants, with numerous non-avoidance episodes occurring due to different ecological factors (Gainer et al., 2022).

Nowadays, behavioural avoidance tests have been conducted using different soil organisms such as earthworms, potworms, springtails, and woodlice (Loureiro et al., 2009; Santos et al., 2010; Lackmann et al., 2023; Puddephatt et al., 2022; Renaud et al., 2022; Malheiro et al., 2023). Despite their advantages, avoidance tests can present a wide variability of responses depending on the type of stressor studied, the model organism selected, and even the type of standard substrate used, whether natural or artificial. Furthermore, they only quantify the impact of stressors on the dispersion of individuals, but do not consider the fragmentation or alteration of individual density at the end of the test: in practice, they do not provide information on how these organisms migrate from a treated soil to a control soil. To compensate for this shortcoming, selecting gregarious organisms, such as terrestrial isopods, is an excellent strategy.

1.5 Terrestrial Isopods as a Non-Conventional Biosensor

Among the main non-target organisms, terrestrial isopods have been proposed as a suitable model organism for behavioural testing, as they are litter detritivores, involved in biogeochemical cycles, and exposed to various exposure pathways, including interstitial water, soil, organic and inorganic matter, air, and food (Lokke and van Gestel, 1998; van Gestel et al., 2018). Currently, there is no established toxicity assessment protocol for this biological model, despite numerous studies demonstrated a greater sensitivity to soil contamination compared to other model organisms (Drobne and Hopkin 1997; Morgado et al., 2016; Maria et al., 2024). The main reason for this is their longer and more complex life cycle compared to other edaphic organisms, such as springtails or earthworms. This makes it difficult to obtain individuals that are synchronized in terms of age, which would be useful for obtaining more homogeneous and comparable results (van Gestel et al., 2018).

At the same time, terrestrial isopods exhibit unique and well-defined eco-ethological traits, the analysis of which suggests that this species could serve as a promising model organism for assessing the impact of stressors on soil communities. Among these traits, the *gregarious behaviour* and the relative increase in population density are considered an ecological feature of considerable adaptive value. Aggregation confers several advantages to individuals, including enhanced protection against predators, improved access to food resources, and greater reproductive opportunities. Most aggregation behaviours are mediated by pheromones (semiochemicals) (Takeda, 1980), which play a crucial role in animal infochemical communication by eliciting behavioural modifications not only among conspecifics but also across taxonomic boundaries. This mechanism is particularly relevant in cryptic ecosystems such as soil, where chemical signals prove more effective than visual or auditory cues (Cardé & Millar, 2009). From an evolutionary point of view, aggregation behaviour represents a pivotal adaptive strategy developed by terrestrial isopods to cope with terrestrial conditions and to mitigate water evapotranspiration under environmental stress. This adaptation is especially vital during moulting periods, which require an increase in haemolymph hydrostatic pressure (Hornung, 2011). Furthermore, the evolution of biphasic ecdysis and nocturnal activity constitute additional physiological and behavioural adaptations that enhance desiccation resistance in these organisms (Devigne et al., 2011; Broly et al., 2016; Nako et al., 2018). Due to their vagile and gregarious nature, woodlice can be used to assess the impact of soil stressors on population-level avoidance and aggregation patterns, as alterations in dispersion or gregariousness represent a sensitive indicator of soil quality. This endpoint acts at population level, therefore is ecologically relevant, as lower individual density reduces litter decomposition rates and increases risk of dehydration, but it is also advantageous because it is rapid and not time-consuming. The proposal of defined and replicable disaggregation indices may also allow for better quantification and standardization of stress effects compared to measurements based solely on the number of individuals in contact with each other (Cividini and Montesanto, 2018; Ďurajková et al., 2022; Delhoumi et al., 2023). As a consequence, alterations in gregariousness represent relevant and standardized indicators, in line with the European Commission's Soil Monitoring Law (Panagos et al., 2025). In this perspective, the development of biosensors based on the behaviour of terrestrial isopods represents a key goal to be achieved in order to identify and prioritize soils subject to depletion, and has been proposed in this doctoral thesis within the Multi-layered Urban Sustainability Action (MUSA) project.

1.6 The Multi-layered Urban Sustainability Action (MUSA) project

The protection of soil health lines with the MUSA (Multi-layered Urban Sustainability Action) project, a research and innovation program funded by the Italian Ministry of University and Research (MIUR) as part of the National Recovery and Resilience Plan (PNRR) adopted and approved by the EC with the aim of supporting and guiding the transformation of metropolitan urban areas towards the creation of an Innovation Ecosystem through an integrated multidisciplinary approach. The project is structured around six areas of intervention (spokes), each divided into different task forces composed of specialized working groups. Specifically, soil protection in terms of habitat function falls under Spoke 1, dedicated to urban regeneration and the design of the 'city of tomorrow', with a particular focus on environmental improvement through monitoring, the protection of ecosystem services, and the implementation of strategies to mitigate the risk associated with contaminants through phytoremediation techniques. Spoke 1 projects include various tasks focusing on strategies and monitoring of phytoremediation processes, as well as analysis of interactions between plants, organisms, and soil microbiota. In particular, Task 5 (Organisms and stress factors) focuses on the effects of contaminants on soil organisms through experimental methodologies that include both field and laboratory investigations, using advanced expertise in ecotoxicology and stress ecology. This project therefore studied and developed an integrated and scalable strategy for monitoring ecological quality in terms of biodiversity, functionality, and contamination of urban soils subjected to greening activities. This strategy required the development of a rapid, economical, and low-impact ecological indicator to identify soils subject to depopulation and with limited habitat function. To this end, this doctoral project proposed the use of terrestrial isopods, in particular the species *Porcellionides pruinosus*, as a model organism on which to develop, for the first time, a new ecological and ecotoxicological endpoint that can consider variations in gregariousness as indicators of soil quality and health.

1.7 PhD Thesis Outline

The loss of soil ecosystem functions and services poses a serious threat to the health of the environment and humans. Given the large scale of degradation, it is necessary to develop new indicators and screening tools that are ecologically relevant, economical, and rapid for monitoring, identifying, and prioritizing unhealthy soils. These indicators cannot be based solely on physical-chemical parameters concerning soil organic matter content, soluble salt concentration, or pH range variations, but must be based above all on the actual biological and ecological responses of soil fauna, primarily those concerning population dynamics and structures. For this reason, it is believed that behavioural tests on gregarious terrestrial organisms, such as *Porcellionides pruinosus* (Ord. Oniscidea), can fill this knowledge gap.

This PhD thesis challenges these gaps by providing new insights into the following topics:

1) Assessing a new ecotoxicological endpoint referring to the joint avoidance and disaggregation effects
2) Investigate the versatility of the new endpoint under different abiotic and biotic stressors
3) Findings some physiochemical descriptors involved in behavioural alterations
4) Linking such behavioural responses to the metrics of soil biodiversity levels in real soils
5) Performing the new endpoint for assessing quality of amended such as biochar

This thesis consists of eight chapters. The current Introduction provides an overview of the state of the art in soil health, the main European standards designed to protect against soil loss, the main challenges relating to the use of ecologically relevant indicators, and the proposal to use terrestrial isopods in behavioural tests. Next, a collection of seven articles is presented (one published review, four research articles already published or submitted, and one ready to be submitted pending patent filing), while the last chapter provides a summary of the overall results and proposes further recommendations and future prospects.

Chapter 2 provides a detailed review of tire particles (TPs) as one of the main sources of micro- and nano-plastics in the environment. It highlights that, although most TPs are deposited in soil or freshwater sediments and accumulate in organisms, research has predominantly focused on the toxicity of leachates, overlooking the direct effects of the particles and their ecotoxicological impact. The chapter also emphasizes that while studies have mainly concentrated on aquatic ecosystems, significant knowledge gaps remain regarding the potential harmful effects of TPs on soil fauna, despite soil being a major plastic sink. Various aspects are analysed, including tire composition and degradation, the transport and deposition of particles in different environments with particular attention to soil, the toxicological effects on soil fauna, potential markers and detection methods for environmental monitoring, an initial risk characterization through the case study of Forlanini Urban Park in Milan, and finally possible risk mitigation measures to promote future sustainability. This review highlights a clearly elevated risk associated with tire particles (TPs) and benzothiazole (BT), a co-formulant used in rubber vulcanization. Additionally, behavioural avoidance tests have proven particularly sensitive to TP contamination. As a result, these two contaminants, both physical (TPs) and chemical (BT), were selected as primary targets for developing the novel behavioural ecotoxicological endpoint.

In chapter 3, a new ecotoxicological endpoint related to the disruption of aggregation behaviour in the gregarious soil species *Porcellionides pruinosus* is developed and evaluated. While avoidance behaviour tests are commonly used for rapid soil quality screening, the impact of soil contamination on the species' adaptive aggregation ability had not been previously investigated. Using tire particles (TPs) and benzothiazole (BT) as representative physical and chemical contaminants, the chapter introduces the disaggregation index (DI) and disaggregation groups (DG) as measures to quantify population fragmentation under contaminant exposure. Results showed that woodlice exposed to certain concentrations of TPs and BT avoided contaminated soil but exhibited reduced aggregation behaviour, indicating population fragmentation. Notably, disaggregation occurred at higher contaminant concentrations than avoidance, suggesting an effect on the population's adaptive capacity even when individuals relocate to uncontaminated soil. These findings highlight that combining avoidance behaviour and disaggregation endpoints may provide more precise and reliable assessments for environmental risk evaluations.

In Chapter 4, we investigate how shifts in soil abiotic and biotic factors influence the aggregation behaviour of *P. pruinosus* and potentially compromise soil habitat function. This study evaluates the effects of temperature, soil moisture, salinity, and pH as abiotic stressors, and edaphic interference, oleic acid (necromone signalling), and microbial community dilution as biotic one. These factors were selected and analysed with the aim of identifying those capable of inducing disaggregation in earth pigs, defining the threshold levels at which these effects occur, and verifying whether it is possible to group the various environmental stress factors according to their intensity and relative impact. The study revealed that temperature and soil moisture produced U-shaped disaggregation patterns, with fragmentation at both low and high levels, while salinity and biotic stressors followed a sigmoidal response. Only pH showed a bimodal distribution, with increased aggregation at both extremes. Such distinct responses were confirmed by K-means cluster analysis, suggesting a distinct behavioural patterns and magnitudes of alteration. These findings demonstrate that *P. pruinosus* aggregation represents a sensitive biosensor of soil environmental changes, providing valuable insights for assessing soil health and for the standardization of laboratory bioassays aimed at monitoring soil habitat function.

Chapter 5 investigates the role of specific physicochemical properties of contaminants on the onset of avoidance and disaggregation responses in terrestrial isopods. A series of twenty-five chemicals related to plant protection products (PPPs), pharmaceutical and personal care products (PPCPs) and perfluoroalkyl acids (PFCAs), all of which are particularly prevalent and dangerous to soil health, were selected as test substances. For each chemical, several nominal concentrations expressed in mg/kg dry soil (d.w.) were selected based on the respective maximum solubilities (g/L) of each compound. Based on the effects observed, the substances were classified into three categories: those that induce both avoidance and disaggregation, those that cause only one of these responses, and inactive substances. Regression models revealed a significant positive correlation between disaggregation and Henry's law constant, identifying a threshold value of 1×10^{-3} $Pa\cdot m^3\cdot mol^{-1}$, beyond which aggregation ceases. These findings suggest that disaggregation effects worsen as substance volatility increases, and that Henry's law constant can serve as a prognostic threshold in regulatory contexts to predict the impact of substances on gregarious populations. In contrast, no predictive descriptors for avoidance responses were identified, indicating that the mechanisms driving migration remain unclear. Given the heterogeneity of behavioural responses, with avoidance and disaggregation responses occurring synchronously or separately, the combined use of the two endpoints in a single bioassay is emphasised to gain a more comprehensive understanding of the ecological impact of soil contaminants.

In Chapter 6, we investigate the role of behavioural bioassays as rapid and integrative tools for assessing and predicting soil biodiversity levels. The study aims to link the behavioural responses of soil invertebrates with biodiversity patterns in invertebrate and bacterial communities across three different urban soils under greening strategy at University of Milano Bicocca. Using a comparative, multi-species, and multi-method approach, model organisms such as earthworms (*Eisenia fetida*), collembolans (*Folsomia candida*), and terrestrial isopods (*Porcellionides pruinosus*) were used to identify soils with reduced habitat function and lower biological diversity. Behavioural results were compared with soil physical-chemical parameters and with ecological indices describing the structural and functional diversity of faunal and microbial communities. The behavioural responses of the test organisms consistently matched reductions in invertebrate biodiversity, indicating habitat depopulation and fragmentation. However, similar patterns were not observed in microbial communities, highlighting a discrepancy that requires further investigation. Overall, the chapter shows that behavioural bioassays, combined with invertebrate diversity assessments, provide an efficient early-warning system for detecting and identifying biodiversity loss in real soils. This integrative approach improves monitoring effectiveness and supports targeted actions for soil conservation and ecosystem restoration.

In conclusion, in Chapter 7, we apply the behavioural responses of soil invertebrates to biochar amendments, a carbon-rich product of biomass pyrolysis, is applied under EU regulations to enhance soil fertility and sequester carbon. While it can improve soil structure and immobilize contaminants, its impact on soil fauna remains debated. This study assessed the responses of collembolans (*Folsomia candida*) and terrestrial isopods (*Porcellionides pruinosus*) to soils amended with biochar derived from *Ricinus sp.* and *Brassica sp.*, pyrolyzed at 400°C and 600°C, and applied at 1% and 10% w/w. After 48 hours, *F. candida* avoided *Ricinus* biochar at high concentrations, suggesting that dosage had a stronger effect than pyrolysis temperature. In contrast, *P. pruinosus* was attracted to *Brassica* biochar at 400°C but avoided it at 600°C, highlighting the combined influence of feedstock type and pyrolysis conditions. Avoidance behaviour in collembolans strongly correlated with increased soil pH and conductivity, indicating alkaline and osmotic stress, whereas these parameters only partially explained isopod responses, implying other contributing factors. To investigate potential mitigating mechanisms, the study tested whether interspecific interactions modify biochar avoidance. Specifically, the presence of woodlice was evaluated for its effect on collembolan behaviour via ecological facilitation, quantified by the proportional of collembolans in biochar amended soil pre and post colonization. Remarkably, collembolans no longer avoided soils previously inhabited by terrestrial isopods, suggesting facilitation mediated by bioturbation, microbial conditioning, or faecal deposition. Overall, Chapter 7 demonstrates that the effects of biochar on soil fauna are shaped by multiple factors, including species identity, biochar feedstock, application rate, pyrolysis temperature, and interspecific interactions. Incorporating facilitation processes into ecotoxicological assessments can provide a more realistic understanding of natural mitigation mechanisms and their role in preserving soil biodiversity.

References

1. Ågerstrand, M., Arnold, K., Balshine, S., Brodin, T., Brooks, B. W., Maack, G., McCallum, E. S., Pyle, G., Saaristo, M., Ford, A. T. 2020. Emerging investigator series: use of behavioural endpoints in the regulation of chemicals. *ESPI*, 22(1), 49-65. https://doi.org/10.1039/C9EM00463G
2. Beach, T., Dunning, N., Luzzadder-Beach, S., Cook, D. E., Lohse, J. 2006. Impacts of the ancient Maya on soils and soil erosion in the central Maya Lowlands. *Catena*, 65(2), 166-178. https://doi.org/10.1016/j.catena.2005.11.007
3. Blum, W. E. 2005. Functions of soil for society and the environment. *RESB*, 4(3), 75-79. https://doi.org/10.1007/s11157-005-2236-x
4. Boiteau G., MacKinley P., 2013. Role of avoidance behavior in the response of *Folsomia candida* to above-freezing cooling temperatures. *Entomol. Exp. Appl.*, 147(1), 50-60. https://doi.org/10.1111/eea.12048
5. Borrelli, P., Van Oost, K., Meusburger, K., Alewell, C., Lugato, E., Panagos, P. 2018. A step towards a holistic assessment of soil degradation in Europe: Coupling on-site erosion with sediment transfer and carbon fluxes. *Environ. Res.*, 161, 291-298. https://doi.org/10.1016/j.envres.2017.11.009
6. Brevik, E. C., Pereg, L., Steffan, J. J., Burgess, L. C. 2018. Soil ecosystem services and human health. *COSUST*, 5, 87-92. https://doi.org/10.1016/j.coesh.2018.07.003
7. Broly, P., Ectors, Q., Decuyper, G., Nicolis, S.C., Deneubourg, J.L. 2016. Sensitivity of density-dependent threshold to species composition in arthropod aggregates. *Sci. Rep.*, 6, 32576. https://doi.org/10.1038/srep32576
8. Broothaerts, N., Panagos, P., Jones, A. 2024. A proposal for soil health indicators at EU-level. Publications Office of the European Union, Luxembourg. https://dx.doi.org/10.2760/8953204
9. Cardé, R.T., Millar, J.G. 2009. Pheromones. In V. H. Resh & R. T. Cardé (Eds.), *Encyclopedia of Insects* (2nd ed.). Academic Press.
10. Čavoški, A. 2020. An ambitious and climate-focused Commission agenda for post COVID-19 EU. *Environ. Polit*., 29(6), 1112-1117. https://doi.org/10.1080/09644016.2020.1784010
11. Cividini, S., Montesanto, G. 2018. Aggregative behavior and intraspecific communication mediated by substrate-borne vibrations in terrestrial arthropods: An exploratory study in two species of woodlice. *Behav. Process*., 157, 422-430. https://doi.org/10.1016/j.beproc.2018.07.006
12. Coyle D. R., Nagendra U. J., Taylor M. K., Campbell J. H., Cunard C. E., Joslin A. H., Mundepid A., Phillips C.A., Callaham Jr M. A., 2017. Soil fauna responses to natural disturbances, invasive species, and global climate change: Current state of the science and a call to action. *Soil Biol. Biochem*., 110, 116-133. https://doi.org/10.1016/j.soilbio.2017.03.008
13. De Vries F. T., Liiri M. E., Bjørnlund L., Bowker M. A., Christensen S., Setälä H. M., Bardgett R. D., 2012. Land use alters the resistance and resilience of soil food webs to drought. *Nat. Clim. Chang*., 2(4), 276-280. https://doi.org/10.1038/nclimate1368
14. Delhoumi, M., Bouslama, M. F., Achouri, M. S. 2023. Effects of moisture decrease on behavioural and life history traits in three species of terrestrial isopods. *Eur. Zool. J.*, 90(2), 832-839. https://doi.org/10.1080/24750263.2023.2273969
15. Devigne, C., Broly, P., Deneubourg, J.L. 2011. Individual preferences and social interactions determine the aggregation of woodlice. *PLoS ONE*, 6(5), e17389. https://doi.org/10.1371/journal.pone.0017389
16. Drobne, D., Hopkin, S. P. 1994. Ecotoxicological laboratory test for assessing the effects of chemicals on terrestrial isopods. *BECT*, 53(3), 390-397. https://doi.org/10.1007/BF00197231
17. Ďurajková, B., Hladký, R., Tuf, I. H. 2022. Higher temperature and substrate vibrations as stress factors for terrestrial isopods–model species matter. *ZooKeys*, 1101, 71. https://doi.org/10.3897/zookeys.1101.77549
18. EC, 2006. Communication from the Commission to the Council, the European Parliament, the European Economic and Social Committee and the Committee of the Regions - Thematic

Strategy for Soil Protection [SEC(2006)620] [SEC(2006)1165] https://eur-lex.europa.eu/legal-content/EN/AUTO/?uri=celex:52006DC0231

19. EC, 2013. Regulation (EU) No 1307/2013 of the European Parliament and of the Council of 17 December 2013 establishing rules for direct payments to farmers under support schemes within the framework of the common agricultural policy and repealing Council Regulation (EC) No 637/2008 and Council Regulation (EC) No 73/2009 http://data.europa.eu/eli/reg/2013/1307/oj
20. EC, 2019. Communication from the Commission to the European Parliament, the European Council, the Council, the European Economic and Social Committee and the Committee of the Regions. The European Green Deal (COM/2019/640 final) https://eur-lex.europa.eu/legal-content/EN/TXT/?uri=celex:52019DC0640
21. EC, 2020. Communication from the Commission to the European Parliament, the Council, the European Economic and Social Committee and the Committee of the Regions — EU Biodiversity Strategy for 2030 — Bringing nature back into our lives (COM(2020) 380 final, 20.5.2020) https://eur-lex.europa.eu/legal-content/EN/TXT/?uri=CELEX:52020DC0380
22. EC, 2021a. Communication from the Commission to the European Parliament, the Council, the European Economic and Social Committee and the Committee of the Regions. Pathway to a Healthy Planet for All EU Action Plan: 'Towards Zero Pollution for Air, Water and Soil' (COM/2021/400 final) https://eur-lex.europa.eu/legal-content/EN/TXT/HTML/?uri=CELEX:52021DC0400
23. EC, 2021b. Communication from the Commission to the European Parliament, the Council, the European Economic and Social Committee and the Committee of the Regions. Forging a climate-resilient Europe - the new EU Strategy on Adaptation to Climate Change (COM/2021/82 final) https://eur-lex.europa.eu/legal-content/EN/TXT/?uri=celex:52021DC0082
24. EC, 2021c. Regulation (EU) 2021/695 of the European Parliament and of the Council of 28 April 2021 establishing Horizon Europe – the Framework Programme for Research and Innovation, laying down its rules for participation and dissemination, and repealing Regulations (EU) No 1290/2013 and (EU) No 1291/2013 (Text with EEA relevance) (PE/12/2021/INIT) http://data.europa.eu/eli/reg/2021/695/oj
25. EC, 2024. Regulation (EU) 2024/1991 of the European Parliament and of the Council of 24 June 2024 on nature restoration and amending Regulation (EU) 2022/869 (Text with EEA relevance) (PE/74/2023/REV/1) http://data.europa.eu/eli/reg/2024/1991/oj
26. EC, 2025. P9_TA(2024)0204 – Soil Monitoring and Resilience (Soil Monitoring Directive) – European Parliament legislative resolution of 10 April 2024 on the proposal for a directive of the European Parliament and of the Council on Soil Monitoring and Resilience (Soil Monitoring Law) (COM(2023)0416 – C9-0234/2023 – 2023/0232(COD)) (Ordinary legislative procedure: first reading) http://data.europa.eu/eli/C/2025/1312/oj
27. ECHA. (2024). Key Areas of Regulatory Challenge. https://doi.org/10.2823/858284
28. Edwards, C. A. 1969. Soil pollutants and soil animals. *Sci. Am.*, 220(4), 88-99. https://www.jstor.org/stable/24926336
29. FAO and ITPS 2015. Status of the World’s Soil Resources (SWSR) – Main Report. https://openknowledge.fao.org/handle/20.500.14283/i5199e
30. FAO, ITPS, GSBI, SCBD, EC. 2020. State of knowledge of soil biodiversity - Status, challenges and potentialities, Report 2020. Rome, FAO. https://doi.org/10.4060/cb1928en
31. Forbes, V. E., Palmqvist, A., Bach, L. 2006. The use and misuse of biomarkers in ecotoxicology. *ETC*, 25(1), 272-280. https://doi.org/10.1897/05-257R.1
32. Ford, A. T., Ågerstrand, M., Brooks, B. W., Allen, J., Bertram, M. G., Brodin, T., Dang, Z. C., Duquesne, S., Sahm, R., Hoffmann, F., Hollert, H., Jacob, S., Klüver, N., Lazorchak, J. M., Ledesma, M., Melvin, S. D., Mohr, S., Padilla, S., Pyle, G. G., Scholz, S., Saaristo, M., Smit, E., Steevens, J. A., van den Berg, S., Kloas, W., Wong, B. B. M., Ziegler, M., Maack, G. 2021. The role of behavioral ecotoxicology in environmental protection. *Environ. Sci. Technol.*, 55(9), 5620-5628. https://doi.org/10.1021/acs.est.0c06493

33. Ford, A. T., Ågerstrand, M., Börjeson, N., Brodin, T., Brooks, B. W., Maack, G., Lazorchak, J. M., Saaristo, M., Wong, B. B. 2025. Perceptions about the use of behavioral (eco) toxicology to protect human health and the environment. *IEAM*, vjaf123. https://doi.org/10.1093/inteam/vjaf123
34. Fox, C. J. S. 1964. The effects of five herbicides on the numbers of certain invertebrate animals in grassland soil. *Can. J. Plant Sci*., 44(5), 405-409. https://doi.org/10.4141/cjps64-080
35. Ghabbour S.I., Imam M. 1967. The effect of five herbicides on three Oligochaete species. *Rev. Écol. Biol. Sol.*, 4: 119-122
36. Gainer, A., Owojori, O., Maboeta, M. 2022. Use of soil invertebrate avoidance tests as an emerging tool in soil ecotoxicology. *Rev. Environ. Contam. Toxicol.*, 260. https://doi.org/10.1007/s44169-021-00004-4
37. Gimbutas, M. A. 1991. The language of the Goddess. *Harper and Row*, San Francisco, California.
38. Gimbutas, M. A. 2001. The living Goddesses. *University of California Press*, Los Angeles, California.
39. Hilgendorf, J., Lautrette-Quinveros, H., Chan, W. S., Del Puerto, O., Ge, J., Noort, K. J., Ronanki, S., Loureiro, S., Spurgeon, D., Selck, H. 2025. Rethinking chemical hazard: an AOP-guided approach to non-conventional endpoints in the environmental assessment of neurotoxic, immunotoxic and metabolic toxic compounds. *Env. Sci. Eur*. https://doi.org/10.1186/s12302-025-01292-6
40. Hornung E., 2011. Evolutionary adaptation of oniscidean isopods to terrestrial life: structure, physiology and behavior. *Terr. Arthropod Rev.*, 4, 95. http://dx.doi.org/10.1163/187498311X576262
41. IPBES 2018: Summary for policymakers of the assessment report on land degradation and restoration of the Intergovernmental Science Policy Platform on Biodiversity and Ecosystem Services. R. Scholes, L. Montanarella, A. Brainich, N. Barger, B. ten Brink, M. Cantele, B. Erasmus, J. Fisher, T. Gardner, T. G. Holland, F. Kohler, J. S. Kotiaho, G. Von Maltitz, G. Nangendo, R. Pandit, J. Parrotta, M. D. Potts, S. Prince, M. Sankaran and L. Willemen (eds.). IPBES secretariat, Bonn, Germany. 44 pages. https://files.ipbes.net/ipbes-web-prod-public-files/spm_3bi_ldr_digital.pdf
42. ISO, 2020a. Soil quality - Avoidance test for determining the quality of soils and effects of chemicals on behaviour - Part 1: Test with earthworms (*Eisenia fetida* and *Eisenia andrei*). ISO 7512-2:2020.
43. ISO, 2020b. Soil quality - Avoidance test for determining the quality of soils and effects of chemicals on behaviour - Part 2: Test with collembolans (*Folsomia candida*). ISO 7512-2:2020.
44. Jensen J, Mesman M (Eds) 2006. Ecological Risk Assessment of Contaminated Land. Decision Support for Site Specific Investigations. *National Institute for Public Health and the Environment*, RIVM, Bilthoven, The Netherlands.
45. Kopittke, P. M., Dalal, R. C., Finn, D., Menzies, N. W. 2017. Global changes in soil stocks of carbon, nitrogen, phosphorus, and sulphur as influenced by long-term agricultural production. *Glob. Chang. Biol*., 23(6), 2509-2519. https://doi.org/10.1111/gcb.13513
46. Kraamwinkel, C. T., Beaulieu, A., Dias, T., Howison, R. A. 2021. Planetary limits to soil degradation. *Commun. Earth Environ.*, 2(1), 249. https://doi.org/10.1038/s43247-021-00323-3
47. Lackmann, C., Šimić, A., Ečimović, S., Mikuška, A., Seiler, T. B., Hollert, H., Velki, M. 2023. Subcellular responses and avoidance behavior in earthworm *Eisenia andrei* exposed to pesticides in the artificial soil. *Agriculture*, 13(2), 271. https://doi.org/10.3390/agriculture13020271
48. Legradi, J. B., Di Paolo, C., Kraak, M. H. S., van der Geest, H. G., Schymanski, E. L., Williams, A. J., Dingemans, M. M. L., Massei, R., Brack, W., Cousin, X., Begout, M.-L., van der Oost, R., Carion, A., Suarez-Ulloa, V., Silvestre, F., Escher, B. I., Engwall, M., Nilén, G.,

Keiter, S. H., Pollet, D., Waldmann, P., Kienle, C., Werner, I., Haigis, A.-C., Knapen, D., Vergauwen, L., Spehr, M., Schulz, W., Busch, W., Leuthold, D., Scholz, S., vom Berg, C. M., Basu, N., Murphy, C. A., Lampert, A., Kuckelkorn, J., Grummt, T., Hollert, H. 2018. An ecotoxicological view on neurotoxicity assessment. *Environ. Sci. Eur*., 30(1), 46. https://doi.org/10.1186/s12302-018-0173-2

49. Lokke, H., Gestel, C. V. (Eds.) 1998. Handbook of soil invertebrate toxicity tests (pp. xxii+-281). *Ecological & Environmental Toxicology Series*, Wiley.
50. Loureiro, S., Amorim, M. J., Campos, B., Rodrigues, S. M., Soares, A. M. 2009. Assessing joint toxicity of chemicals in *Enchytraeus albidus* (Enchytraeidae) and *Porcellionides pruinosus* (Isopoda) using avoidance behaviour as an endpoint. *Environ. Poll*., 157, 625–636. https://doi.org/10.1016/j.envpol.2008.08.010
51. Malheiro, C., Prodana, M., Cardoso, D., Soares, A., Morgado, R., Loureiro, S. 2023. Soil habitat function after innovative nanoagriproducts application: Effect of ageing on the avoidance behaviour of the soil invertebrates *Enchytraeus crypticus* and *Folsomia candida*. *STOTEN*, 901, 165955. https://doi.org/10.1016/j.scitotenv.2023.165955
52. Maria, V. L., Santos, J., Prodana, M., Cardoso, D. N., Morgado, R. G., Amorim, M. J., Barreto, A. 2024. Toxicity mechanisms of plastic nanoparticles in three terrestrial species: antioxidant system imbalance and neurotoxicity. *Nanotoxicology*, 18(3), 299-313. https://doi.org/10.1080/17435390.2024.2358781
53. McAfee A., Metz B. N., Milone J. P., Foster L. J., Tarpy D. R., 2022. Drone honey bees are disproportionately sensitive to abiotic stressors despite expressing high levels of stress response proteins. *Commun. Biol*., 5(1), 141. https://doi.org/10.1101/2021.08.28.456261
54. Montgomery, D. R. 2007. Soil erosion and agricultural sustainability. *PNAS*, 104(33), 13268-13272. https://doi.org/10.1073/pnas.0611508104
55. Morgado R., Ferreira N. G., Cardoso D. N., Soares A. M., Loureiro S., 2015. Abiotic factors affect the performance of the terrestrial isopod *Porcellionides pruinosus*. *Appl. Soil Ecol*., 95, 161-170. http://dx.doi.org/10.1016/j.apsoil.2015.06.012
56. Morgado, R. G., Gomes, P. A., Ferreira, N. G., Cardoso, D. N., Santos, M. J., Soares, A. M., Loureiro, S. 2016. Toxicity interaction between chlorpyrifos, mancozeb and soil moisture to the terrestrial isopod *Porcellionides pruinosus*. *Chemosphere*, 144, 1845-1853. https://doi.org/10.1016/j.chemosphere.2015.10.034
57. Nako, J.D., Lee, N.S., Wright, J.C. 2018. Water vapor absorption allows for volume expansion during molting in *Armadillidium vulgare* and *Porcellio dilatatus* (Crustacea, Isopoda, Oniscidea). *ZooKeys*, 801, 459–479. https://doi.org/10.3897/zookeys.801.23344
58. Neumann, E. 1981. An Analysis of the Archetype. *Princeton University Press*, Princeton, New Jersey.
59. OECD 1984. OECD Guidelines for the Testing of Chemicals No. 207. Earthworm, Acute toxicity tests. Organisation for Economic Co-operation and Development, Paris.
60. Orgiazzi, A., Panagos, P., Yigini, Y., Dunbar, M. B., Gardi, C., Montanarella, L., Ballabio, C. 2016. A knowledge-based approach to estimating the magnitude and spatial patterns of potential threats to soil biodiversity. *STOTEN*, 545, 11-20. https://doi.org/10.1016/j.scitotenv.2015.12.092
61. Panagos, P., Montanarella, L., Barbero, M., Schneegans, A., Aguglia, L., Jones, A. 2022. Soil priorities in the European Union. *GEODRS*, 29, e00510. https://doi.org/10.1016/j.geodrs.2022.e00510
62. Panagos, P., Borrelli, P., Jones, A., Robinson, D. A. 2024. A 1-billion-euro mission: A Soil Deal for Europe. *EJSS*, 75(1), e13466. https://doi.org/10.1111/ejss.13466
63. Perlin, J. 1991. A forest journey. The role of wood in the development of civilization. 2nd ed. *Harvard University Press*, Cambridge/Mass., London.
64. Pulleman, M., Creamer, R., Hamer, U., Helder, J., Pelosi, C., Peres, G., Rutgers, M. 2012. Soil biodiversity, biological indicators and soil ecosystem services—an overview of European approaches. *COSUST*, 4(5), 529-538. https://doi.org/10.1016/j.cosust.2012.10.009

65. Puddephatt, K. J., McCarthy, L. H., Serre, B. M. 2022. Is land-applying biosolids to agricultural areas "sustainable"? Part one: Assessing the potential chronic, sublethal and lethal ecotoxicity of biosolids on *Folsomia candida* and *Lumbricus terrestris*. *Res. Sq*. https://doi.org/10.21203/rs.3.rs-1350257/v1
66. Renaud, M., Natal-da Luz, T., Ribeiro, R., Sousa, J. P. 2022. The recolonization concentration concept: Using avoidance assays with soil organisms to predict the recolonization potential of contaminated sites. *Toxics*, 10(3), 127. https://doi.org/10.3390/toxics10030127
67. Sachs, J. D. 2012. From millennium development goals to sustainable development goals. *The Lancet*, 379 (9832), 2206-2211. https://doi.org/10.1016/S0140-6736(12)60685-0
68. Santos, M. J., Soares, A. M., Loureiro, S. 2010. Joint effects of three plant protection products on the terrestrial isopod *Porcellionides pruinosus* and the collembolan *Folsomia candida*. *Chemosphere*, 80, 1021–1030. https://doi.org/10.1016/j.chemosphere.2010.05.031
69. Schäffer A., Groh K. J., Sigmund G., Azoulay D., Backhaus T., Bertram M. G., Carney Almroth B., Cousins I. T., Ford A. T., Grimalt J. O., Guida Y., Hansson M. C., Jeong Y., Lohmann R., Michaels D., Mueller L., Muncke J., Öberg G., Orellana M. A., Scheringer M. 2023. Conflicts of interest in the assessment of chemicals, waste, and pollution. *Environ. Sci. Technol*., 57, 19066–19077. https://doi.org/10.1021/acs.est.3c04213
70. Scopes N.E.A., Lichtenstein E.P. 1967. The use of *Folsomia fimetaria* and *Drosophila melanogaster* as test insects for the detection of insecticide residues. *J. Econ. Entomol.* 60: 1539-1541. https://doi.org/10.1093/jee/60.6.1539
71. Takeda, N. 1980. The aggregation pheromone of some terrestrial isopod crustaceans. *Experientia*, 36, 1296–1297. https://doi.org/10.3897/zookeys.801.23533
72. Trigos-Peral G., Maák I. E., Schmid S., Chudzik P., Czaczkes T. J., Witek M., Casacci LP, Sánchez-García D., Lőrincz Á., Kochanowski M., Heinze J., 2024. Urban abiotic stressors drive changes in the foraging activity and colony growth of the black garden ant *Lasius niger*. *STOTEN*, 915, 170157. https://doi.org/10.1016/j.scitotenv.2024.170157
73. van Gestel, C. A. 2012. Soil ecotoxicology: state of the art and future directions. *ZooKeys*, (176), 275. https://doi.org/10.3897/zookeys.176.2275
74. van Gestel, C. A., Loureiro, S., Zidar, P. 2018. Terrestrial isopods as model organisms in soil ecotoxicology: a review. *ZooKeys*, (801), 127. https://doi.org/10.3897/zookeys.801.21970
75. van Straalen, N.M., 2003. Ecotoxicology becomes stress ecology. *Environ. Sci. Technol*., 37, 324A–330A. https://doi.org/10.1021/es0325720
76. Veerman, C. P. 2023. Activity update of the Mission Board of European Union on soil health and food. *SEH*, 1(2), 100018. https://doi.org/10.1016/j.seh.2023.100018
77. Vighi, M., Finizio, A., Villa, S. 2006. The evolution of the environmental quality concept: from the US EPA Red Book to the European Water Framework Directive. *ESPR*, 13(1), 9-14. https://doi.org/10.1065/espr2006.01.003

Chapter 2

Silent Contamination: The State of the Art, Knowledge Gaps, and a Preliminary Risk Assessment of Tire Particles in Urban Parks

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toxics

Review

Silent Contamination: The State of the Art, Knowledge Gaps, and a Preliminary Risk Assessment of Tire Particles in Urban Parks

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Abstract: Tire particles (TPs) are one of the main emission sources of micro- and nano-plastics into the environment. Although most TPs are deposited in the soil or in the sediments of freshwater and although they have been demonstrated to accumulate in organisms, most research has focused on the toxicity of leachate, neglecting the potential effects of particles and their ecotoxicological impact on the environment. In addition, studies have focused on the impact on aquatic systems and there are many gaps in the biological and ecotoxicological information on the possible harmful effects of the particles on edaphic fauna, despite the soil ecosystem becoming a large plastic sink. The aim of the present study is to review the environmental contamination of TPs, paying particular attention to the composition and degradation of tires (I), transport and deposition in different environments, especially in soil (II), the toxicological effects on edaphic fauna (III), potential markers and detection in environmental samples for monitoring (IV), preliminary risk characterization, using Forlanini Urban Park, Milan (Italy), as an example of an urban park (V), and risk mitigation measures as possible future proposals for sustainability (VI).

Keywords: tire particles; soil pollution; ecotoxicology; environmental risk assessment

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1. Introduction

Highway and road runoff are one of the main sources of pollution of the environment [1], contributing to the release of a plethora of contaminants such as hydrocarbons (HCs), heavy metals (HMs), micro-plastics or nano-plastics (MPs and NPs), and airborne particulate matter (PM) [2]. However, road traffic contributes to the release of insidious and silent contaminants into the environment, such as MPs produced by tire tread wear: tire particles (TPs).

This contamination was understated for a long time due to a poor consideration of synthetic rubbers in the definition of plastic; in fact, for the International Organization for Standardization (ISO), plastic is defined as a “material which contains as an essential ingredient a high polymer and which, at some point in its transformation into finished products, can be shaped by flow” [3]. Except for thermoplastics and thermosets, elastomers such as rubbers, which compose the tread of tires, have been excluded from this definition. However, Hartmann et al. [4] elaborated an inclusive plastic debris classification, including elastomers (synthetic rubber), heavily modified natural polymers or vulcanized natural rubber, and inorganic and hybrid polymers contained in the tire rubber. Therefore, tire wear particles are considered one of the main hidden sources of MPs and NPs which will require more insights in the future. [5–9].

Recent studies have shown that TPs could be responsible for about 40% by weight of the total amount of MPs in rivers in Europe, with concentrations up to 179 mg/L in sediments flowing from rivers [10]. TPs are furthermore responsible for more than 50% of the contamination of the soil, as shown by the fraction content in the urban and rural soils of China [11].

Globally, it has been estimated that more than 6 million tonnes of TPs per year are released, of which 1 million tonnes per year are from the European Union and the United States [12–14]. In general, TPs are found in every environment, especially in the soil ecosystem, which is strongly polluted by TPs with an estimated annual release of plastic from 4 to 23 times higher than that in the ocean [15]. Only 12% of TPs eventually reach the surface of the water through sewers; despite this, the large environmental load of TPs in the soil is underestimated and deserves to be investigated. The crucial role of particles produced by tire degradation and their human health impact have been well documented in recent studies [16–20], while others have been concerned with the potential environmental risks associated with using tire fill material [21,22]. However, most of these studies have focused only on the chemical leachate derived from the TPs and most of the observed effects have rarely come down to the molecular level.

The aim of this review is to sift through, especially for the soil ecosystem, the current knowledge about the environmental contamination of TPs, paying particular attention to the chemical composition and degradation processes of tires (I), the transport and deposition in the environment according to their size and density (II), the potential markers and detectors in environmental samples for monitoring (III), the toxicological effects in edaphic organisms (VI), a preliminary risk characterization, paying particular attention to the TPs emission in Forlanini Park, Milan (Italy), as a sampling area (V), and some risk mitigation measures as possible future proposals for sustainability (VI).

2. Materials and Methods

This review was drafted by searching the literature for keywords such as “tire particles”, “soil”, “ecotoxicology”, “risk assessment”, and “sustainability”. The use of datasets such as Web of Science or Minerva and Prometheus (University of Milano-Bicocca) was utilized for this research. For a coherent review supported by scientific data, recent (less than ten years) and old (more than ten years) articles and reviews were considered for a general overview of the composition, fate, and environmental markers of TPs. A further criterion for the literature selection was applied for the ecotoxicological effect section, skimming the time frame and selecting only suitable items from 2020 until January 2023 in order to report recent studies. Overall, about 119 articles of the 137 open-access articles related to TPs in soil were selected. Only peer-reviewed articles were included.

3. Definition and Chemical Composition of Tire and Road Wear Particles

TPs occur due to mechanical friction between tires and the road surface during driving, acceleration, or braking, and the heat generated alters the original chemical composition of the wear particles [23,24]. Factors such as the climate, the type of tire, the road surface, the nature of the contact, the speed, the weight of the vehicle, and the driving style could affect their production [13,25,26]. It is estimated that an average car tire lasts between 20–50,000 km before wearing out, releasing about 10–30% of the tread rubber into the environment (at least 1–2 kg) [27].

When they come into contact with asphalt, the TPs undergo a morphological and dimensional change due to the incorporation of the road surface material and the increasing particle core size [28,29]. These aggregates are defined as tire and road wear particles (TRWPs), which have a coating of 10–50% by volume [24] and significantly change their density from 1.13–1.16 g/m^3 to 1.5–2.2 g/cm^3 [30,31]. The most abundant percentage mass (about 90–95%) of TRWPs is made up of heavy particles defined as ‘coarse particles’ [32], which are deposited on soil, sediment, or freshwater environments and not suspended in the air, while small amounts (maximum 10% of the total mass) are made up of a more volatile fraction and emitted into the air, and they are defined as ‘fine particles’ [28,33,34]. Particle size distribution and fractionation during transport into the environment is not currently known but could range from 10 nm to <5 mm [6–9,35,36].

The average percentages of a car tire's composition are shown in Figure 1. The tread of a tire has a wide variety of chemical compounds, mainly:

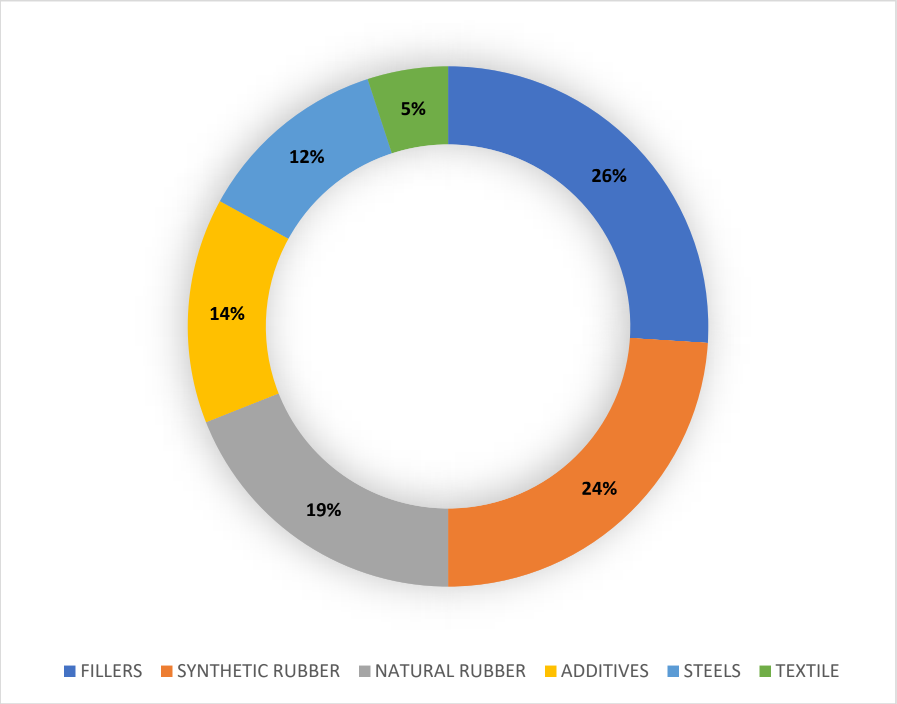

Figure 1. Donut chart relating to the percentage average composition of car tire treads. The data come from the literature [37,38].

- Rubbers: Components typically consist of blends of styrene-butadiene rubber (SBR), such as polybutadiene (PBD), and isoprene rubber (IR), the forerunner of natural rubber (NR), mixed with carbon black or silica (as a reinforcing agent/filler), oils (as softeners and extenders), and curing chemicals. In the past, tires were made only of natural rubber, such as that extracted from the Brazilian rubber tree *Hevea brasiliensis* [39]; nowadays, also for ecological reasons, a mixture of natural and synthetic rubbers is used. Such SBRs describe families of synthetic rubbers derived from emulsion-polymerized (E-SBR, more widely used) or solution-polymerized (S-SBR) styrene and butadiene. The styrene/butadiene ratio affects the properties of the rubber: if the styrene content is high, the rubbers are harder and less rubbery. Normally, 23.5% of the rubber consists of styrene and the remaining 76.5% consists of butadiene [40].
- Organic chemicals: Benzoic acid (BZA) and N-nitrosodiphenylamine (NDphA) are burning retarders and slow down the vulcanization process [34]. Diamines and waxes are also used as anti-degradants by oxidizing agents (oxygen or ozone) and by heat [41,42]. Studies on TRWPs leachate confirmed that they are a potential source of benzothiazoles (BTs), as accelerators of vulcanization, and 1-octanethiol (1-OT) [43], phthalates (PTEs), additives, such as bisphenol A (BSA), and polycyclic aromatic hydrocarbons (PAHs), such as benzo-γ-perylene, fluorene, benzo-α-pyrene, benzo-β-fluoranthene, phenanthrene, benzo-κ-fluoranthene, pyrene, anthracene, and fluoranthene [35,44–47]. Other substances released in leachate are N-(1,3-dimethylbutyl)-N′-phenyl-p-phenylenediamine (6-PPD) and its ozonation product 6-PPD-quinone [48], used as stabilizing additives, hexa(methoxymethyl)melamine (HMMM) [49–51], and N,N′-diphenylguanidine (DPG), an accelerator of vulcanization [14,52].
- Heavy metals (HM): Trace elements such as Zn, Al, Fe, Cd, Cr, Ni, Hg, and Cu are present on TRWPs [43]; consequently, tire wear contributes to the release of HMs into the environment. About 1% of zinc oxide (ZnO) is used as a catalyst to vulcanize the rubber mixtures, transforming them into highly elastic matter; consequently, TRWPs

are considered to be among the main sources of Zn in the environment [53]. Other accelerators are sulfur, sulphenamides, and thiazoles [34].

- Fillers: Most of the components of a tire tread consist of fillers, mainly black carbon (22–40%) finely pulverized by incomplete combustion and added for making the tire resistant to UV rays. In recent years, carbon is sometimes replaced by nanometer glass spheres of silica, which gives the tread strong adhesion and resistance to tearing, heat, and ageing [13].

4. Environmental Fate of TRWPs

4.1. Transport and Deposition Pathways

Once produced, TRWPs deposited on roads can be mobilized and transported by wind, traffic-induced air currents or washed away by rainwater action to other compartments, such as topsoil, air, waste waters, sediment, water surfaces, or other road sections [24,54]. They may potentially drift, accumulate, aggregate, persist, leach, or degrade, affecting the stability of the exposed ecosystems such as those reported in Figure 2 [32,55].

Transport distances depend on particle size and density; coarse particles tend to settle very close to the roadside, within 30 m [34], while fine particles can remain suspended in the air for a long time before settling over many meters [1,2]. Transport of TRWPs can also be influenced by aggregation events both with natural particulates (homo-aggregation) and anthropic particulates (hetero-aggregation) [56–59]. Most of the TRWPs produced accumulate in the soil (about 67%), while the remainder accumulates in the air (3–5%) and in wastewater treatment systems (30%), where they settle in the purification sludge, are transported in freshwater, bioaccumulate, or biodegrade [13]. Only 12% of TRWPs eventually reach the surface of the water through sewers [35], while about 18–22% settle in sediment [60,61]. TRWPs can also bioaccumulate in soil organisms through the food web [62–64].

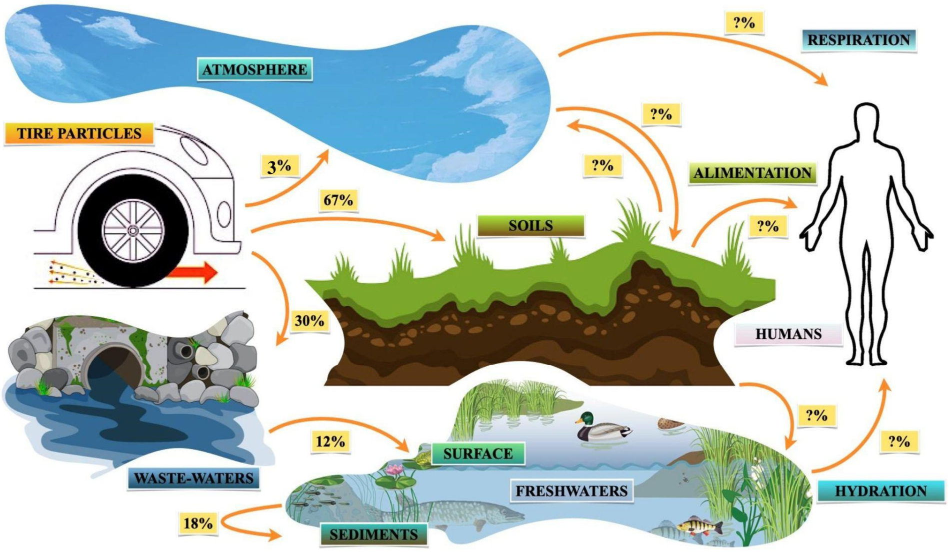

Figure 2. Pathways of the transport, deposition, and fate of TRWPs in environments such as soils, the atmosphere, wastewaters, sediments, and freshwater [13,60,61]. Most of the TRWPs accumulate in the topsoil (67%), while during storm events, they end up accumulating in wastewater (30%) through road surface sewers, where they are transported and accumulate in freshwater such as rivers and lakes, settling in sediments (18%) or remaining on the surface (12%); only a small fraction of the 'fine particles' end up in the atmosphere (3%), but they are also the most unstable, as they can settle after a long time and be resuspended or not at all. The pathways of accumulation in organisms through respiration, feeding, or drinking are potentially viable, which makes these TRWPs particularly dangerous and treacherous.

4.1.1. Soil

Soil is the main ecosystem affected by TRWPs contamination. These particles are dispersed from the road surface, where the concentrations are between 0.7 and 210 g/kg d.w. [65–67]; similarly, coarse TRWPs concentrations in the soil range from 0.6 to 117 g/kg d.w. [66,68–70]. Soil concentrations of TRWPs decrease rapidly with the increase in the distance from the road, with a reduction of more than 80% at a 30 m distance from the road [66,69] and it decreased by 30–40% at a 10–30 cm depth in the soil [66], but the estimated concentrations depend on the type of environmental marker used for detecting these particles and the type of sampling carried out whether it was conducted in-depth or adjacent to busy roads. In addition, the distribution can vary according to various climatic (wind, water, and UV) and anthropic (vehicle traffic) variables; as degradation in the soil is slow, it is possible that they are resuspended in the air by wind erosion or washed away by runoff [69].

4.1.2. Air

The finest particulates, with a diameter generally greater than a micrometer, are emitted directly into the air [71]. These have much longer residence times in the atmosphere than coarse particles due to their low specific weight and reduced density. In addition, once deposited even many kilometers away, they can be resuspended in the atmosphere by wind or the air turbulence of vehicular traffic [71]. The road air can contain about 0.4–11 μg/m^3 of TRWPs [65,66,69,70,72], but estimated concentrations are a function of the sampling distance: in general, the concentration of fine TRWPs increases by 40–50% at about 18 m away from the roadside [66]. The scattering distance depends on wind action, particle size and density, vehicular traffic, and even temperature, ranging from a maximum distance of 30 m [69] to a distance of 86 m from the road [73]. The particle size distribution is variable: the dominant size is between 2.5–10 μm (PM10), while the portion below 2.5 (PM2.5) constitutes only 0.68% in reference to the mass [24,74].

4.1.3. Sewer Systems, Freshwater, and Sediments

A major source of runoff from the road surface, after wind action, is rainwater, which contributes to the transport of TRWPs into the aquatic environment. In sewage systems connected to urban roads, the estimated concentration ranges from 0.3 to 179 mg/L [34,64,65,75]. In rivers, the concentrations during storm events range from 0.09 to 3.6 mg/L, while concentrations under dry conditions are below the limits of detection [65,76]. In sediments, TRWP concentrations are between 0.3 to 155 g/kg d.w., with the greatest concentration being observed in heavily trafficked areas [16,34,64]; however, some of the TRWP concentrations in sediments reported in the literature are based on the use of benzothiazoles (BTs) as a marker, which, being soluble in water, could underestimate the actual amount of TPs accumulating in the sediment [77].

Table 1 reports and summarizes the TRWP concentrations in different ecosystems.

Table 1. Concentrations of TRWPs in different environmental compartments and relative references.

Compartments	[TRWPs]	References
Road face	0.7–210 g/kg [1]	[60–63]
Soil	0.6–117 g/kg [1]	[62,64–66]
Sediment	0.3–155 g/kg [1]	[16,34,60,63]
Sewage	0.3–179 mg/L	[34,60,61,63,71]
Freshwater	0.09–6.4 mg/L	[34,61,72,73]
Air	0.4–11 μg/m^3	[61,62,64,66,68–70]

[1] dry soil.

4.2. Degradation Processes

The degradation of TRWPs (Figure 3), which depends on the susceptibility of photodegradation and biodegradation processes [32], presents a degradation rate of 0.15% per day [69].

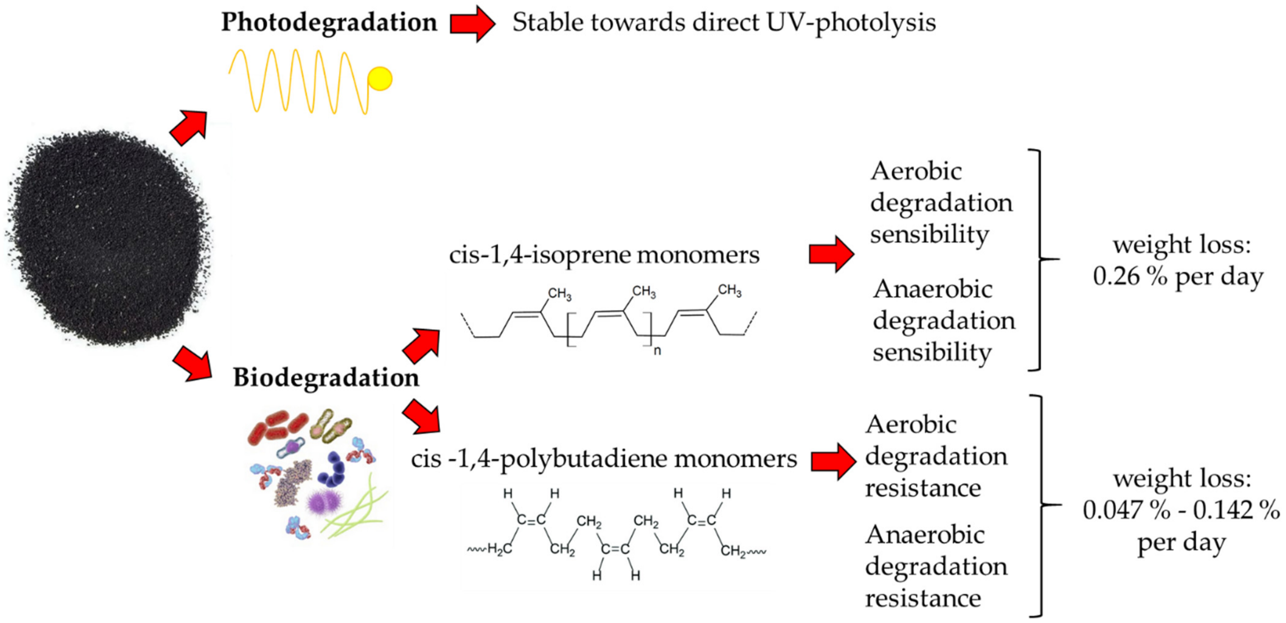

Figure 3. Diagram relating to the main degradative processes in which TRWPs are involved [32,78–80]. The photodegradation processes of rubbers are minimal and do not provide quantitative information, while biodegradation processes depend on the type of monomer involved (cis-1,4-isoprene or cis-1,4-polybutadiene) and the laboratory conditions.

Data relating to the photodegradation processes of rubbers are minimal and do not provide quantitative information [78]. Furthermore, the polymeric double bonds of rubbers can only be lysed by high-energy UV radiation (UV–C, 100–280 nm) and, consequently, are considered stable to direct UV photolysis [79]. Embrittlement due to UV radiation in the range of 290–400 nm is also prevented by protective agents such as antiozonants and antithermoxidants [79]. Concerning to biodegradation processes, cis-1,4-isoprene monomers, typical of natural and synthetic isoprene rubbers, are among the most sensitive to aerobic and anaerobic biodegradation, with a weight loss of about 0.26% per day [32,80]. This is because, during the vulcanization processes, the polyisoprene chains are covalently linked via sulfide bridges, which can be severed by aerobic and anaerobic microbes and modified, with an increase in hydrophilicity and unsaturated bonds [79]. Cis-1,4-polybutadiene monomers, typical of butadiene and styrene–butadiene rubbers, are resistant to biotic and abiotic oxidation, with a loss of 0.047% or 0.142% per day, respectively, depending on the absence or presence of styrene [32]. It has been demonstrated that *Nocardia* spp. 835A-Rc can degrade pneumatic dust under laboratory conditions [81]. Complete biodegradation of TRWPs is believed to be inhibited by the presence of co-formulates, such as Zn salts, BT, and 6-PPD [79,81].

4.3. Environmental Markers of TRWPs

For detecting TRWP pollution in soils and other ecosystems, different chemical markers can be used, as reported in Table 2.

Table 2. Summary table of the most used markers for the detection of tire particles in the environment, their specificity, and relative literature references.

Markers	Methods	Specificity	References
SBR	Thermal extraction desorption gas chromatography–mass spectrometry (TED-GC/MS) Fourier transform infrared spectroscopy (FTIR) Raman spectroscopy	High	[25,34,62,65,66,69]
BTs	Thermal extraction desorption gas chromatography–mass spectrometry (TED-GC/MS)	Medium	[34,60,61,70,72,78]
Zn	Inductively coupled plasma–optical emission spectroscopy (ICP-OES)	Medium/low	[16,34,79–83]

Elastomers such as styrene–butadiene (SBR) or natural rubber (NR) can be used as a marker to identify TRWPs in different environments [34,66,69,70]; indeed, 70% and 75% of the SBR and NR produced globally are totally employed in the production of tires, respectively [42].

BTs, used as vulcanization accelerators in tread manufacturing processes [77], are additional environmental markers for monitoring TRWPs in soil or sediment [34,64,65,73,76,83]. The main BTs include 24MoBT (2-(4-morpholinyl) benzothiazole), HOBT (2-hydroxybenzothiazole), and NCBA (Ncyclohexyl-2-benzothiazolamine); however, the use of BTs in industrial processes has decreased in less than two centuries [65]; in addition, BTs detected in the environment could have different origins, such as antifreeze products [34].

One of the most used environmental markers in the identification of TRWPs is certainly Zn, coming from the ZnO used as a catalyst in the tread vulcanization processes [34,53,84–86]. Despite its wide use, Zn appears to be the most generalist environmental marker of TRWPs, with it having different emission sources and being soluble in particulates, returning less precise vertical and horizontal content gradients, which may underestimate the real concentration of TRWPs in the environment [84]. Although Zn may appear to be a generic marker, methods have been developed to quantify extractable organic Zn as a marker of TRWPs in various environmental matrices [84].

In general, the detection of TRWPs can be performed by thermal extraction desorption gas chromatography–mass spectrometry (TED-GC/MS), quantifying the mass of MPs in soil samples produced after the thermal degradation of SBR, or by density separation processes. Zn can be quantified by inductively coupled plasma–optical emission spectroscopy (ICP-OES) after acid digestion of soil samples with hydrofluoric (HF) or nitric (HNO3) acid [84]. Spectroscopic methods are not recommended, because added carbon black causes total absorbance or fluorescence interferences [14]; however, Workek et al. [25] developed a spectroscopic method which combined Fourier transform infrared spectroscopy (FTIR) with a Raman spectrometer, capable of analyzing particles below 10 µm, to identify synthetic rubber.

In any case, it is legitimate to underline once again that environmental markers for the detection of TRWPs have limitations; in fact, some of these chemical markers are subject once leached to seizure or degradation, such as BT [38], which could lead to underestimation, but, at the same time, different emission sources could lead to an overestimation of the concentrations of these environmental markers of TRWPs [34].

5. Toxicity Effects on Edaphic Fauna and Plants

TRWPs have demonstrated important and often underestimated toxic effects and are starting to be an important stressor for ecotoxicological investigations [87,88]. In the literature, most of the studies conducted on TRWPs have focused on the leachate's effects, mainly in aquatic environments [14,17,43,89–91]; only a few studies have investigated the effect of particles, despite previous studies showing that particles can be as toxic as the leachate [43,68,87,92–94]. Furthermore, studies on TRWP toxicity conducted in soil are still in the infancy stage and require more attention.

As reported by Khan et al. [94], a different toxicological trend of the particulate and of the leachate could not be excluded, perhaps following a dose-dependent trend in the first case and a non-sigmoidal trend in the other and suggesting an important factor of danger in the ingestion of particles and the release of chemicals into the digestive tract.

To date, a proper risk assessment of TRWPs is challenging due to a lack of data. In this regard, it should be essential to draw up a repeatable and comparable experimental design, which takes into account the effects of tire particles as well as leachate, in order to provide more realistic ecological data. One of the main causes of this scarce and non-standardized data plethora is probably due to the lack of attention paid in the past to soils.

Currently, in the literature, in addition to particles, the main factors responsible for the toxicity of TRWPs are co-formulates (PAHs, BTs, and HMs), and their overall toxicity depends on the size and density of the particles themselves, their composition, their aging, and the model organisms used during the experimental tests. Below, the major soil model organisms and plants exposed to TRWPs are reviewed (Table 3).

Table 3. Summary table of the main soil organisms exposed to TRWPs. For each experiment, the dimensions of the particles, the duration of exposure, the type of soil used, and the physical parameters were kept constant, and the analyses carried out are specified. N.A.: data not available.

Species		**TRWPs' Average Size (µm)**	**[TRWPs'] Range (mg/kd d.w.)**	**Exposition Time (Days)**	**Soil**	**Physical Parameters**	**Analyses**	**Ref.**
Edaphic fauna	*C. elegans*	125 µm	1–10.000 mg/kg	Acute test: 2 days Chronic test: 12 days	Texture: sand 93.3%, silt 5.0%, and clay 1.7% pH: 6.7 ± 0.2	Temperature: 20 ± 2 °C WHC: 0.34 ± 0.10 mL/g Photoperiod: in the dark	Endpoint (%): Survival rate, body length, and brood size Chemical analysis: ICP-OES	[95]
	E. andrei	<600 µm	1–1000 mg/kg	Acute test: 48 h Chronic test: 28 days	Texture: fine sand 95.5%, silt and clay 4.5% Organic Carbon: 2.5% pH: N.A.	Temperature: 20 ± 2 °C WHC: 50% Photoperiod: N.A.	Endpoint (%): Net response (avoidance test) and brood size Biochemical parameters: Protein content, ROS, GST, CAT, GR, AChE, CES, GPx, and MXR	[96]
	E. fetida	606.25 µm	10,000–200,000 mg/kg	Chronic test: 14–28 days	Standard soil (OECD 220 guideline): kaolinite clay 20%, quartz sand 70%, and sphagnum peat 10% Organic carbon: 5% Nitrogen: 0.098% pH: 6.9	Temperature: 20 ± 2 °C WHC: 25% Photoperiod: 12:12 h	Biochemical parameters: SOD, CAT, POD, GSH, and MDA Chemical analysis: ICP-MS, ATR-FTIR, and SEM	[65]
	E. crypticus	443.25 µm	48–30,000 mg/kg	Chronic test: 21 days	LUFA 2.2 soil: loamy sand Organic carbon: 1.73% Nitrogen: 0.19% pH: 5.6–4.94	Temperature: 20 °C WHC: 50–60% Photoperiod: 16:8 h	Endpoint (%): Survival, brood size, and gut microbial alteration Chemical analysis: GC-MS and ICP-MS	[68,97]
	F. candida	382.5 µm	200–15,000 mg/kg	Chronic test: 28 days	LUFA 2.2 soil: loamy sand Organic carbon: 1.73% Nitrogen: 0.19% pH: 5.6–4.94	Temperature: 20 °C WHC: 50% Photoperiod: 16:8 h	Endpoint (%): Survival, body length, and brood size	[62,68,98]
	P. scaber	141.45 µm	200–15,000 mg/kg	Chronic test: 21 days	LUFA 2.2 soil: loamy sand Organic carbon: 1.73% Nitrogen: 0.19% pH: 5.6–4.94	Temperature: 20 °C WHC: 40% Photoperiod: in the dark	Biochemical analysis: AChE and ETS Genetic analysis: Expression of immune-related genes in hemocytes and the digestive gland and hepatopancreas	[98,99]

Table 3. *Cont.*

Species		TRWPs′ Average Size (µm)	[TRWPs′] Range (mg/kd d.w.)	Exposition Time (Days)	Soil	Physical Parameters	Analyses	Ref.
Plants	*A. porrum*	125 µm	10,000–160,000 mg/kg	Chronic test: 42 days	Albic Luvisol: loamy sand Organic carbon: 1.87% Nitrogen: 0.12% pH: 5.41	Temperature: 22–18 °C WHC: 40% Photoperiod: 12:12 h	Endpoint (%): Effects on plant growth, change soil pH, and alteration in litter decomposition and respiration	[100]
	V. radiata	326.5 µm	1–10 g/kg	Chronic test: 28 days	Loamy sand organic matter (SOM): 0.9% pH: 5.4	Temperature: 26 °C WHC: 80% Photoperiod: 16:8 h	Endpoint (%): Growth rate of the shoots and leaves and root length Biochemical analyses: Content of polyphenolic compounds (anthocyanins, chlorophyll, flavonoids, and nitrogen balance index), and photosynthetic activities	[62]

5.1. Earthworms

Regarding earthworms, *Enchytraeus crypticus*, *Eisenia andrei*, and *Eisenia fetida* have recently been used for the effect assessment of TRWPs alone or in mixtures with other substances, often showing ambiguous results [64,68,96,97].

Studies conducted on *E. crypticus* exposed to TRWPs have shown both a survival and reproduction decrease, but it is not yet clear whether this decrease is dose-dependent or not. In Ding et al.'s study [97], exposure to TRWPs ≥ 240 mg/kg significantly reduced enchytraeid survival, while exposure at concentrations ≥ 48 mg/kg reduced their reproductive rates, showing dose-dependent toxicity; on the contrary, in Selonen et al.'s study [68], the reproduction rates of enchytraeids decreased only at the lowest (200 mg/kg) and highest (15,000 mg/kg) concentrations, showing a non-dose-dependent trend. Maybe, the different results depend on the size of particles, the chemical conditions of the soil, such as the pH, or the type of tire used. Future studies will help us to better understand these results.

Exposure to TRWPs tests conducted on the Lumbricidae family, such as *E. andrei* and *E. fetida*, did not show any mortality or effects on reproduction or avoidance behavior, while the activity of some oxidative stress biomarkers, such as ROS, GST, and CAT, showed variable trends during the exposure time [63,96,97]. The biochemical response of antioxidant enzymes, such as CAT, or secondary detoxification systems, such as GST, represent important early defense systems that different organisms activate against any disturbing factor; the increase in reactive oxygen species (ROS), on the contrary, underlines a strong condition of oxidative stress usually caused by a general breakdown of the biochemical defense systems [63]. Even if considered early defense systems, molecular biomarkers can warn about the effects that a contaminant or any stressor can have both at an individual level and at a population level, such as an effect of an endocrine disruptor, the consequences of which can affect the entire dynamics of a population species.

Earthworms have also been shown to ingest tire particles, modifying their surface and favoring the release of Zn in the digestive tract, emphasizing, once again, how the particles play a crucial role as leachate [97]. It is probable that, due to the morphological simplicity of the digestive tract, the retention time of these particles is short [101]; however, ingestion can alter gut microbial diversity, which could the change physiology and resistance to stress over time [97].

These results suggest that the accumulation pathways and toxic effects of these TRWPs are not yet fully understood in earthworms, which requires the implementation of standard, replicable, and more ecologically realistic methods that consider the concentrations of TRWPs to use, their size, their vehicular origin, and the effects of long-term exposure, also at the microbial level.

5.2. Nematodes

In the literature, the only study conducted on the nematode *Caenorhabditis elegans* has shown that the toxicity of TRWPs may depend on the exposure time and the aging period of the contaminated soils, altering survival, growth, and brood [95]. In short-term tests on *C. elegans*, it was observed that aged soils (soil incubated with TRWPs for 30 and 75 days) showed effects on growth and brood size at 1 mg/kg d.w., while the same effects in soils not incubated with TRWPs occur at 100 mg/kg and 10,000 mg/kg, respectively. Similarly, long-term exposure tests showed early effects on survival already by the 6th day (10,000 mg/kg) in aged soils, rather than by the 8th day (10 mg/kg) in unaged soils. The authors suggest that the increase in toxicity in aged soils depends on an increase in the leachate of chemicals. Among these substances, the concentrations of metals leached into interstitial waters may not show a significant variation between treatments, demonstrating that the presence of HMs may not be the main cause of the high measured toxicity; conversely, PAHs or other organic chemical compounds may be mainly responsible for the measured toxic effects, but these studies require further investigation [95]. For the authors, it is not to be excluded that these values may depend on the low origin concentration of metals in the tire tread, too high soil pH, or too short an exposure period [95].

5.3. Springtails

Springtails such as *Folsomia candida* are often used in ecotoxicological tests related to soil contamination, with them being organisms of high ecological value and easy to grow in laboratory conditions.

In the literature, exposure to TRWPs in *F. candida* has shown different effects. In Selonen et al.'s study [68], nominal exposure concentrations of only tire particles from 0 to 15,000 mg/kg d.w. showed a decreasing trend in survival and reproduction, but not significantly. In Kim et al.'s study [62], on the other hand, TRWPs appear to reduce the growth rate in springtails at the concentration of 10,000 mg/kg d.w., probably due to the engulfment of soil pores, which stimulates springtails to expend more energy in movement or to expel ingested particles; furthermore, reproduction rates appeared to decrease in TRWP treatments, albeit not in a statistically significant way. Springtails have also been shown to ingest them, showing the potential role of the particles' toxicity.

TRWPs can also modify the bioavailability for edaphic organisms of other contaminants; in springtails, it was demonstrated that TRWPs decrease the toxicity of insecticide chlorpyrifos, reducing its lethality (but not significantly) and its reproduction effects according to the sigmoidal concentration–response [98]. These results may be related to a seizure of chlorpyrifos by the tire particles, probably due to their lipophilicity; future tests could focus on mixture studies involving organic chemicals or metals in order to understand the mechanisms of seizure or release by TRWPs under different environmental conditions for a real risk assessment.

5.4. Woodlice

Woodlice are important edaphic organisms in litter and they are used as test species in studies of ecotoxicity and ecophysiology [102,103].

For springtails, in Selonen et al.'s study [98], the effect of mixing chlorpyrifos with low and high concentrations of tire particles reduced mortality and the interference on acetylcholinesterase (AchE) in *Porcellio scaber*, demonstrating reduced bioavailability of chlorpyrifos by TRWPs. AChE, an enzyme involved in neurotransmission in the cholinergic nervous system, is a target for specific and nonspecific neuro-inhibitors, such as organic and inorganic compounds; as Zn and BT are present in high concentrations, they are likely to inhibit AChE activity [98].

Other preliminary studies conducted on *P. scaber* exposed to TRWPs suggest that these induce the activation of immune-related genes in hemocytes and the hepatopancreas, modulating the immune system and the types of hemocytes in the hemolymph; in Dolar et al.'s study [99] increased ETS activity and impaired differential hemocyte count (DHC) were observed at the maximum concentration of 300 mg/kg, but hemocyte alteration was restored after the 7th day of exposure, showing immune recovery in the woodlice.

5.5. Plants

TRWPs have been shown to negatively affect plant growth by inducing an alteration in photosynthetic activity and polyphenolic composition, modifying soil pH, and changing litter respiration and decomposition processes [62,100].

The seeds of *Allium porrum*, once sterilized (0.5% solution of NaClO-bleach for 10 min and 70% ethanol for 40 s) and germinated in soils contaminated by TRWPs, in a range of 0–160,000 mg/kg according to a progression per step of 10,000 mg/kg, showed a reduction in leaf and root growth already at concentrations of 10,000 mg/kg, with this stabilizing at around 60,000 mg/kg [100]. Litter decomposition, consisting of tea leaf sachets (Lipton Green Tea, Sencha Exclusive Selection), slightly increased at low concentrations of TRWPs, but reversed its trend at concentrations > 60,000 mg/kg, decreasing slightly, underlying how the presence of TRWPs profoundly alters biogeochemical cycles and soil decomposition rates [100]. Furthermore, the soil respiration rate, as well as the pH and leached Zn levels, continued to increase until the end of the test [100]. The increase in leached Zn seems to be one of the main disturbing elements for plant growth, but its bioavailability is influenced by abiotic parameters, such as pH; in fact, heavy metals are easily absorbed at an acidic pH, while their absorption is already inhibited at more alkaline pHs. It is therefore important to know the abiotic conditions of soil to understand how TRWP contamination affects soil processes and plant homeostasis.

Similar effects were observed in the leaf and shoot growth of *Vigna radiata* exposed to TRWPs from three different vehicles (car, bike, and e-scooter) under dry soil concentrations of 1 and 10 g/kg for 28 days [62]. Specifically, TRWPs from different vehicles have been observed to alter plant homeostasis differently; bikes and e-scooters in fact reduce leaf and sprout growth, as well as altering polyphenolic biomarkers, reducing anthocyanin and flavonoid levels, and increasing nitrogen balance [62]. In addition, the photosynthetic activity of some parameters of photosystem II (PSII) was altered differently according to the TRWPs: the steady-state PSII efficacy (QYLss), the photochemical hardening coefficient (qP), and the fraction of open PSII centers (qL) were reduced in *V. radiata* leaves exposed to TRWPs from cars and bikes, while they increased in those exposed to TRWPs from e-scooters [62]. These three parameters are related to the photosynthesis of PSII and generally decrease when the photosynthetic activity of chlorophyll-a in PSII is inhibited; instead, polyphenolic compounds, such as anthocyanins and flavonoids, protect plants from reactive oxygen species by neutralizing cellular ROS levels. The decrease in anthocyanin and flavonoid levels, as well as the reduction in the photosynthetic activity of PSII, demonstrates that TRWPs induce oxidative stress in plants and that this stress factor depends on the type of tire compound, considering that tire particles emitted by different vehicles show different effects. This underlines how even the composition of the tread can influence the expected effects on plants.

6. Preliminary Risk Assessment and Future Projections for Urban Soils: The 'Milan Case'

For estimating a preliminary risk related to TRWPs and co-formulates, a preliminary risk assessment was applied to a green area as an example in the city of Milan, Italy: Forlanini Park. This park is a huge Milanese urban green area located close to Forlanini Avenue, a long speedway that connects the city to the nearest Linate airport; therefore, being a busy road, TRWPs and associated contaminants could potentially be released into the adjacent Forlanini Park, which was chosen in this review as a reference area to assess the preliminary risk associated with TRWPs transport.

Parco Forlanini was also selected as a green area in Milan subject to reforestation by the ForestaMi Project, promoted by the Municipality of Milan and Co., which involves the planting of 3 million trees by 2030 in all green areas of Milan in order to purify the air, improve living conditions in the wider area, and mitigate the effects of climate change; consequently, Parco Forlanini can be considered as an outdoor laboratory for monitoring the impact of contaminants, such as TRWPs, and the effects of climate change.

In order to estimate the environmental risk of TRWPs and co-formulates, the risk quotient (RQ) was calculated by the ratio of the predicted environmental concentration (PEC) and the predicted no effect concentration (PNEC):

$$\mathrm{RQ} = \mathrm{PEC}/\mathrm{PNEC}, \tag{1}$$

Consequently, all $\mathrm{RQ} > 1$ values were considered as an unacceptable risk factor, while $\mathrm{RQ} < 1$ values were considered as acceptable risk factors. For evaluating the PEC, an estimation method based on the ratios between the emission factors (EFs) of tire particles or associated compounds and urban soil mass was applied:

$$\mathrm{PEC}_x = \mathrm{EF}_x/\mathrm{M}_x, \tag{2}$$

PEC_x = predicted environmental concentration of the x contaminant
EF_x = mg of the emission factor of the x contaminant emitted by registered cars
M = kilograms of urban soil considered.

6.1. TRWP Emission Factors

For quantifying the emissions factors, three principal methods have been developed in the literature [13,35,100]. The most widely used method for estimating $\mathrm{EF}_{\mathrm{TRWPs}}$ and considered in this review combines the emission factors of tire wear by vehicle type, the number of vehicles on the road, and the annual kilometers driven [13,35,104]:

$$\mathrm{EF}_{\mathrm{TRWPs}} = \mathrm{EF}_{\mathrm{V}} \times \mathrm{N}_{\mathrm{V}} \times \mathrm{d}, \tag{3}$$

$\mathrm{EF}_{\mathrm{TRWPs}}$ = emission factors of TRWPs (mg x vehicles/y);
EF_{v} = vehicle-specific emission factors of TRWPs (mg/km);
N_{v} = number of vehicles on the road
d = distance in km/y

The tire wear EFs by vehicle category and track type are shown in Table 4 [13,35,104].

Table 4. Tire wear emission factors (mg/km) of some different vehicles, depending on the type of road (urban, rural, or highway) [13,35,104].

Vehicles	**$\mathrm{EF}_{\mathrm{TRWPs}}$ (mg/km)**		
	Urban	**Rural**	**Highway**
Car	1.32×10^2	8.5×10^1	1.0×10^2
Bus	4.2×10^2	2.7×10^2	3.3×10^2
Motorcycle	6.0×10^1	3.9×10^1	4.7×10^1
Truck	6.6×10^2	4.2×10^2	5.2×10^2
Lorry	8.5×10^2	5.5×10^2	6.7×10^2

To estimate the EFs of TRWPs in Forlanini Park, only the emissions from cars were considered. Considering a Milanese car fleet of 688,223 since 2020, an emission factor of cars equal to 132 mg/km [13,35,104], and the length of Forlanini Avenue of approximately 3 km, the $\mathrm{EF}_{\mathrm{FOR}}$ is equal to:

$$\mathrm{EF}_{\mathrm{FOR}} = (\mathrm{EF}_{\mathrm{CAR}}) \times (\mathrm{N}_{\mathrm{V}}) \times (\mathrm{l}_{\mathrm{FOR}}) = (1.32 \times 10^2\ \mathrm{mg/km}) \times (688{,}223) \times (3\ \mathrm{km}) = 27.2 \times 10^7\ \mathrm{mg}, \tag{4}$$

EF_{FOR} = Maximum emission of TRWPs produced by car fleet in 3 km of Forlanini Avenue
EF_{CAR} = Estimated emission factor of TRWPs per kilometer (mg/km) from a common car (Table 4)
V_{MI} = Milan's registered cars
l_{FOR} = Forlanini Avenue's length (3 km) adjacent to the park (Figure 4a).


(**a**) (**b**)

Figure 4. Satellite image of Forlanini Park; the portion of land considered in this review is marked in purple (**a**). Image of Forlanini Avenue, where it is possible to notice the proximity without obstacles between the road and the park (**b**).

6.2. Mass of Considered Soil

To evaluate the PEC_{TRWPs} from Equation (2), the soil's volume to Forlanini Park of soil susceptible to contamination by TRWPs was estimated, since this is potentially limited to the first 30 m from the roadside and to the first 10 cm of depth [66,69]; estimating that Forlanini Avenue (Figure 4) is about 3 km long, the volume of land affected by TRWPs is equal to:

$$V_{SOIL} = l_{FOR} \times w_{FOR} \times d_{FOR} = (3000 \text{ m}) \times (30 \text{ m}) \times (0.1 \text{ m}) = 9.0 \times 10^3 \text{ m}^3, \quad (5)$$

V_{SOIL} = Total volume of Forlanini Park soil potentially impacted by TRWPs
l_{FOR} = Forlanini Avenue's length adjacent to the park
w_{FOR} = Width of soil impacted by TWRP transport [66,69]
d_{FOR} = Depth of soil potentially contaminated by TRWPs [66]

Estimating a general soil density of about $\rho = 1.4 \times 10^3$ kg/m^3, the mass of soil volume affected by TRWPs is equal to:

$$M_{soil} = (\rho) \times (V_{soil}) = (1.4 \times 10^3 \text{ kg/m}^3) \times (9.0 \times 10^3 \text{ m}^3) = 1.26 \times 10^7 \text{ kg d.w.}, \quad (6)$$

M_{soil} = kg d.w. of the Forlanini soil mass considered
ρ = kg/m^3 of the soil's density
V_{SOIL} = m^3 of the total volume of Forlanini Park's soil from Equation (5)

6.3. PEC of TRWPs

By relating the number of maximum emissions of TRWPs produced by car fleets within 3 km of Forlanini Avenue to the mass of the soil potentially subject to such contamination in Forlanini Park, PEC_{TRWPs} was obtained by Equation (2):

$$PEC_{TRWPs} = (EF3_{MI})/(M_{soil}) = (27.2 \times 10^{7}\ \text{mg})/(1.26 \times 10^{7}\ \text{kg}) = 21.62\ \text{mg/kg d.w.} \quad (7)$$

In addition, the PECs relating to some of the most common contaminants traced in the TRWPs (organic chemicals and metals) were calculated through a proportion, using the average tire particle concentrations known in the literature (Table 5) [23,43,68,97,105–108].

Table 5. PNEC (mg/kg d.w.), PEC (mg/kg d.w.), and RQ related to the car tire particles and some co-formulates estimated based on the concentrations reported in the literature [23,43,68,97,105–108] and potentially released in the first 30 m of topsoil of Forlanini Park, Milan. (*) = high risk; (-) = no data.

Substances		$PNEC_{SOIL}$ [mg/kg d.w.]	PEC_{SOIL} [mg/kg d.w.]	RQ
Tire Particles		10 [4]	21.62	2.16 *
Organic Chemicals (non-PAHs)	Benzothiazole	0.017 [2]	0.060	3.58 *
	1-indanone	-	0.002	-
	1-octanethiol	-	0.0008	-
Organic Chemicals (PAHs)	Pyrene	1 [2]	0.037	0.037
	Fluoranthene	1.5 [2]	0.018	0.012
	Phenanthrene	1.8 [2]	0.009	0.005
	Benzo[ghi]perylene	0.17 [2]	0.004	0.024
	Anthracene	0.13 [2]	0.002	0.022
	Acenaphthylene	0.29 [2]	0.0004	0.001
	Benzo(b)fluoranthene	0.28 [2]	0.006	0.022
	Chrysene	0.55 [2]	0.012	0.023
	Indeno(1.2.3-cd)pyrene	0.13 [2]	0.002	0.022
	Fluorene	1 [2]	0.004	0.004
	Naphthalene	1 [2]	0.0002	0.0002
	Benzo[a]anthracene	0.079 [2]	0.008	0.105
	Benzo(a)pyrene	0.053 [2]	0.004	0.075
	Acenaphthene	0.038 [2]	0.0001	0.005
	Benzo(k)fluoranthene	0.27 [2]	0.001	0.007
	Dibenzo(a.h)anthracene	0.054 [2]	0.001	0.034
Metals	Zn	83.1 [1]	19.62	0.23
	Fe	-	0.37	-
	Al	-	0.76	-
	Cu	65 [1,3]	0.42	0.006
	Cr	21.1 [1]	0.06	0.003
	Ni	29.9 [1]	0.06	0.002
	Pb	212 [1]	0.06	0.0003
	Hg	0.022 [1]	0.0003	0.018
	Cd	0.9 [1]	0.001	0.002

[1] ECHA website; [2] EU website; [3] ref. [105]; [4] From this review.

The PNEC values related to the compounds have been reported in the literature (ECHA website; EU website, ref. [105]) and the PNEC for the TRWPs was determined starting from the most conservative data found in the literature (10,000 mg/kg on growth in *F. candida*) [62] and applying an assessment factor (AF) of 1000, as reported by the ECHA guidelines. It was not possible to determine the PNEC of some co-formulas, due to the paucity of data in the literature on the toxic effects of these substances in soil.

Finally, the RQs related to the TRWPs and co-formulates were determined by Equation (1).

7. Discussion

The results showed an unacceptably high risk for TRWPs (RQ = 2.16) and BTs (RQ = 3.58). A low risk emerges for all the priority PAHs, from which it is also possible to identify whether the soil is heavily or slightly contaminated by summing the estimated environmental concentrations of them [109]; according to the results obtained in this review, $\sum PEC_{PAHs}$ is equivalent to 0.115 mg/kg, which would correspond to Forlanini Park's soil not being considered as contaminated by PAHs coming from the TRWPs of cars. Regarding HMs, a high risk did not emerge, but it should be remembered that these results can be influenced and modified by important chemical parameters, such as pH; it was not possible to define an RQ for important HMs such as Fe and Al due to the absence of soil PNEC data, which will require further investigation in the future. Despite these results, on the one hand, the tire particles seem to induce a risk related to the particles and the effects of the mixture, whereas on the other hand, they could reduce the bioavailability of some co-formulates [98]. Further investigations will help us to understand these aspects and fill some gaps in the literature.

It should be noted that the RQs evaluated for Forlanini Park, as well as any value reported in this review, are modeling results and do not take into account the real environmental contamination in that park by these TRWPs. The entire vehicle fleet of the city of Milan was also considered, as there are no data relating to the actual vehicular circulation in Forlanini Avenue, and, consequently, the value of TRWPs emitted could be overestimated; furthermore, only tire particles emitted by cars were evaluated in this review, thus underestimating the real PEC of TRWPs in Forlanini Park. In addition, the EFs and mileage depend on local factors, such as the climate conditions, type of road, driving speed, the type of vehicle, and the weight of them [110]; regarding the last one, it has been demonstrated that heavy cars increase the friction with the asphalt and, as a result, purportedly greener and heavy electric and hybrid cars release TRWPs in the same way as other petrol-powered heavy cars. This aspect should not be underestimated for functional ecological transition, and a thorough investigation is suggested for the future.

However, this review could offer an initial and potential corpus of data for a risk assessment of TRWPs in urban soils. Actually, there is not a proper risk characterization for TRWPs contamination in soils yet. On the one hand, this lack depends on historical factors, because the attention paid to tire particle contamination is relatively recent and has mainly affected aquatic environments rather than the soil; on the other hand, this lack depends on technical and replicable factors, such as the sampling areas or type of toxicity tests, which have shown fluctuating results depending on exposure times, particle size, or model organisms.

Moreover, the data in the literature may underestimate the true contamination of TRWPs depending on the type of environmental marker used; in fact, although products of thermal degradation of SBR appear to be the most specific markers, most of the semi-quantitative information in the literature on the accumulation in roadside soils is derived from Zn content measurements [35], which is a generic marker and could return underestimated vertical transport values [16,34,66,84]. The choice of environmental markers is of great importance, as it could affect the actual distribution of TRWPs in the environment, and, consequently, future studies of these estimates should be investigated using more reliable markers.

8. Sustainability and Risk Mitigation Measures

Risk mitigation measures (RMMs) can be proposed to reduce the impact of TRWPs and associated chemicals. As suggested by Kumar et al. [111], potential risk mitigation measures (RMMs) to reduce TRWP pollution could be contamination source reduction, environmental fate capture, and end-of-sewage treatment; most of these aspects, however, have so far been dealt with only for aquatic environments, both sewage and freshwater [35,112,113]. Hence, an accurate future study is necessary for characterization of the risk to the soil ecosystem.

An initial intervention could be to act at the source level, promoting technologies to reduce and minimize the friction between tires and roads or promoting less use of heavy vehicles, which would help to increase the contact between the tread and the asphalt and, consequently, the release of TRWPs. Further attention could be placed on the distance of highways or roads from the soil ecosystem, increasing the width of sidewalks or, in general, reducing the contact between the soil and the road as much as possible; it is also possible to think of fairly high structures capable of blocking and retaining these rubber particles, preventing them from dispersing into the surrounding soil. However, such research has not been taken into consideration yet.

One RMM could also be formulating new sustainable tires; in fact, tires alone contribute to 20–30% of pollution from the automotive industry [114] and many of the petrochemical components, such as synthetic rubber polymers, carbon black, and adjuvants, are not renewable [115]. Several alternative solutions regarding tire compounds have already been proposed, such as latex from guayule (*Parthenium argentatum*), as alternative and sustainable sources of polyisoprene [116] and recycled plastic bottles, as sources of reinforcing fabrics such as polyethylene terephthalate (PET) [117]. Fillers such as carbon black and silica can be obtained more sustainably through the recycling of used tires in the first case or from rice husk silica in the second case [118]. Vegetable oils, such as soybean, flax, and guayule, could replace adjuvant petrogenic oils, such as naphthenic and paraffinic oils, giving tires greater performance and making them more sustainable, with them being biodegradable and safer oils [115,119].

9. Conclusions

This review has analyzed different aspects of TRWPs, focusing on their chemical composition, their environmental fate, their detection techniques, their toxicity, and the associated risk, taking as an example the case study of Forlanini Park. It was found that TRWPs and BTs could constitute a high risk, which will require further confirmation through field studies. These data reinforce those reported in the literature about the potential hazard of TRWPs and associated chemicals, placing more attention on soil ecosystems and particle contamination, as well as leachate.

In conclusion, we can define these points as future goals of the research and understanding of TRWPs contamination in soil:

(a) Greater and deeper attention to soil contamination by TRWPs;
(b) Standardization in the detection of these contaminants, with greater awareness of the choice of environmental markers;
(c) Standardization in toxicity tests, using efficient model organisms sensitive to TRWPs;
(d) Analyze the effects determined not only by the leachate but also by the particles to understand the environmental toxicity of TRWPs;
(e) Carry out interventions at the urban level to reduce both the contact between the tire tread and the asphalt and between the road and the surrounding soil;
(f) Promoting new sustainable tires as an efficient strategy to reduce TRWPs.

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References

1. Karagulian, F.; Belis, C.A.; Dora, C.F.C.; Prüss-Ustün, A.M.; Bonjour, S.; Adair-Rohani, H.; Amann, M. Contributions to Cities' Ambient Particulate Matter (PM): A Systematic Review of Local Source Contributions at Global Level. *Atmos. Environ.* **2015**, *120*, 475–483. [CrossRef]
2. Samek, L.; Stegowski, Z.; Styszko, K.; Furman, L.; Zimnoch, M.; Skiba, A.; Kistler, M.; Kasper-Giebl, A.; Rozanski, K.; Konduracka, E. Seasonal Variations of Chemical Composition of PM2.5 Fraction in the Urban Area of Krakow, Poland: PMF Source Attribution. *Air Qual. Atmos. Health* **2020**, *13*, 89–96. [CrossRef]
3. ISO International Organization for Standardization. Plastics—Vocabulary (ISO 472:2013). 2013. Available online: https://www.iso.org/obp/ui/#iso:std:iso:472:ed-4:v1:en (accessed on 28 January 2023).
4. Hartmann, N.B.; Huffer, T.; Thompson, R.C.; Hassellov, M.; Verschoor, A.; Daugaard, S.; Rist, T.; Karlsson, N.; Brennholt, M.; Cole, M.P.; et al. Are we speaking the same language? Recommendations for a definition and categorization framework for plastic debris Environ. *Sci. Technol.* **2019**, *53*, 1039–1047.
5. Arias, A.H.; Alfonso, M.B.; Girones, L.; Piccolo, M.C.; Marcovecchio, J.E. Synthetic Microfibers and Tyre Wear Particles Pollution in Aquatic Systems: Relevance and Mitigation Strategies. *Environ. Pollut.* **2021**, *295*, 118607. [PubMed]
6. Sundt, P.; Schulze, P.-E.; Syversen, F. Sources of Microplastics-Pollution to the Marine Environment. *Mepex Nor. Environ. Agency* **2014**, *86*, 20.
7. Lassen, C.; Foss Hansen, S.; Magnusson, K.; Norén, F.; Bloch Hartmann, N.I.; Rehne Jensen, P.; Nielsen, T.G.; Brinch, A. *Microplastics—Occurrence, Effects and Sources of Releases to the Environment in Denmark Danish*; Environmental Protection Agency: Copenhagen, Denmark, 2015; Volume 29, p. 1401.
8. Magnusson, K.; Eliasson, K.; Fråne, A.; Haikonen, K.; Hultén, J.; Olshammar, M.; Stadmark, J.; Voisin, A. *Swedish Sources and Pathways for Microplastics to the Marine Environment. A Review of Existing Data*; Report number C 183; Swedish Environmental Protection Agency: Stockholm, Sweden, 2016.
9. Sherrington, C.; Darrah, C.; Hann, S.; Cole, G.; Corbin, M. *Study to Support the Development of Measures to Combat a Range of Marine Litter Sources*; Report for European Commission DG Environment; The Danish Environmental Protection Agency: Copenhagen, Denmark, 2016.
10. Andersson-Sköld, Y.; Johannesson, M.; Gustafsson, M.; Järlskog, I.; Lithner, D.; Polukarova, M.; Strömvall, A.-M. *Microplastics from Tyre and Road Wear*; Digitala Vetenskapliga Arkivet: Stockholm, Sweden, 2020.
11. Zhou, Y.; Liu, X.; Wang, J. Characterization of Microplastics and the Association of Heavy Metals with Microplastics in Suburban Soil of Central China. *Sci. Total Environ.* **2019**, *694*, 3798. [CrossRef]
12. Boucher, J.; Friot, D. *Primary Microplastics in the Oceans: A Global Evaluation of Sources, Primary Microplastics in the Oceans: A Global Evaluation of Sources*; IUCN International Union for Conservation of Nature and Natural Resources (IUCN): Gland, Switzerland, 2017; p. 43.
13. Kole, J.P.; Löhr, A.J.; van Belleghem, F.G.A.J.; Ragas, A.M.J. Wear and tear of tyres: A stealthy source of microplastics in the environment. *Int. J. Environ. Res. Public Health* **2017**, *14*, 1265.
14. Wagner, S.; Hüffer, T.; Klöckner, P.; Wehrhahn, M.; Hofmann, T.; Reemtsma, T. Tire wear particles in the aquatic environment—A review on generation, analysis, occurrence, fate and effects. *Water Res.* **2018**, *139*, 83–100. [CrossRef]
15. Horton, A.A.; Walton, A.; Spurgeon, D.J.; Lahive, E.; Svendsen, C. Microplastics in freshwater and terrestrial environments: Evaluating the current understanding to identify the knowledge gaps and future research priorities. *Sci. Total Environ.* **2017**, *586*, 127–141. [CrossRef]
16. Wik, A.; Lycken, J.; Dave, G. Sediment quality assessment of road runoff detention systems in Sweden and the potential contribution of tire wear. *Water Air Soil Pollut.* **2008**, *194*, 301–314. [CrossRef]
17. Wik, A. Toxic components leaching from tire rubber. *Bull. Environ. Contam. Toxicol.* **2007**, *79*, 114–119. [CrossRef]
18. Wik, A.; Dave, G. Acute toxicity of tire rubber leachates to Daphnia magna—Variability and toxic components. *Chemosphere* **2006**, *64*, 1777–1784. [CrossRef] [PubMed]
19. Benevento, S.; Draper, A. Analysis of tire rubber leachate with a bacterial mutagenesis assay. In Proceedings of the SETAC North America 26th Annual Meeting, Baltimore, MD, USA, 13–17 November 2005; pp. 13–17.
20. Gualtieri, M.; Andrioletti, M.; Vismara, C.; Milani, M.; Camatini, M. Toxicity of tire debris leachates. *Environ. Int.* **2005**, *31*, 723–730. [CrossRef]
21. Humphrey, D.N.; Katz, L.E. Water-quality effects of tire shreds placed above the water table—Five-year field study. *Transp. Res. Rec.* **2000**, *1714*, 18–24. [CrossRef]
22. Sheehan, P.J.; Warmerdam, J.M.; Ogle, S.; Humphrey, D.N.; Patenaude, S.M. Evaluating the risk to aquatic ecosystems posed by leachate from tire shred fill in roads using toxicity tests, toxicity identification evaluations, and groundwater modeling. *Environ. Toxicol. Chem.* **2006**, *25*, 400–411. [CrossRef] [PubMed]

23. Kreider, M.L.; Panko, J.M.; McAtee, B.L.; Sweet, L.I.; Finley, B.L. Physical and chemical characterization of tire-related particles: Comparison of particles generated using different methodologies. *Sci. Total Environ.* **2010**, *408*, 652–659. [CrossRef]
24. Sommer, F.; Dietze, V.; Baum, A.; Sauer, J.; Gilge, S.; Maschowski, C.; Giere, R. Tire abrasion as a major source of microplastics in the environment. *Aerosol Air Qual. Res.* **2018**, *18*, 2014–2028. [CrossRef]
25. Workek, J.; Badura, X.; Białas, A.; Chwiej, J.; Kawoń, K.; Styszko, K. Pollution from Transport: Detection of Tyre Particles in Environmental Samples. *Energies* **2022**, *15*, 2816. [CrossRef]
26. Alexandrova, O.; Kaloush, K.E.; Allen, J.O. Impact of Asphalt Rubber Friction Course Overlays on Tire Wear Emissions and Air Quality Models for Phoenix, Arizona, Airshed. *Transp. Res. Rec.* **2007**, *2011*, 98–106. [CrossRef]
27. Grigoratos, T.; Martini, G. Brake Wear Particle Emissions: A Review. *Environ. Sci. Pollut. Res.* **2015**, *22*, 2491–2504. [CrossRef]
28. Panko, J.M.; Chu, J.; Kreider, M.L.; Unice, K.M. Measurement of airborne concentrations of tire and road wear particles in urban and rural areas of France, Japan, and the United States. *Atmos. Environ.* **2013**, *72*, 192–199. [CrossRef]
29. Adachi, K.; Tainosho, Y. Characterization of heavy metal particles embedded in tire dust. *Environ. Int.* **2004**, *30*, 1009–1017. [CrossRef] [PubMed]
30. Kayhanian, M.; McKenzie, E.R.; Leatherbarrow, J.E.; Young, T.M. Characteristics of road sediment fractionated particles captured from paved surfaces, surface run-off and detention basins. *Sci. Total Environ.* **2012**, *439*, 172–186.
31. Rhodes, E.P.; Ren, Z.Y.; Mays, D.C. Zinc leaching from tire crumb rubber. *Environ. Sci. Technol.* **2012**, *46*, 12856–12863. [CrossRef] [PubMed]
32. Baensch-Baltruschat, B.; Kocher, B.; Stock, F.; Reifferscheid, G. Tyre and road wear particles (TRWP)—A review of generation, properties, emissions, human health risk, ecotoxicity, and fate in the environment. *Sci. Total Environ.* **2020**, *733*, 137823. [CrossRef]
33. Park, I.; Kim, H.; Lee, S. Characteristics of tire wear particles generated in a laboratory simulation of tire/road contact conditions. *J. Aerosol Sci.* **2018**, *124*, 30–40. [CrossRef]
34. Wik, A.; Dave, G. Occurrence and effects of tire wear particles in the environment—A critical review and an initial risk assessment. *Environ. Pollut.* **2009**, *157*, 1–11. [CrossRef]
35. Baensch-Baltruschat, B.; Kocher, B.; Kochleus, C.; Stock, F.; Reifferscheid, G. Tyre and road wear particles—A calculation of generation, transport and release to water and soil with special regard to German roads. *Sci. Total Environ.* **2021**, *752*, 141939. [CrossRef]
36. Bertling, J.; Bertling, R.; Hamann, L. Kunststoffe in der Umwelt: Mikro-und Makroplastik. In *Ursachen, Mengen, Umweltschicksale, Wirkungen, Lösungsansätze, Empfehlungen. Kurzfassung der Konsortialstudie, Fraunhofer UMSICHT*; Fraunhofer-Institut für Umwelt-, Sicherheits- und Energietechnik UMSICHT: Oberhausen, Germany, 2018.
37. United States Tires Manufactures Association—USTMA 2017. Available online: https://www.ustires.org/whats-tire-0 (accessed on 28 January 2023).
38. European Chemical Agency—ECHA, Annex XV Report—Rubber Granules Evaluation—Ver 1.01. 2017. Available online: https://echa.europa.eu/documents/10162/17220/annexes_to_axv_report_rubber+granules_en.pdf/f3cc9f58-8ab3-8e4a-0258-51466817f0fd?t=1488792289876 (accessed on 28 January 2023).
39. Onokpise, O.U. Natural rubber, Hevea brasiliensis (Willd. ex A. Juss.) Müll. Arg, germplasm collection in the Amazon Basin, Brazil: A retrospective. *Econ. Bot.* **2004**, *58*, 544–555. [CrossRef]
40. Obrecht, W.; Lambert, J.P.; Happ, M.; Oppenheimer-Stix, C.; Dunn, J.; Krüger, R. Rubber, 4. Emulsion Rubbers. In *Ullmann's Encyclopedia of Industrial Chemistry*; Verlag Chemie: Hoboken, NJ, USA, 2012.
41. Ahlbom, J.; Duus, U. *Nya Hjulspår-En Produktstudie av Gummidäck*; Report 6/94; Swedish Chemicals Agency: Solna, Sweden, 1994; p. 77.
42. Barbin, W.W.; Rodgers, M.B. The science of rubber compounding. In *Science and Technology of Rubber*, 2nd ed.; Mark, J.E., Erman, B., Eirich, F.R., Eds.; Academic Press: San Diego, CA, USA, 1994; pp. 419–469.
43. Halle, L.L.; Palmqvist, A.; Kampmann, K.; Jensen, A.; Hansen, T.; Khan, F.R. Tire wear particle and leachate exposures from a pristine and road-worn tire to Hyalella azteca: Comparison of chemical content and biological effects. *Aquat. Toxicol.* **2021**, *232*, 105769.
44. Lamprea, K.; Bressy, A.; Mirande-Bret, C.; Caupos, E.; Gromaire, M.C. Alkylphenol and bisphenol A contamination of urban runoff: An evaluation of the emission potentials of various construction materials and automotive supplies. *Environ. Sci. Poll. Res.* **2018**, *25*, 21887–21900. [CrossRef]
45. Celeiro, M.; Dagnac, T.; Llompart, M. Determination of priority and other hazardous substances in football fields of synthetic turf by gas chromatography-mass spectrometry: A health and environmental concern. *Chemosphere* **2018**, *195*, 201–211. [CrossRef] [PubMed]
46. Celeiro, M.; Lamas, J.P.; Garcia-Jares, C.; Dagnac, T.; Ramos, L.; Llompart, M. Investigation of PAH and other hazardous contaminant occurrence in recycled tyre rubber surfaces. case study: Restaurant playground in an indoor shopping center. *Int. J. Environ. Anal. Chem.* **2014**, *94*, 1264–1271. [CrossRef]
47. Krüger, O.; Kalbe, U.; Berger, W.; Nordhau, K.; Christoph, G.; Walzel, H.P. Comparison of batch and column tests for the elution of artificial turf system components. *Environ. Sci. Technol.* **2012**, *46*, 13085–13092. [CrossRef] [PubMed]
48. Tian, Z.; Zhao, H.; Peter, K.T.; Gonzalez, M.; Weel, J.; Wu, C.; Hu, X.; Prat, J.; Mudrock, E.; Heinger, R.; et al. A ubiquitous tire rubber–derived chemical induces acute mortality in coho salmon. *Science* **2021**, *371*, 185–189. [CrossRef]

49. Alhelou, R.; Seiwert, B.; Reemtsma, T. Hexamethoxymethylmelamine—A precursor of persistent and mobile contaminants in municipal wastewater and the water cycle. *Water Res.* **2019**, *165*, 114973. [CrossRef]
50. Johannessen, C.; Helm, P.; Metcalfe, C.D. Detection of selected tire wear compounds in urban receiving waters. *Environ. Pollut.* **2021**, *287*, 117659. [CrossRef]
51. Peter, K.T.; Tian, Z.; Wu, C.; Lin, P.; White, S.; Du, B.; McIntyre, J.K.; Scholz, N.L.; Kolodziej, E.P. Using high-resolution mass spectrometry to identify organic contaminants linked to urban stormwater mortality syndrome in coho Salmon. *Environ. Sci. Technol.* **2018**, *52*, 10317–10327. [CrossRef]
52. Seiwert, B.; Klöckner, P.; Wagner, S.; Reemtsma, T. Source-related smart suspect screening in the aqueous environment: Search for tire-derived persistent and mobile trace organic contaminants in surface waters. *Anal. Bioanal. Chem.* **2020**, *412*, 4909–4919. [CrossRef]
53. Councell, T.B.; Duckenfield, K.U.; Landa, E.R.; Callender, E. Tire-Wear Particles as a Source of Zinc to the Environment. *Environ. Sci. Technol.* **2004**, *38*, 4206–4214. [CrossRef]
54. Hillenbrand, T.; Toussaint, D.; Böhm, E.; Fuchs, S.; Scherer, U.; Kreissig, J.; Kotz, C. Einträge von Kupfer, Zink und Blei in Gewässer und Böden—Analyse der Emissionspfade und möglicher. In *Emissionsminderungsmaßnahmen*; Umweltbundesamt: Dessau, Germany, 2005.
55. Knight, L.J.; Parker-Jurd, F.N.F.; Al-SidCheikh, M.; Thompson, R.C. Tyre wear particles: An abundant yet widely unreported microplastic? *Environ. Sci. Pollut. Res. Int.* **2020**, *27*, 18345–18354. [CrossRef] [PubMed]
56. Wang, X.; Bolan, N.; Tsang, D.C.W.; Sarkar, B.; Bradney, L.; Li, Y. A review of microplastics aggregation in aquatic environments: Influence factors, analytical methods, and environmental implications. *J. Hazard. Mater.* **2021**, *402*, 123496. [CrossRef] [PubMed]
57. Cai, L.; He, L.; Peng, S.; Li, M.; Tong, M. Influence of titanium dioxide nanoparticles on the transport and deposition of microplastics in quartz sand. *Environ. Pollut.* **2019**, *253*, 351–357. [CrossRef] [PubMed]
58. Long, M.; Paul-Pont, I.; Hegaret, H.; Moriceau, B.; Lambert, C.; Huvet, A.; Soudant, P. Interactions between polystyrene microplastics and marine phytoplankton lead to species-specific hetero-aggregation. *Environ. Pollut.* **2017**, *228*, 454–463. [CrossRef] [PubMed]
59. Zhao, J.; Liu, F.F.; Wang, Z.Y.; Cao, X.S.; Xing, B.S. Heteroaggregation of graphene oxide with minerals in aqueous phase. *Environ. Sci. Technol.* **2015**, *49*, 2849–2857. [CrossRef]
60. Sieber, R.; Kawecki, D.; Nowack, B. Dynamic probabilistic material flow analysis of rubber release from tires into the environment. *Environ. Pollut.* **2020**, *258*, 113573.
61. Unice, K.M.; Kreider, M.L.; Panko, J.M. Comparison of tire and road wear particle concentrations in sediment for watersheds in France, Japan, and the United States by quantitative pyrolysis GC/MS analysis. *Environ. Sci. Technol.* **2013**, *47*, 8138–8147.
62. Kim, L.; Lee, T.Y.; Kim, H.; An, Y.J. Toxicity assessment of tire particles released from personal mobilities (bicycles, cars, and electric scooters) on soil organisms. *J. Hazard. Mater.* **2022**, *437*, 129362.
63. Sheng, Y.; Liu, Y.; Wang, K.; Cizdziel, J.C.; Wu, Y.; Zhou, Y. Ecotoxicological effects of micronized car tire wear particles and their heavy metals on the earthworm (*Eisenia fetida*) in soil. *Sci. Total Environ.* **2021**, *793*, 148613.
64. Kumata, H.; Yamada, J.; Masuda, K.; Takada, H.; Sato, Y.; Sakurai, T.; Fujiwara, K. Benzothiazolamines as tire-derived molecular markers: Sorptive behavior in street runoff and application to source apportioning. *Environ. Sci. Technol.* **2002**, *36*, 702–708.
65. Kumata, H.; Sanada, Y.; Takada, H.; Ueno, T. Historical trends of n-cyclohexyl-2-benzothiazoleamine, 2-(4 morpholinyl)benzothiazole, and other anthropogenic contaminants in the urban reservoir sediment core. *Environ. Sci. Technol.* **2000**, *34*, 246–253. [CrossRef]
66. Fauser, P.; Tjell, J.C.; Mosbaek, H.; Pilegaard, K. Quantification of tire-tread particles using extractable organic zinc as tracer. *Rubber Chem. Technol.* **1999**, *72*, 969–977. [CrossRef]
67. Reddy, C.M.; Quinn, J.G. Environmental chemistry of benzothiazoles derived from rubber. *Environ. Sci. Technol.* **1997**, *31*, 2847–2853. [CrossRef]
68. Selonen, S.; Dolar, A.; Kokalj, A.J.; Sackey, L.N.; Skalar, T.; Fernandes, V.C.; Rede, D.; Delerue-Matos, C.; Hurley, R.; Nizzetto, L.; et al. Exploring the impacts of microplastics and associated chemicals in the terrestrial environment—Exposure of soil invertebrates to tire particles. *Environ. Res.* **2021**, *201*, 111495. [CrossRef]
69. Cadle, S.H.; Williams, R.L. Gas and particle emissions from automobile tires in laboratory and field studies. *Rubber Chem. Technol.* **1978**, *52*, 146–158. [CrossRef]
70. Pierson, W.R.; Brachaczek, W.W. Airborne particulate debris from rubber tires. *Rubber Chem. Technol.* **1974**, *47*, 1275–1299. [CrossRef]
71. Boulter, P. *A Review of Emission Factors and Models for Road Vehicle Non-Exhaust Particulate Matter*; TRL Limited: Bracknell, UK, 2005.
72. Cardina, J.A. Particle size determination of tire-tread rubber in atmospheric dust. *Rubber Chem. Technol.* **1974**, *47*, 1005–1010. [CrossRef]
73. Kim, M.G.; Yagawa, K.; Inoue, H.; Lee, Y.K.; Shirai, T. Measurement of tire tread in urban air by pyrolysis—Gas chromatography with flame photometric detection. *Atmos. Environ. Part A—Gen. Top.* **1990**, *24*, 1417–1422. [CrossRef]
74. Panko, J.M.; Hitchcock, K.M.; Fuller, G.W.; Green, D. Evaluation of tire wear contribution to PM2.5 in urban environments. *Atmosphere* **2019**, *10*, 99. [CrossRef]
75. Zeng, E.Y.; Tran, K.; Young, D. Evaluation of potential molecular markers for urban stormwater runoff. *Environ. Monit. Assess.* **2004**, *90*, 23–43. [CrossRef]

76. Ni, H.-G.; Lu, F.-H.; Luo, X.-L.; Tian, H.-Y.; Zeng, E.Y. Occurrence, phase distribution, and mass loadings of benzothiazoles in riverine runoff of the Pearl River Delta, China. *Environ. Sci. Technol.* **2008**, *42*, 1892–1897. [CrossRef]
77. Brownlee, B.G.; Carey, J.H.; MacInnis, G.A.; Pellizzari, I.T. Aquatic environmental chemistry of 2-(thiocyanomethylthio)benzothiazole and related benzothiazoles. *Environ. Toxicol. Chem.* **1992**, *11*, 1153–1168. [CrossRef]
78. Wypych, G. *Handbook of UV Degradation and Stabilization*, 3rd ed.; Wypych, G., Ed.; Elsevier: Toronto, ON, Canada, 2020; Volume 7, p. 321.
79. Wagner, S.; Klöckner, P.; Reemtsma, T. Aging of tire and road wear particles in terrestrial and freshwater environments—A review on processes, testing, analysis and impact. *Chemosphere* **2022**, *288*, 132467. [CrossRef]
80. Rose, K.; Steinbüchel, A. Biodegradation of Natural Rubber and Related Compounds: Recent Insights into a Hardly Understood Catabolic Capability of Microorganisms. *Appl. Environ. Microbiol.* **2005**, *71*, 2803–2812. [CrossRef]
81. Tsuchii, A.; Tokiwa, Y. Microbial degradation of tyre rubber particles. *Biotechnol. Lett.* **2001**, *23*, 963–969. [CrossRef]
82. Stevenson, K.; Stallwood, B.; Hart, A.G. Tire rubber recycling and bioremediation: A review. *Bioremediation J.* **2008**, *12*, 1–11. [CrossRef]
83. Spies, R.B.; Andresen, B.D.; Rice, D.W. Benzothiazoles in estuarine sediments as indicators of street runoff. *Nature* **1987**, *327*, 697–699. [CrossRef]
84. Müller, A.; Kocher, B.; Altmann, K.; Braun, U. Determination of tire wear markers in soil samples and their distribution in a roadside soil. *Chemosphere* **2022**, *294*, 133653. [CrossRef]
85. Hjortenkrans, D.S.T.; Bergback, B.G.; Haggerud, A.V. Metal emissions from brake linings and tires: Case studies of Stockholm, Sweden 1995/1998 and 2005. *Environ. Sci. Technol.* **2007**, *41*, 5224–5230. [CrossRef]
86. Davis, A.P.; Shokouhian, M.; Ni, S. Loading estimates of lead, copper, cadmium, and zinc in urban runoff from specific sources. *Chemosphere* **2001**, *44*, 997–1009. [CrossRef]
87. Dolar, A.; Selonen, S.; Van Gestel, C.A.; Perc, V.; Drobne, D.; Kokalj, A.J. Microplastics, chlorpyrifos and their mixtures modulate immune processes in the terrestrial crustacean Porcellio scaber. *Sci. Total Environ.* **2021**, *772*, 144900. [CrossRef] [PubMed]
88. Selonen, S.; Dolar, A.; Kokalj, A.J.; Skalar, T.; Dolcet, L.P.; Hurley, R.; van Gestel, C.A.M. Exploring the impacts of plastics in soil–the effects of polyester textile fibers on soil invertebrates. *Sci. Total Environ.* **2020**, *700*, 134451. [CrossRef] [PubMed]
89. Halle, L.L.; Palmqvist, A.; Kampmann, K.; Khan, F.R. Ecotoxicology of micronized tire rubber: Past, present and future considerations. *Sci. Total Environ.* **2020**, *706*, 135694. [CrossRef] [PubMed]
90. Marwood, C.; McAtee, B.; Kreider, M.; Ogle, R.S.; Finley, B.; Sweet, L.; Panko, J. Acute aquatic toxicity of tire and road wear particles to alga, daphnid, and fish. *Ecotoxicology* **2011**, *20*, 2079–2089. [CrossRef]
91. Turner, A.; Rice, L. Toxicity of tire wear particle leachate to the marine macroalga, Ulva lactuca. *Environ. Pollut.* **2010**, *158*, 3650–3654. [CrossRef]
92. Cunningham, B.; Harper, B.; Brander, S.; Harper, S. Toxicity of micro and nano tire particles and leachate for model freshwater organisms. *J. Hazard. Mater.* **2022**, *429*, 128319. [CrossRef]
93. Carrasco-Navarro, V.; Muñiz-González, A.B.; Sorvari, J.; Martínez-Guitarte, J.L. Altered gene expression in Chironomus riparius (insecta) in response to tire rubber and polystyrene microplastics. *Environ. Pollut.* **2021**, *285*, 117462. [CrossRef]
94. Khan, F.R.; Louise Lynn Halle, L.L.; Palmqvist, A. Acute and long-term toxicity of micronized car tire wear particles to Hyalella azteca. *Aquat. Toxicol.* **2019**, *213*, 105216. [CrossRef]
95. Kim, S.W.; Leifheit, E.F.; Maaß, S.; Rillig, M.C. Time-Dependent Toxicity of Tire Particles on Soil Nematodes. *Front. Environ. Sci.* **2021**, *9*, 744668. [CrossRef]
96. Lackmann, C.; Velki, M.; Šimić, A.; Müller, A.; Braun, U.; Ečimović, S.; Hollert, H. Two types of microplastics (polystyrene-HBCD and car tire abrasion) affect oxidative stress-related biomarkers in earthworm Eisenia andrei in a time-dependent manner. *Environ. Int.* **2022**, *163*, 107190. [CrossRef]
97. Ding, J.; Zhu, D.; Wang, H.T.; Lassen, S.B.; Chen, Q.L.; Li, G.; Lv, M.; Zhu, Y.G. Dysbiosis in the gut microbiota of soil fauna explains the toxicity of tire tread particles. *Environ. Sci. Technol.* **2020**, *54*, 7450–7460. [CrossRef] [PubMed]
98. Selonen, S.; Kokalj, A.J.; Benguedouara, H.; Petroodya, S.S.A.; Dolar, A.; Drobnec, D.; van Gestel, C.A.M. Modulation of chlorpyrifos toxicity to soil arthropods by simultaneous exposure to polyester microfibers or tire particle microplastics. *Appl. Soil Ecol.* **2023**, *181*, 104657. [CrossRef]
99. Dolar, A.; Drobne, D.; Dolenec, M.; Marinšek, M.; Kokalj, A.J. Time-dependent immune response in Porcellio scaber following exposure to microplastics and natural particles. *Sci. Total Environ.* **2022**, *818*, 151816. [CrossRef] [PubMed]
100. Leifheit, E.F.; Kissenerl, H.L.; Faltin, E.; Ryo, M.; Rillig, M.C. Tire abrasion particles negatively affect plant growth even at low concentrations and alter soil biogeochemical cycling. *Soil Ecol. Lett.* **2022**, *4*, 409–415. [CrossRef]
101. Jager, T.; Fleuren, R.H.L.J.; Hogendoorn, E.A.; de Korte, G. Elucidating the Routes of Exposure for Organic Chemicals in the Earthworm, Eisenia andrei (Oligochaeta). *Environ. Sci. Technol.* **2003**, *37*, 3399–3404. [CrossRef]
102. Van Gestel, C.A.M.; Loureiro, S.; Zidar, P. Terrestrial isopods as model organisms in soil ecotoxicology: A review. *ZooKeys* **2018**, *801*, 127–162. [CrossRef]
103. Loureiro, S.; Sampaio, A.; Brandão, A.; Nogueira, A.J.A.; Soares, A.M.V.M. Feeding behavior of the terrestrial isopod Porcellionides pruinosus Brandt, 1833 (Crustacea, Isopoda) in response to changes in food quality and contamination. *Sci. Total Environ.* **2006**, *369*, 119–128. [CrossRef]

104. Unice, K.M.; Weeber, M.P.; Abramson, M.M.; Reid, R.C.D.; van Gils, J.A.G.; Markus, A.A.; Vethaak, A.D.; Panko, J.M. Characterizing export of land-based microplastics to the estuary—Part II: Sensitivity analysis of an integrated geospatial microplastic transport modeling assessment of tire and road wear particles. *Sci. Total Environ.* **2019**, *646*, 1650–1659. [CrossRef]
105. Caetano, A.L.; Marques, C.R.; Gonçalves, F.; da Silva, E.F.; Pereira, R. Copper toxicity in a natural reference soil: Ecotoxicological data for the derivation of preliminary soil screening values. *Ecotoxicology* **2016**, *25*, 163–177. [CrossRef]
106. Klöckner, P.; Reemtsma, T.; Eisentraut, P.; Braun, U.; Ruhl, A.S.; Wagner, S. Tire and road wear particles in road environment—Quantification and assessment of particle dynamics by Zn determination after density separation. *Chemosphere* **2019**, *222*, 714–721. [CrossRef]
107. Redondo-Hasselerharm, P.E.; de Ruijter, V.N.; Mintenig, S.M.; Verschoor, A.; Koelmans, A.A. Ingestion and Chronic Effects of Car Tire Tread Particles on Freshwater Benthic Macroinvertebrates. *Environ. Sci. Technol.* **2018**, *52*, 13986–13994. [CrossRef] [PubMed]
108. Asheim, J.; Vike-Jonas, K.; Gonzalez, S.V.; Lierhagen, S.; Venkatraman, V.; Veivåg, I.L.S.; Snilsberg, B.; Flaten, T.P.; Asimakopoulos, A.G. Benzotriazoles, benzothiazoles and trace elements in an urban road setting in Trondheim, Norway: Re-visiting the chemical markers of traffic pollution. *Sci. Total Environ.* **2019**, *649*, 703–711. [CrossRef] [PubMed]
109. Maliszewska-Kordybach, B. Polycyclic aromatic hydrocarbons in agricultural soils in Poland: Preliminary proposals for criteria to evaluate the level of soil contamination. *Appl. Geochem.* **1996**, *11*, 121–127. [CrossRef]
110. Jekel, M. *Scientific Report on Tyre and Road Wear Particles, TRWP, in the Aquatic Environment*; European Tyre Rubber Manufactures Association (ETRMA): Brussels, Belgium, 2019; pp. 1–35.
111. Luo, Z.; Zhou, X.; Su, Y.; Wang, H.; Yu, R.; Zhou, S.; Xu, E.G.; Xing, B. Environmental occurrence, fate, impact, and potential solution of tire microplastics: Similarities and differences with tire wear particles. *Sci. Total Environ.* **2021**, *795*, 148902. [CrossRef] [PubMed]
112. Kumar, M.; Xiong, X.; He, M.; Tsang, D.C.; Gupta, J.; Khan, E.; Bolan, N.S. Microplastics as pollutants in agricultural soils. *Environ. Pollut.* **2020**, *265*, 114980. [CrossRef] [PubMed]
113. Verschoor, A.J.; Van Gelderen, A.; Hofstra, U. Fate of recycled tyre granulate used on artificial turf. *Environ. Sci. Eur.* **2021**, *33*, 27. [CrossRef]
114. Araujo-Morera, J.; Lopez-Manchado, M.A.; Verdejo, R.; Hernandez, S.M. Sustainable mobility: The route of tires through the circular economy model. *Waste Manag.* **2021**, *126*, 309–322. [CrossRef]
115. Jomin, T.; Renuka, P. Road to Sustainable Tire Materials: Current State-of-the-Art and Future Prospectives. *Environ. Sci. Technol.* **2023**, *57*, 2209–2216.
116. Ren, X.; Barrera, C.S.; Tardiff, J.L.; Gil, A.; Cornish, K. Liquid Guayule Natural Rubber, a Renewable and Crosslinkable Processing Aid in Natural and Synthetic Rubber Compounds. *J. Clean. Prod.* **2020**, *276*, 122933. [CrossRef]
117. Rodgers, S.; Meng, F.; Poulston, S.; Conradie, A.; McKechnie, J. Renewable Butadiene: A Case for Hybrid Processing via Bio- and Chemo-Catalysis. *J. Clean. Prod.* **2022**, *364*, 132614. [CrossRef]
118. Bartoli, M.; Rosi, L.; Frediani, M.; Undri, A.; Frediani, P. Depolymerization of Polystyrene at Reduced Pressure through a Microwave Assisted Pyrolysis. *J. Anal. Appl. Pyrolysis* **2015**, *113*, 281–287. [CrossRef]
119. Abdulrahman, A.S.; Jabrail, F.H. Treatment of Scrap Tire for Rubber and Carbon Black Recovery. *Recycling* **2022**, *7*, 27. [CrossRef]

Chapter 3

Disaggregation behavior in the terrestrial isopod *Porcellionides pruinosus* as a new ecotoxicological endpoint for assessing infochemical disrupting activity

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Original Articles

Disaggregation behavior in the terrestrial isopod *Porcellionides pruinosus* as a new ecotoxicological endpoint for assessing infochemical disrupting activity

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ABSTRACT

Among rapid ecotoxicological bioassays for screening soil quality, avoidance behavior tests on gregarious edaphic species such as *Porcellionides pruinosus* are widely used. However, the effect of soil contamination on adaptive aggregation ability has not been investigated. The aim of this study was to develop a new ecotoxicological endpoint related to the disaggregation effect under infochemical disruption at the population level during an avoidance behavior test. This new endpoint was evaluated using tire particles (TPs) and benzothiazole (BT) as preliminary physical and chemical substances. The disaggregation index (DI) and disaggregation groups (DG) are presented as measures of fragmentation of the population to quantify the effect of contaminants on aggregation behavior. Aggregation disruption in a group of ten individuals was assessed alongside the sub-lethal avoidance test after a 48 h exposure. The degree of disaggregation is measured by the number of subgroups formed. The DI and DG indices range from 0 to 1, representing the highest degree of aggregation and disaggregation, respectively, achieved at the end of the test.

Our results show that all woodlice exposed to TPs $\geqslant$1,250 mg/kg d.w. and BT $\geqslant$500 mg/kg d.w. successfully avoided contaminated soil, but failed to show gregarious behavior, indicating fragmentation within the population, even if in uncontaminated soil. The disaggregation effects in woodlice occurred at higher concentrations (TPs $\geqslant$7,500 mg/kg d.w.; BT $=$ 1,000 mg/kg d.w.) than the avoidance ones, suggesting a possible effect on the adaptive capabilities of the population even if they move to the control soil. These results suggest a combination of avoidance behavior and disaggregation in individuals of *P. pruinosus*. Consideration of both aspects may provide more accurate and robust results for environmental risk assessment.

1. Introduction

In recent years, there has been a notable increase in the interest surrounding the investigation of the impact of contaminants and stressors on the dynamics of soil populations (van Straalen and van Gestel, 2008; Lima et al., 2015; Morgado et al., 2015; Ferreira et al., 2016; Bandeira et al., 2020; Sengupta et al., 2023), in line with the ecotoxicological paradigm that has been developed into stress ecology (van Straalen, 2003; van Gestel, 2012). Therefore, understanding the ethological traits associated with the migration or communication of edaphic populations and the effects of contaminants and multiple stressors on their ecological niche contributes to enhancing ecological realism in the ecotoxicological risk assessment framework.

Among the relevant, rapid, and cost-effective ecotoxicological bioassays, the avoidance behavior test is considered a sensitive screening tool with improved ecological realism for evaluating the ”limited habitat function” of the soil ecosystem (ISO, 2008), using soil organisms' ability to choose or avoid harmful substances within the soil (Loureiro et al., 2005; Gainer et al., 2022). This test provides both prognostic and diagnostic information, allowing to assess the suitability of chemicals and materials introduced into the environment, such as compost and biochar, and identify the effects of contaminants and environmental stressors on population distributions in anthropized soils. However, although easy to perform and highly versatile, the avoidance behavior of soil invertebrates varies between species and contaminants, with numerous non-avoidance episodes occurring for different ecological

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factors (Gainer et al., 2022). Nowadays, behavioral avoidance tests have been carried out using different soil organisms such as earthworms, potworms, springtails and woodlice, underlining the versatility of the tests (Loureiro et al., 2009; Santos et al., 2010; Lackmann et al., 2023; Renaud et al., 2022; Malheiro et al., 2023; Puddephatt et al., 2022).

Particularly, the terrestrial isopod *Porcellionides pruinosus* is considered a relevant model organism (Loureiro et al., 2005; van Gestel et al., 2018), due to the possibility of studying different exposure pathways (soil, interstitial water, air, and food) and determining different endpoints (Warburg, 1993). Being epiedaphic detritivores, they are also considered keystone species in maintaining soil ecosystem processes, such as biogeochemical cycles, and ecosystem services, such as regulating services (Mészárosné Póss et al., 2022). Notwithstanding, there is currently no established toxicity assessment protocol for this biological model, due to the longer and more complex life cycle than other edaphic organisms, such as springtails or earthworms, which makes complicated to obtain age-synchronized individuals, which would be useful to obtain more homogeneous results (van Gestel et al., 2018).

Likewise, terrestrial isopods such as *P. pruinosus* possess some unique and evident eco-ethological traits, the analyzes of which would allow this species to be considered as a potential model organism for assessing the impact of stressors on edaphic communities. Among these traits, the ability of these organisms to aggregate and increase the population density is considered an interesting trait of high ecological value (Takeda, 1980). Aggregation offers individuals a series of benefits, such as protection against predators, availability of food or accessibility to reproduction. Most aggregation behaviors are driven by pheromones (semiochemicals), which perform important functions in animal infochemical communication by inducing behavioral alterations between individuals of the same species and beyond, especially in cryptic ecosystems such as soil, where chemical signals are more useful than other senses (Lee and Frost, 2022; Cardé and Millar, 2009). From an evolutionary point of view, aggregation behavior is a strategy that woodlice have selected to adapt to terrestrial life and to reduce water loss under conditions of environmental stress, particularly during molting periods where an increase in hemolymphatic hydrostatic pressure is required (Allee, 1926; Elisabeth, 2011). The evolution of a biphasic ecdysis, as well as nocturnal adaptation, are other important physiological and behavioral characteristics evolved by woodlice to counteract dehydration (Devigne et al., 2011; Broly et al., 2016; Nako et al., 2018).

Although aggregation plays an important ecological role, the impact of soil contaminants on such behavior in woodlice and how such alteration impacts population dynamics is an unexplored area of research. In literature, there are some different considerations about the aggregation impact on avoidance assessment. Zidar and Fiser (2022) reported that the aggregation behavior could suppress the avoidance leading to an underestimation of soil contaminants, while Loureiro et al. (2005) have observed that the number of organisms does not produce differences in the avoidance responses. Within this framework, we support the usefulness of aggregation behavior as a tool for a more ecological soil stressor effect. The aim of this study is to propose the assessment of the disaggregation effect in individuals of *P. pruinosus* exposed to soil contaminants as a new ecotoxicological endpoint to be evaluated jointly with the avoidance behavior. We propose a methodology to evaluate the alteration of gregariousness, introducing two new disaggregation indexes, namely the disaggregation index (DI) and the disaggregation in groups index (DG). This protocol was therefore tested using two types of soil contaminants: tire particles (TPs) and benzothiazole (BT). Fragmentation of the population on individuals of *P. pruinosus* is then proposed as a new parameter at the population level for understanding the effects of hazardous physicals or chemicals on the infochemical activity of individuals.

2. Materials and Methods

2.1. Test organism and culture procedure

Organisms of the species *Porcellionides pruinosus* Brandt (1833) were kindly provided by the Laboratory of Applied Ecology and Ecotoxicology, CESAM, at the University of Aveiro (Portugal), and kept in the facility at the University of Milano-Bicocca, Milan (Italy). Isopod cultures were maintained in moist soil (water holding capacity - WHC 40 %), at 21 °C temperature, photoperiod of 16:8 h (light–dark) and were fed ad *libitum* with gruel made from alder leaves (*Alnus glutinosa*), potato peels and rabbit vegetable chow (Løkke and van Gestel, 1998; van Gestel et al., 2018; Loureiro et al., 2006). The cultures were sprayed with ultrapure water twice a week and food was provided. Only adults with a wet weight of 14–30 mg were used during the experiment, regardless of sex. The distinction between adults and juveniles was made on phenotypic traits, in particular by the individual size and color of the cuticle (Ismail, 2021). Molting animals, abnormal individuals, pregnant females and individuals lacking antennae were excluded. The last one is considered essential as the aggregation pheromone receptors are placed in the antennae.

2.2. Soil and test substances

The standard LUFA 2.2 sandy loam soil (Speyer, Germany) was used in all the experiments. The properties of this soil include a pH $= 5.5 \pm 0.2$ (0.01 M $CaCl2$), WHC $= 41.8 \pm 3.0$ (g/100 g), C $= 1.77 \pm 0.2$ (%), N $= 0.17 \pm 0.02$, texture $= 7.3 \pm 1.2$ (%) clay; 13.8 ± 2.7 (%) silt and 78.9 ± 3.5 (%) sand.

To develop the method for assessing disaggregation and avoidance behaviors, two different types of contaminants were chosen: a composite material made of tire particles (TPs), and a pure substance, benzothiazole (BT), which is a co-formulate of TPs used as accelerators of vulcanization (Federico et al., 2023). Benzothiazole was purchased from Merck Millipore ($C7H5NS$ 96% purity, CAS: 95–16-9). A stock solution of 50 mg/mL was prepared in methanol (Merck Millipore) to allow chemical solubilisation and was stored at 4 °C until use. Serial dilution in Milli-Q Water was performed to obtain the final eight test concentrations (0, 30, 60, 125, 250, 500, 750, and 1,000 mg/kg dry weight - d.w.). In the final test solutions, the maximum percentage of co-solvent used was 0.02 % in volume (v/v %). This percentage was below the level suggested by the OECD guideline based on aquatic testing (OECD, 2019), used in this work as threshold value for test dilutions.

Tire particles (TPs), black in color and size less than 180 μm diameter, were provided by the Department of Earth and Environmental Sciences, University of Milano-Bicocca (Italy), and details were provided elsewhere (Gualtieri et al., 2008). Eight nominal concentrations of TPs (0, 300, 600, 1,250, 2,500, 5,000, 7,500, and 10,000 mg/kg d.w.) were tested. These nominal concentrations correspond to concentrations detected or predicted in the environment (Federico et al., 2023).

2.3. Range finding tests

Before the final avoidance and disaggregation behavior test, range-finding avoidance tests with only one individual of *P. pruinosus* were performed to assist in the selection of appropriate concentrations to be used, as suggested by OECD guidelines (i.e. OECD, 2019), while assessing the effect of compounds and/or materials of unknown toxicity. These tests were performed to avoid interference induced by the aggregation pheromone during the experiment and to provide a new method for the standardization of the model organism. The tests were monitored at two different times, T1 $= 24$ h and T2 $= 48$ h, in order to assess whether this behavior could also occur at 24 h within the standard 48 h of the avoidance test.

A removable plastic divider was placed in plastic Petri dishes 100 × 10 mm along the longest diagonal of the dish and 10 g d.w. LUFA 2.2 soil was placed on each side. Uncontaminated soil was added to one side of the dish and treated soil to the other. Three nominal concentrations for each substance (TPs = 100, 1,000 and 10,000 mg/kg d.w.; BT = 10, 100 and 1,000 mg/kg d.w.) were chosen. Dual controls with LUFA 2.2 soil on both sides of the Petri dishes were also carried out.

TPs were added directly to dry soil and mixed gently, while BT from a stock solution of 50 mg/mL were diluted in distilled water and spiked on the treated soil. The experiments on the two substances were performed separately. Both control and treated soils for each substance were moistened to achieve a WHC = 40%. Five replicates were carried out for each concentration. The tests were carried out in thermostatic chambers at 21 ± 1 °C, 16:8 h (light:dark) photoperiod. After 24 h and 48 h, the number of individuals present on the two sides of the Petri dishes was counted.

2.4. *Behavioral tests*

The avoidance and disaggregation behaviors tests were performed to evaluate the ability of edaphic organisms to avoid contaminated soil towards clean soil (ISO/CD, 2003) and to determine a potential infochemical disruption on aggregation behavior during the avoidance behavior.

Nine replicates were performed for each treatment with ten individuals of *P. pruinosus* per replicate, which were gently introduced by spoon in the midline of each test box.

Plastic boxes (170 × 120 mm) were divided into two compartments using a removable plastic split and filled with 50 g d.w. of LUFA 2.2 soil per each side. A part of the soil was treated with the test substances, while the other part was left uncontaminated. Dual controls were performed with LUFA 2.2 soil on both sides of the box to infer the homogeneous distribution of the organisms in the box. In the TPs experiments, TPs were added directly into the soil and mixed uniformly with the highest concentration tested being 10,000 mg/kg d.w. In BT experiments, the BT highest concentration used was 1,000 mg/kg d.w. Both test soils and control soils were spiked to reach a WHC of 40%.

Nine replicates were performed for each treatment with ten individuals of *P. pruinosus* per replicate. The tests were carried out in thermostatic chambers at 21 ± 1 °C, 16:8 h (light:dark) photoperiod. After 48 h, plastic boxes were gently removed from the thermostatic chambers and left to rest for 30 min. All these precautions were implemented to avoid any external disturbances that could induce individuals to move and dissociate from any clusters formed during the experiment. High resolution color pictures were therefore taken and processed by the Image J software.

2.5. *Statistical methods for data analysis*

Avoidance behavior A% was calculated by the equation:

$$A\% = \frac{n_C - n_T}{n} \times 100, \tag{1}$$

where n_C is the number of individuals on the control soil, n_T is the number of individuals on the test soil, and n is the total number of individuals retrieved at the end of the experiment ($n = n_C + n_T$) for each level of concentration per replicate. A positive net response indicates avoidance whilst a negative net response indicates attraction to the contaminated soil (Gainer et al., 2022).

Non-avoidance occurs when the distribution of organisms is approximately equal (50% plus or minus 10) between treated and control soils (Gainer et al., 2022). When the total number of individuals in treated soils is less than 20%, it means that more than 80% of individuals avoided it, according to the ”limited habit function” (ISO/CD, 2003; ISO, 2008).

The avoidance response was ascertained in two ways. At first, we tested the proportion of organisms on the uncontaminated soil at the end of the experiment p_C (proportion of organisms in control soil) against a fixed value of 0.5 for each concentration by means of a robust test and using an M-estimator of central tendency (Mair and Wilcox, 2020). We provided also a bootstrap based 95% confidence interval for p_C conditioning to a fixed concentration level. Second, we used avoidance data, aggregating the replicate results for each concentration level, to estimate a concentration–response curve with the aim to derive effective concentration EC50, namely the estimated concentrations required to obtain a 50% of an avoidance response in the organisms. We obtained 95% confidence intervals for EC50 via the delta method.

Several dose–response curves were estimated (Finney, 1979). In particular we considered three common nonlinear regression functions namely: log–logistic (L), log-normal or probit (P) and Weibull (W) models, and a simple linear regression to the log-transformed toxicity data values and logit transformed endpoint values (backwards log–logistic (BL)) and a simple linear regression applied to the log-transformed toxicity data values and probit transformed endpoint values (backwards probit (BP)). Table 1 reports the list of model functions used to fit the concentration–response data (Seber and Wild, 1998). The coefficient b denotes the slope of the concentration–response curve, and $\Phi()$ is the cumulative distribution function of a standard normal distribution. Nicely, for log–logistic model e parameter corresponds to the effective concentration EC50 (i.e. concentration triggering the 50% of response). Maximum likelihood estimation is used for fitting concentration–response models. A robust version of BL (BLR) and BP (BPR), using MM-type estimators for linear (regression) models are also considered.

Note that we considered only models with two parameters to not incur in overfitting, i.e., when the statistical models are too complex in view of the limited available data.

Akaike Information Criterion (AIC) and Bayesian Information Criterion (BIC) were used for model selection: models with the lowest AIC and BIC values were considered the “best”.

To measure the aggregation behavior of individuals of *P. pruinosus*, we considered the number of groups or clusters formed at the end of the experiment. In our analysis we define a “cluster” as a group of individuals whose bodies touch at least at one point. If an individual is completely detached from the others, it is referred to as a singlet group. Closed individuals, but without interaction, weren’t considered as part of the same clusters. Specifically, at the end of the experiments, the boxes were gently opened and released for 15 min before counting off the clusters, in order to avoid any movement and consequently cluster’s disruption. Several color pictures were taken and analyzed by ImageJ software. The number of clusters was evaluated both in control and treated soils.

As a measure of the disaggregation behavior effect, we propose two disaggregation indexes, the disaggregation index (DI) and the disaggregation in groups index (DG) as follows:

$$\mathrm{DI} = \frac{s + 2d}{n} \qquad \text{and} \qquad \mathrm{DG} = \frac{g}{n}, \tag{2}$$

where s is the number of singlet groups at the end of the experiment, d the number of doublets at the end of the experiment, g is the number of identified groups, and n is the number of individuals at the end of the

Table 1

Concentration–response regression models. List of regression models for the response ($y = f(x)$) as a function of the concentration (x) of a given contaminant.

Regression Model	Model equation
log–logistic (L)	$f(x) = \frac{1}{1 + \exp\{b(\log(x) - \log(e)\}}$
probit (P)	$f(x) = \Phi(b(\log(x) - \log(e)))$
Weibull (W)	$f(x) = \exp\{-\exp\{b(\log(x) - \log(e))\}\}$
backward log–logistic (BL)	$\log\frac{f(x)}{1 - f(x)} = a + b\log(x)$
backward probit (BP)	$\Phi(f(x)) = a + b\log(x)$

experiment per replicate.

Both indexes can assume a value in the range $(0,1)$, where 1 indicates a maximum degree of disaggregation, while 0 indicates aggregation in only one cluster.

The indexes could be calculated considering only the organisms in the uncontaminated soil at the end of the experiment but also referring to all animals alive at the end of the experiment considering the entire box.

The rationale for the DI proposal is based on the assumption that groups formed by a single individual or at most two individuals are indicators of population fragmentation, and this trend could seriously affect the probability of regional or spot density reduction of isopod population. From an ecological point of view, singlet groups represent the maximum degree of fragmentation of the population, while doublet groups represent the minimum condition in which there could be a 50% chance that the two individuals are sexually compatible and that they can consequently increase the fitness of the population. The DI index measures the disaggregation behavior as a weighted mean of the number of singlets and doublets where the weights are equal to the group size, and it ignores higher aggregation orders.

DG index is equal to the ratio between the total number of identified clusters and the total number of alive organisms at the end of the experiment. In the case in which all the organisms are disaggregated, i.e., each forms a cluster, the DG index will be equal to 1, while if all the organisms are aggregated, i.e., there is only one big cluster, the index assumes its minimum value $1/n$, which in case of a large sample size, tends to zero.

The two proposed indexes both measure the level of disaggregation, but from two different perspectives: while the first one is closely linked to groups formed by a single individual or at most two individuals, the second index is a more general measure of the degree of granularity of the final aggregation. Moreover, due to the complexity of the phenomenon under analysis, we believe that an approach based on multiple indicators is preferable to using a single indicator, as this can reduce measurement error and lead to more precise results.

As a further step, we considered the disaggregation index data calculated for each concentration level to estimate a linear regression model with the aim to derive the median effective concentration EC50, as, by convention, a value of 0.5 has been assigned as the threshold above which organisms show disaggregation behavior. This assigned value has an ecological meaning because if, for example, the disaggregation index DI value is equal to 0.6, it means that, out of 10 total individuals collected at the end of the test, six groups composed of one or, e.g. three groups formed by two individuals were counted (or four singlets and a doublet), while only four individuals, which means less than half, maintained a gregarious behavior.

The data were considered statistically significant for values of $p < 0.05$. All statistical analyses were performed using R 4.3.0 (R Core Team, 2023).

3. Results

3.1. Range finding test at one individual

Results about the avoidance behavior tests on single individuals of *P. pruinosus* are reported in supplementary material (Table S1).

Single individuals of *P. pruinosus* showed a net avoidance response at concentrations of TPs = 10,000 mg/kg d.w. and BT = 1,000 mg/kg d.w., which were therefore considered the maximum concentrations to test.

However, in the final tests on ten individuals, it was decided to test lower sub-lethal concentrations which did not determine avoidance responses, in order to obtain a greater number of points for the representation of the concentration–response curves of the individual contaminants during the data analysis.

3.2. Avoidance behavior tests

All avoidance behavior tests performed presented less than 20% of mortality and a stochastic distribution in the controls, which are essential conditions for the validity of tests (Loureiro et al., 2005).

Isopods showed a significant avoidance already at TP concentration of 1,250 mg/kg d.w. and at BT concentration of 500 mg/kg d.w., meaning that less than 20% of individuals were in the treated soil (Fig. 1).

The robust proportional test (p_C) against a fixed value of 0.5 and 95% confidence intervals confirmed the depopulation of the test soils, showing a greater number of individuals in the control soils from a concentration level of at least of 1,250 and 500 mg/kg d.w. for TPs and BT, respectively (Fig. 2).

According to the AIC and BIC selection criteria, the Weibull model (AIC: −6.48; BIC: −6.242) resulted in the best fit one (Fig. 3 -left) for TPs, thus the estimated EC50 concentration level along the related 95% confidence interval (CI) was 1,039 mg/kg d.w. (CI: 527.5–1,550 mg/kg d.w.). For BT we selected the log–logistic model (AIC: −18.6; BIC: −18.36)(Fig. 3 - right), thus the estimated EC50 was 153 mg/kg d.w. (CI: 105.9–200.1 mg/kg d.w.). All the estimated parameters are summarized in Table 2.

3.3. Disaggregation effect tests

In order to standardize the method, a mortality condition of less than 20% and a gregarious condition of not less than 80% were imposed in the controls. In fact, in all control tests, individuals always showed a disaggregation never greater than 20%, emphasizing that 80% of the individuals were always aggregated in a single cluster, thus validating our test. DI and DG indexes confirm the validation of the control tests (Table S2).

The cluster distribution (Fig. 4) indicates that while there was a disaggregation effect in the exposure to TPs and BT, the majority of these fragmented clusters were present in the control soil. This suggests that, although terrestrial isopods avoided contaminated soil, they did not maintain gregarious behavior, which could affect the survival and fitness of the population even in uncontaminated soil. In Fig. 4, for each replicate and for each concentration level the number of identified clusters in uncontaminated soil is shown, with the size-color point being proportional to the cluster size (lighter colors identify larger groups). It is evident that as the concentration increases, the level of disaggregation increases in the control soil, and the sample size of each cluster is also becoming increasingly smaller, e.g., at the highest concentration, in fact, we find many groups made up of a few individuals.

The values of DI and DG indexes (see Eqs. (2)) were used separately as endpoints to derive EC50 estimates along with a 95% confidence interval fitting a linear regression model (Fig. 5).

The two estimated linear models, considering the DI and DG indexes as dependent variables, are both statistically significant and show approximately the same goodness of fit. The estimated EC50 concentration level and related 95% confidence interval for each substance and index are reported in Table 3.

The EC50 estimates derived from the DI and DG indexes used as dependent variables in a regression linear model differ slightly from each other, in particular the use of DG leads to more conservative values for both TPs and BT. However, the resulting confidence intervals at 95% levels for EC50 partially overlap, although the inference from DG is always more precise, resulting in a narrower confidence interval for EC50 at the same confidence level for both TPs and BT.

The composite use of both indexes could help in catching the complex aggregation phenomenon and the partially statistical overlap between these indicators suggests that future research might consider using these indicators to measure gregarious behavior of individuals of *P. pruinosus*.

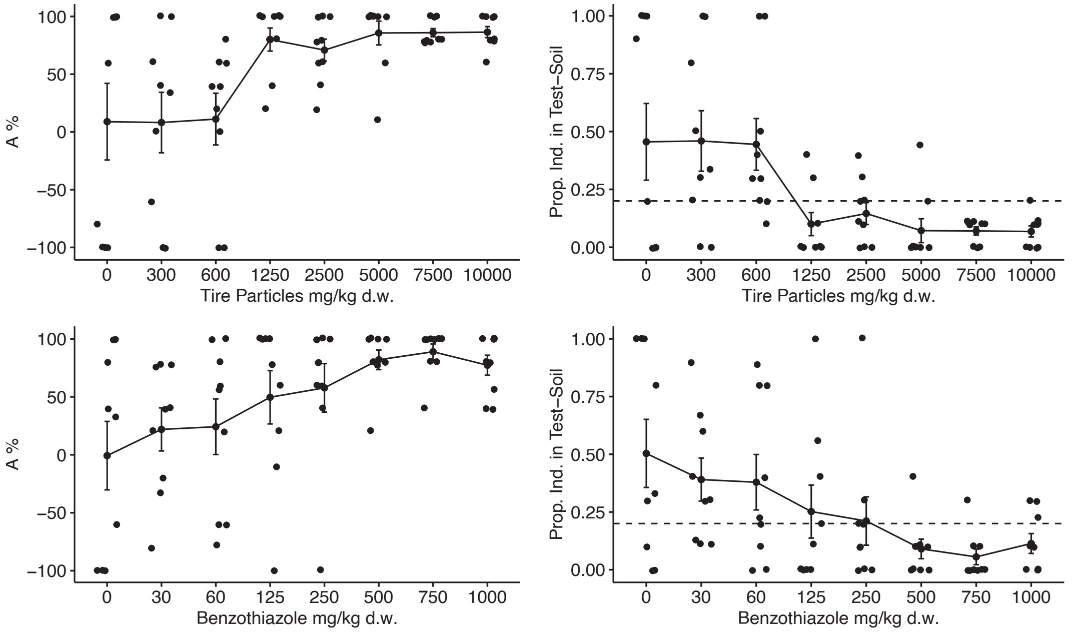

Fig. 1. Avoidance behavior (A%) of *Porcellionides pruinosus* (left) and proportion of individuals in test-soils (right) when exposed to tire particles and benzothiazole (mg/kg d.w.) in LUFA 2.2 soil for 48 h. Avoidance behavior responses are expressed as mean values ± standard error, while the distribution of values relating to each replicate is shown. The dotted line in the right panel refers to the 0.2 corresponding to the "limit habitat function" of test soil.

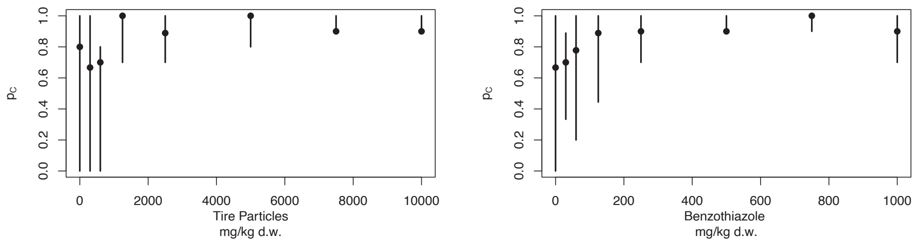

Fig. 2. Robust one-sample test results for the proportion of organisms on uncontaminated soil against a fixed value of 0.5 for each concentration, along with the approximated 95% confidence intervals based on 10,000 bootstrap replicates.

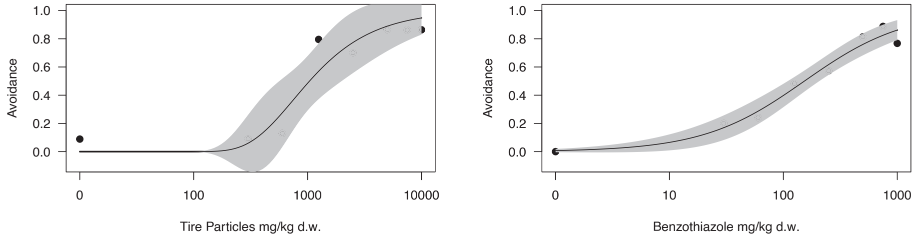

Fig. 3. Dose–response curves for the avoidance behavior of *Porcellionides pruinosus* to the exposure to tire particles (left, Weibull) and benzothiazole (right, log–logistic). Points represent mean observed net response data and line is the fitted regression model (shaded region = 95% confidence bounds).

3.4. Consolidated results

The combined avoidance and disaggregation test yielded the following noteworthy findings:

- The avoidance and disaggregation responses exhibited by *P. pruinosus* individuals occurred at different concentrations. The concentration at which disaggregation can be detected is higher than that at which avoidance occurs.
- The disaggregation effects were observed exclusively among population that avoided the treated soil as the remaining individuals in the treated soil exhibited a percentage of $\leqslant$20%, which is a requisite condition for confirming avoidance.
- The population in the uncontaminated soil demonstrated an increase in the degree of disaggregation, emphasizing that despite the success of avoidance, the individuals exhibited a reduction in their gregarious behavior.

Table 2
Concentration–response models and parameter estimates from the avoidance behavior tests with *Porcellionides pruinosus* exposed to tire particles and benzothiazole for 48 h in Lufa 2.2 soil (Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1).

Contaminants	Model	Parameter	Estimate	SE	p-value
Tire Particles	Weibull	*b*	−1.130	0.387	0.027 *
		e	751.289	157.288	0.003 **
		EC50	1,039 mg/kg d.w.		
Benzothiazole	log–logistic	*b*	−0.974	0.124	0.0002 ***
		e	153	19.2	0.0002 ***
		EC50	153 mg/kg d.w.		

4. Discussion

4.1. Effect of the number of individuals and contaminants on the avoidance behavior response

The avoidance assay using individuals of *P. pruinosus* is a useful and sensitive screening method for appraising contaminated soil. In our work, TPs and BT induced avoidance behavior on the terrestrial isopods following a dose–response function. Referred to TPS, the EC50 estimate is in the same order of the environmental concentrations, which range from 600 to 117,000 mg/kg d.w. (Wik and Dave, 2009; Federico et al., 2023), suggesting a "limited habitat function" (ISO, 2008) and a potential risk of depopulation effect in soils exposed to TPs. There are few studies on avoidance effects of TPs at relevant ecological levels and the results are not always consistent as the effects depend on their physical dimensions, chemical composition and the aging state of the tire (Federico et al., 2023). Nevertheless, Lackmann et al. (2023) reported no avoidance up to a concentration of TPs ($< 600\ \mu$m) of 1,000 mg/kg d.w. on individuals of *Eisenia foetida*. Therefore, our findings demonstrate the higher sensitivity of individuals of *P. pruinosus* to sublethal concentrations of contaminants in avoidance tests, highlighting the significance and importance of standardizing this biological model. The EC50 calculated in this study for BT is higher than the concentrations of BT found in soil, ranging from 0.23 to 99.3 mg/kg d.w. (Zhang et al., 2018; Li et al., 2023). To our knowledge, no avoidance tests involving BT and any organisms have been conducted previously, allowing for fair

Table 3
The estimated EC50 concentration level and related 95% confidence interval for each substance estimated from the fitted linear model for the two different indexes as a function of the contaminant concentration. Data are expressed in mg/kg d.w..

Contaminants	Index	Estimated EC50	95% CI
Tire Particles	DI	8,566	[4,318 - 12,814]
	DG	7,454	[3,754 - 11,154]
Benzothiazole	DI	922	[241 - 1,604]
	DG	712	[269 - 1,154]

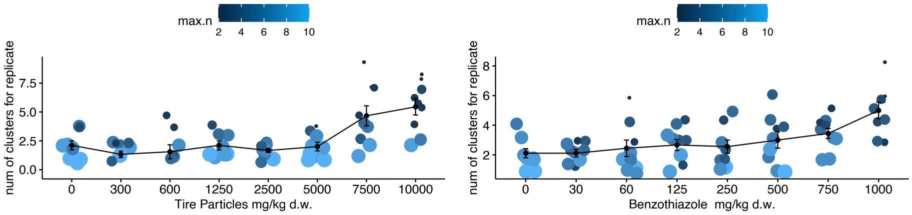

Fig. 4. Identified clusters for each concentration level of tire particles (left) and benzothiazole (right) in control soil. Each point represents the number of clusters identified for each replicate ($n = 9$), and the size-color point is proportional to the cluster size (lighter color identifies larger groups). Black points correspond to the mean size of the clusters identified for each concentration level ± standard error. In the legend max.n refers to the size of the largest group identified in that replication.

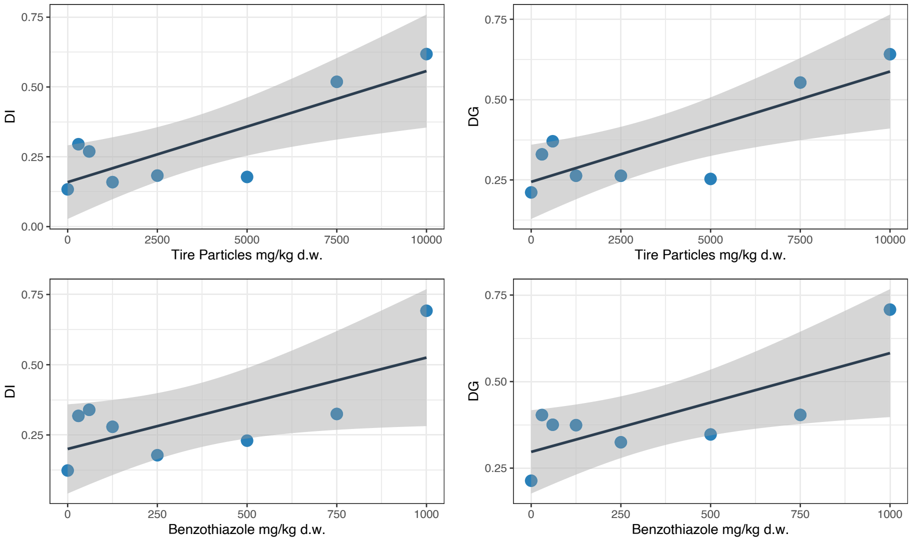

Fig. 5. Linear regression fit (solid line) with the 95% confidence region (shaded region) for DI response (left) and for DG response (right) of *Porcellionides pruinosus* as a function of tire particles and benzothiazole exposure. Points represent mean observed DI and DG data respectively. Both indexes refer to all alive organisms at the end of experiments.

comparisons to be made. In both cases, our study verified the sensitivity of the bioassay, even when carried out with either ten ($n = 10$) or one ($n = 1$) individuals per replicate in tests, as the thresholds for the lack of avoidance behavior remained constant for both sample sizes (Table S1).

This outcome is unexpected because it is commonly understood that interaction with others of the same species can hinder or weaken the capacity to effectively avoid polluted soil. In the study of Zidar and Fiser (2022), avoidance tests performed with paired individuals of *Porcellio scaber* reduced the capability of the groups to avoid soil contaminated by a pyrethrin-based insecticide compared to single individuals, which resulted in an underestimation of the contaminant's impact on the avoidance response of the individuals due to their gregarious behavior, thereby increasing the proportion of individuals in treated soil. In our study, on the contrary, the impact of individual interaction doesn't affect the ability of individuals to avoid contaminated soil, and the net response (A%) in the final ten individual tests was not underestimated by gregariousness, which is also supported by the first study where isopods were used in avoidance behavior tests (Loureiro et al., 2005).

The causes of these outcomes may be influenced by different factors, including the model organism, the size of the sample group, the toxicological characteristics of the substances under investigation, or their physical and chemical properties.

Ecotoxicological testing frequently seeks to draw conclusions about larger ecosystems or populations, therefore a higher number of tested individuals can enhance the ecological extrapolation of test results. Therefore, a small number of individuals during ecotoxicological bioassays may not sufficiently represent ecological realism of dynamic populations. To improve the uniformity of results and standardize toxicity assessments on *P. pruinosus*, we suggest maintaining ten individuals per box for avoidance and disaggregation behavior biotests.

Additionally, using individual tests to preliminarily determine the range finding of substances (chemical or physical) can allow for the avoidance response in the absence of influences determined by the aggregation pheromone. Observing these species and noting any variations in preferences between individuals and groups can provide valuable insights. Another factor that helps explain the observed discrepancy can be the mode of action of the administered substances. Usually, avoidance behavior tests are performed for detecting chemical presence before the induction of toxicity, so it is expected that organisms avoid the treated soil before getting effects. At the same time, it is not possible to underestimate the effects induced by chemicals within 48 h on the ability of exposed organisms to avoid, as demonstrated, for example, by the effect of pharmaceutical such as carbamazepine or fluoxetine which can still induce an effect within the end of the test and, as a result, reduce the ability of organisms to abandon the treated soil as they are unable to do so (Oliveira et al., 2015).

In our work, BT shows a narcotic effect on the ability of individuals of *P. pruinosus* to avoid. As known in literature, BT is an aromatic heterocyclic compound exhibiting fungicidal activity, stimulating apoptosis by synthesizing programmed cell death protein 5, altering F-actin structures and cytoskeleton stability, and reducing gene expression of cytochrome P450 4F5 and glutathione S-transferase associated with detoxification metabolism (Mei et al., 2019). In general, it is expected for BT to not have an arthropod-selective mode of action. In contrast, Zidar and Fiser (2022) tested a product containing pyrethrin as an active principle, which has a specific mode of action on arthropods, preventing the closure of voltage-gated sodium channels in axonal membranes. A challenge in comprehending the mechanism of soil pollutants on collective behavior in woodlice is the lack of characterization of the aggregation pheromone molecule. Therefore, a deeper understanding of the means by which substances induce infochemical changes in the collective behavior of soil organisms and grouping species could enhance our comprehension of the phenomena of aggregation and disaggregation and the impact of compounds on modulating this behavior.

Additionally, our experiments demonstrate that avoidance behavior in individual tests was elicited within 24 h, and the positions of the individuals were unaltered even after 48 h of exposure. Consistent with previous research (Natal-da Luz et al., 2008; Owojori et al., 2011; Frankenbach et al., 2014), reducing exposure duration to 24 h is both feasible and appropriate for individual tests. Subsequently, this technique enhances the timing of the 48 h follow-up assessments on ten individuals, with the goal of evaluating avoidance and disaggregation in a more efficient manner.

4.2. Disaggregation behavior effect as a new ecotoxicological endpoint

The article presents a newly proposed method of developing an expeditious and cost-effective behavioral endpoint to assess the effects of altering gregariousness in the population of *P. pruinosus*. We suggest merging this endpoint with the avoidance behavior test, in order to reinforce the ecological informative meaning of the bioassay. In fact, avoidance behavior only is insufficient to clarify the modality of migration, that is the way in which the population of *P. pruinosus* moves towards the control soil, whether in groups or clusters.

To deal with these issues, the adaptive aggregation behavior in individuals of woodlice may, on the contrary, be helpful for understanding how such avoidance occurs, observing whether the individuals move in groups or if the population is fragmenting.

This behavioral endpoint has ecological relevance, since aggregation behavior has widespread in many edaphic organisms (Verhoef and Nagelkerke, 1977; Salmon and Ponge, 2001; Broly et al., 2014; Chase et al., 1980; Broly et al., 2014; Zirbes et al., 2012), especially in terrestrial isopods, which are the only crustaceans to have fully colonized the mainland (Broly et al., 2013), through morpho-physiological adaptations (Gibbs and Rajpurohit, 2010; Dias et al., 2013; Nako et al., 2018; Sfenthourakis and Hornung, 2018) and behavioral strategies, as being nocturnal or gregarious (Devigne et al., 2011; Broly et al., 2014). As a result of soil contamination, the aggregation ability of these organisms has a significant impact on soil adaptation, primarily by reducing population density and affecting population fitness. Changes in social structure and the dispersal of individuals may affect mating opportunities and offspring survival, as they have distinct sexes. Therefore, contaminant-induced disaggregation can disrupt the reproductive success of woodlice.

Likewise, it cannot be ruled out that fragmented migration and disaggregation behavior in general may be an adaptive strategy for terrestrial isopods themselves in stressful situations, in order to colonize or separately patrol neighboring areas in search of non-stressed soils. This assumption is still conjectural and deserves to be explored further in the future. Despite the many aspects to be explored regarding disaggregation behavior the combination with the avoidance behavior test could help to refine the protocols relating to the standardization of the biological model of *P. pruinosus*, for which there is still no regulatory toxicity assessment yet (van Gestel et al., 2018), and to refine the avoidance tests themselves, currently under development by the International Organization for Standardization (Loureiro et al., 2005), in order to evaluate, through a single and rapid avoidance-disaggregation bioassay, two different and ecologically relevant endpoints at a population level.

To deal with it, this paper introduced two disaggregation indexes (DI and DG) to measure the alteration of gregariousness on individuals of *P. pruinosus* and for extrapolating an EC50 of disaggregation, useful for the purposes of identification and characterization of the risk. Both of these indexes help to highlight an effect of social alteration, taking into consideration both the number of clusters and the numerousness of these, especially those made up of one or a maximum of two individuals, whose formation may not necessarily lead to conditions of population restoration. Specifically, the DI index, although it returns slightly higher EC50 values, has ecologically effects, given that it takes into consideration the number of singlet and doublet clusters, which can be sensitive to those selective bottleneck and genetic drift phenomena that lead to the isolation of the population (Sfenthourakis and Hornung, 2018). These non-Darwinian selection phenomena could profoundly reduce population variability and increase its sensitivity to anthropogenic or environmental stressors. At the same time, the effects on the population dynamics of isolated groups may depend not only on the ”size of bottleneck” but also on the rate of population growth (Nei et al., 1975).

Furthermore, both DI and DG values encompass the assessment of both control and treated soils, along with the entire boxes. Neglecting this aspect would be unwise since prior research indicates that individuals undergoing avoidance bioassay may fail to avoid the treated soil, possibly due to preventing factors or immobilization (Oliveira et al., 2015). In that case, the number of clusters in the control soil will be very low and therefore it would be better to consider the number of clusters over the entire total box. For this reason, it is recommended to use DI or DG indexes considering the entire box.

5. Conclusions

A new ecotoxicological endpoint related to the disaggregation effect was adopted to rapidly detect the presence of infochemicals that can disrupt the intraspecific communication and influence the modality of avoidance behavior. The quantification of the disaggregation index (DI) and disaggregation in groups (DG) also allowed the determination of EC50 values useful in the regulatory context. This work demonstrated how exposures to TPs and BT induced terrestrial isopods to migrate towards the control soil, maintaining an aggregation state within a range of tested concentrations, while higher concentrations induced disaggregation behavior even when the isopods were moved in the control soil.

Future studies will focus on understanding the potential molecular mechanisms underlying the disaggregation effect in individuals of *P. pruinosus*, in order to confirm a disruption of aggregation behavior induced by soil contamination.

Credit authorship contribution statement

LF: Conceptualization, Methodology, Data curation, Investigation, Writing – original draft. **GSM**: Methodology, Software, Formal analysis, Data curation, Writing – review & editing. **SL**: Resources, Validation, Writing – review & editing. **SV**: Conceptualization, Methodology, Project administration, Resources, Supervision, Funding acquisition, Validation, Writing – review & editing.

Declaration of Competing Interest

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

Data availability

Data will be made available on request.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.ecolind.2024.112602.

References

Allee, W.C., 1926. Studies in animal aggregations: Causes and effects of bunching in land isopods. J. Exp. Zool. 45, 255–277. https://doi.org/10.1002/jez.1400450108.

Bandeira, F.O., Lopes Alves, P.R., Hennig, T.B., Toniolo, T., da Luz, T.N., Baretta, D., 2020. Effect of temperature on the toxicity of imidacloprid to Eisenia andrei and Folsomia candida in tropical soils. Environ. Pollut. 267, 115565. https://doi.org/10.1016/j.envpol.2020.115565.

Broly, P., Deneubourg, J.L., Devigne, C., 2013. Benefits of aggregation in woodlice: a factor in the terrestrialization process? Insect. Soc. 60, 419–435. https://doi.org/10.1007/s00040-013-0313-7.

Broly, P., Devigne, L., Deneubourg, J.L., Devigne, C., 2014. Effects of group size on aggregation against desiccation in woodlice (isopoda: Oniscidea). Physiol. Entomol. 39, 165–171. https://doi.org/10.1111/phen.12060.

Broly, P., Ectors, Q., Decuyper, G., Nicolis, S.C., Deneubourg, J.L., 2016. Sensitivity of density-dependent threshold to species composition in arthropod aggregates. Sci. Rep. 6, 32576. https://doi.org/10.1038/srep32576.

Cardé, R.T., Millar, J.G., 2009. Pheromones in Encyclopedia of insects. Second Edition.

Chase, R., Croll, R.P., Zeichner, L.L., 1980. Aggregation in snails, Achatina fulica. Behav. Neural Biol. 30, 218–230. https://doi.org/10.1016/s0163-1047(80)91101-2.

Devigne, C., Broly, P., Deneubourg, J.L., 2011. Individual preferences and social interactions determine the aggregation of woodlice. Plos one 6, 17389. https://doi.org/10.1371/journal.pone.0017389.

Dias, A.C., Krab, E.J., Mariën, J., Zimmer, M., Cornelissen, J.H.C., Ellers, J., Wardle, D.A., Berg, M.P., 2013. Traits underpinning desiccation resistance explain distribution patterns of terrestrial isopods. Oecologia 172, 667–677. https://doi.org/10.1007/s00442-012-2541-3.

Elisabeth, H., 2011. Evolutionary adaptation of oniscidean isopods to terrestrial life: Structure, physiology and behavior. Terr. Arthropod Rev. 4, 95–130. https://doi.org/10.1163/187498311X576262.

Federico, L., Masseroni, A., Rizzi, C., Villa, S., 2023. Silent contamination: The state of the art, knowledge gaps, and a preliminary risk assessment of tire particles in urban parks. Toxics 11. https://doi.org/10.3390/toxics11050445.

Ferreira, N.G., Morgado, R.G., Amaro, A., Machado, A.L., Soares, A.M., Loureiro, S., 2016. The effects of temperature, soil moisture and uv radiation on biomarkers and energy reserves of the isopod Porcellionides pruinosus. App. Soil Ecol. 107, 224–236. https://doi.org/10.1016/j.apsoil.2016.06.007.

Finney, D.J., 1979. Bioassay and the practice of statistical inference. Int. Stat. Rev. 47, 1–12.

Frankenbach, S., Scheffczyk, A., Jänsch, S., Römbke, J., 2014. Duration of the standard earthworm avoidance test: Are 48h necessary? App. Soil Ecol. 83, 238–246. https://doi.org/10.1016/j.apsoil.2014.04.006.

Gainer, A., Owojori, O., Maboeta, M., 2022. Use of soil invertebrate avoidance tests as an emerging tool in soil ecotoxicology. Rev. Environ. Contam. Toxicol. 260 https://doi.org/10.1007/s44169-021-00004-4.

Gibbs, A.G., Rajpurohit, S., 2010. Insect Hydrocarbons: Biology, Biochemistry, and Chemical Ecology. Cambridge University Press. chapter Cuticular lipids and water balance. p. 100–120. doi:10.1017/CBO9780511711909.007.

Gualtieri, M., Mantecca, P., Cetta, F., Camatini, M., 2008. Organic compounds in tire particle induce reactive oxygen species and heat-shock proteins in the human alveolar cell line a549. Environ. Int. 34, 437–442. https://doi.org/10.1016/j.envint.2007.09.010.

Ismail, T.G., 2021. Seasonal shape variations, ontogenetic shape changes, and sexual dimorphism in a population of land isopod Porcellionides pruinosus: a geometric morphometric study. JOBAZ 82, 1–15. https://doi.org/10.1186/s41936-021-00209-y.

ISO, 2008. Soil quality-avoidance test for determining the quality of soils and effects of chemicals on behaviour-part 1: Test with earthworms (Eisenia fetida and Eisenia andrei). International Organization for Standardization, Geneva, Switzerland. ISO 17512–1.

ISO/CD, 2003. Soil quality – avoidance test for testing the quality of soils and the toxicity of chemicals – test with earthworms (Eisenia fetida). ISO 17512. International Organization for Standardization, Geneva, Switzerland.

Lackmann, C., Šimić, A., Ečimović, S., Mikuška, A., Seiler, T.B., Hollert, H., Velki, M., 2023. Subcellular responses and avoidance behavior in earthworm Eisenia andrei exposed to pesticides in the artificial soil. Agriculture 13. https://doi.org/10.3390/agriculture13020271.

Lee, C.E., Frost, B.W., 2022. Morphological stasis in the eurytemora affinis species complex (copepoda: Temoridae). Hydrobiologia 480, 111–128. https://doi.org/10.1023/A:1021293203512.

Li, Z.M., Pal, V.K., Kannan, P., Li, W., Kannan, K., 2023. 1,3-diphenylguanidine, benzothiazole, benzotriazole, and their derivatives in soils collected from northeastern united states. Sci. Total Environ. 887, 164110. https://doi.org/10.1016/j.scitotenv.2023.164110.

Lima, M.P., Cardoso, D.N., Soares, A.M., Loureiro, S., 2015. Carbaryl toxicity prediction to soil organisms under high and low temperature regimes. Ecotoxicol. Environ. Saf. 114, 263–272. https://doi.org/10.1016/j.ecoenv.2014.04.004.

Loureiro, S., Amorim, M.J., Campos, B., Rodrigues, S.M., Soares, A.M., 2009. Assessing joint toxicity of chemicals in Enchytraeus albidus (enchytraeidae) and Porcellionides pruinosus (isopoda) using avoidance behaviour as an endpoint. Environ. Pollut. 157, 625–636. https://doi.org/10.1016/j.envpol.2008.08.010.

Loureiro, S., Sampaio, A., Brandão, A., Nogueira, A.J., Soares, A.M., 2006. Feeding behaviour of the terrestrial isopod Porcellionides pruinosus brandt, 1833 (crustacea, isopoda) in response to changes in food quality and contamination. Sci. Total Environ. 369, 119–128. https://doi.org/10.1016/j.scitotenv.2006.05.023.

Loureiro, S., Soares, A.M., Nogueira, A.J., 2005. Terrestrial avoidance behaviour tests as screening tool to assess soil contamination. Environ. Pollut. 138, 121–131. https://doi.org/10.1016/j.envpol.2005.02.013.

Natal-da Luz, T., Römbke, J., Sousa, J.P., 2008. Avoidance tests in site-specific risk assessment—influence of soil properties on the avoidance response of collembola and earthworms. Environ. Toxicol. Chem. 27, 1112–1117. https://doi.org/10.1897/07-386.1.

Løkke, H., van Gestel, C.A.M., 1998. Handbook of Soil Invertebrate Toxicity Tests. John Wiley & Sons, Chichester, UK.

Mair, P., Wilcox, R., 2020. Robust statistical methods in R using the WRS2 package. Behav. Res. 52, 464–488. https://doi.org/10.3758/s13428-019-01246-w.

Malheiro, C., Prodana, M., Cardoso, D., Soares, A., Morgado, R., Loureiro, S., 2023. Soil habitat function after innovative nanoagriproducts application: Effect of ageing on the avoidance behaviour of the soil invertebrates Enchytraeus crypticus and Folsomia candida. Sci. Total Environ. 901, 165955. https://doi.org/10.1016/j.scitotenv.2023.165955.

Mei, X., Liu, Y., Huang, H., Du, F., Huang, L., Wu, J., Li, Y., Zhu, S., Yang, M., 2019. Benzothiazole inhibits the growth of Phytophthora capsici through inducing apoptosis and suppressing stress responses and metabolic detoxification. Pestic. Biochem. Phys. 154, 7–16. https://doi.org/10.1016/j.pestbp.2018.12.002.

Morgado, R., Ferreira, N.G., Cardoso, D.N., Soares, A.M., Loureiro, S., 2015. Abiotic factors affect the performance of the terrestrial isopod Porcellionides pruinosus. Appl. Soil Ecol. 95, 161–170. https://doi.org/10.1016/j.apsoil.2015.06.012.

Mészárosné Póss, A., Tóthné Bogdányi, F., Tóth, F., 2022. Consumption of fungi-infected fallen pear leaves by the common woodlouse. Acta Phytopathol. 57, 79–91. https://doi.org/10.1556/038.2022.00133.

Nako, J.D., Lee, N.S., Wright, J.C., 2018. Water vapor absorption allows for volume expansion during molting in Armadillidium vulgare and Porcellio dilatatus (crustacea, isopoda, oniscidea). ZooKeys 801, 459–479. https://doi.org/10.3897/zookeys.801.23344.

Nei, M., Maruyama, T., Chakraborty, R., 1975. The bottleneck effect and genetic variability in populations. Evolution 29, 1–10. https://doi.org/10.2307/2407137.

OECD, 2019. Guidance Document on Aquatic Toxicity Testing of Difficult Substances and Mixtures. OECD Series on Testing and Assessment. OECD Publishing, Paris, France.

Oliveira, M., Cardoso, D., Soares, A., Loureiro, S., 2015. Effects of short-term exposure to fluoxetine and carbamazepine to the collembolan Folsomia candida. Chemosphere 120, 86–91. https://doi.org/10.1016/j.chemosphere.2014.06.038.

Owojori, O.J., Healey, J., Princz, J., Siciliano, S.D., 2011. Can avoidance behavior of the mite Oppia nitens be used as a rapid toxicity test for soils contaminated with metals or organic chemicals? Environ. Toxicol. Chem. 30, 2594–2601. https://doi.org/10.1002/etc.658.

Puddephatt, K.J., McCarthy, L.H., Serre, B.M., 2022. Is land-applying biosolids to agricultural areas sustainable? part one: Assessing the potential chronic, sublethal and lethal ecotoxicity of biosolids on Folsomia candida and Lumbricus terrestris. doi: 10.21203/rs.3.rs-1350257/v1.

R Core Team, 2023. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria. URL: https://www.R-project.org/.

Renaud, M., Natal-da Luz, T., Ribeiro, R., Sousa, J.P., 2022. The recolonization concentration concept: Using avoidance assays with soil organisms to predict the recolonization potential of contaminated sites. Toxics 10. https://doi.org/10.3390/toxics10030127.

Salmon, S., Ponge, J.F., 2001. Earthworm excreta attract soil springtails: laboratory experiments on Heteromurus nitidus (collembola: Entomobryidae). Soil Biol. Biochem. 33, 1959–1969. https://doi.org/10.1016/S0038-0717(01)00129-8.

Santos, M.J., Soares, A.M., Loureiro, S., 2010. Joint effects of three plant protection products to the terrestrial isopod Porcellionides pruinosus and the collembolan Folsomia candida. Chemosphere 80, 1021–1030. https://doi.org/10.1016/j.chemosphere.2010.05.031.

Seber, G.A.F., Wild, C.J., 1998. Nonlinear Regression. John Wiley & Sons, New York.

Sengupta, S., Leinaas, H.P., van Gestel, C.A., Jager, T., Rundberget, T., Borgå, K., 2023. High sensitivity to dietary imidacloprid exposure in early life stages of Folsomia quadrioculata (collembola) populations from contrasting climates. Appl. Soil Ecol. 187, 104880. https://doi.org/10.1016/j.apsoil.2023.104880.

Sfenthourakis, S., Hornung, E., 2018. Isopod distribution and climate change. ZooKeys 801, 25–61. https://doi.org/10.3897/zookeys.801.23533 arXiv:https://doi.org/10.3897/zookeys.801.23533.

Takeda, N., 1980. The aggregation pheromone of some terrestrial isopod crustaceans. Experientia 36, 1296–1297. https://doi.org/10.3897/zookeys.801.23533.

van Gestel, C.A., 2012. Soil ecotoxicology: state of the art and future directions. ZooKeys 176, 275–296. https://doi.org/10.3897/zookeys.176.2275.

van Gestel, C.A., Loureiro, S., Zidar, P., 2018. Terrestrial isopods as model organisms in soil ecotoxicology: a review. ZooKeys 801, 127–162. https://doi.org/10.3897/zookeys.801.21970.

van Straalen, N.M., 2003. Ecotoxicology becomes stress ecology. Environ. Sci. Technol. 37, 324A–330A. https://doi.org/10.1021/es0325720.

van Straalen, N.M., van Gestel, C.A., 2008. A stress ecology framework for comprehensive risk assessment of diffuse pollution. Sci. Total Environ. 406, 479–483. https://doi.org/10.1016/j.scitotenv.2008.06.054.

Verhoef, H.A., Nagelkerke, C.J., 1977. Formation and ecological significance of aggregations in collembola. an experimental study. Oecologia 31, 215–226.

Warburg, M.R., 1993. Evolutionary biology of land isopods. Springer-Verlag, Berlin.

Wik, A., Dave, G., 2009. Occurrence and effects of tire wear particles in the environment – a critical review and an initial risk assessment. Environ. Pollut. 157, 1–11. https://doi.org/10.1016/j.envpol.2008.09.028.

Zhang, J., Zhang, X., Wu, L., Wang, T., Zhao, J., Zhang, Y., Men, Z., Mao, H., 2018. Occurrence of benzothiazole and its derivates in tire wear, road dust, and roadside soil. Chemosphere 201, 310–317. https://doi.org/10.1016/j.chemosphere.2018.03.007.

Zidar, P., Fiser, Z., 2022. Avoidance behaviour toxicity tests should account for animal gregariousness: a case study on the terrestrial isopod Porcellio scaber. ZooKeys 1101, 87–108. https://doi.org/10.3897/zookeys.1101.76711.

Zirbes, L., Brostaux, Y., Mescher, M., Jason, M., Haubruge, E., Deneubourg, J.L., 2012. Self-assemblage and quorum in the earthworm Eisenia fetida (oligochaete, lumbricidae). PloS one 7, e32564. https://doi.org/10.1371/journal.pone.0032564.

Supplementary Material

Disaggregation behavior in the terrestrial isopod *Porcellionides pruinosus* as a new ecotoxicological endpoint for assessing infochemical disrupting activity

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Table S1. Range finding of avoidance behavior tests with one individual of *P. pruinosus* at three nominal concentrations of TPs (100, 1,000, 10,000 mg/kg d.w.) and BT (10, 100, 1,000 mg/kg d.w.). The table shows the replicates, the percentage of avoidance (A%) for each replicate and the mean value.

	mg/kg d.w.	Replicates	control soil	treated soil	number of individuals	A %	Mean
[TPs]	100	1	0	1	1	-100	-20
	100	2	0	1	1	-100	
	100	3	0	1	1	-100	
	100	4	1	0	1	100	

	100	5	1	0	1	100	
	1	1	1	0	1	100	60
	1	2	1	0	1	100	
	1	3	1	0	1	100	
	1	4	1	0	1	100	
	1	5	0	1	1	-100	
	10	1	1	0	1	100	100
	10	2	1	0	1	100	
	10	3	1	0	1	100	
	10	4	1	0	1	100	
	10	5	1	0	1	100	
[BT]	10	1	1	0	1	100	20
	10	2	1	0	1	100	
	10	3	0	1	1	-100	
	10	4	0	1	1	-100	
	10	5	1	0	1	100	
	100	1	1	0	1	100	20
	100	2	0	1	1	-100	
	100	3	1	0	1	100	

100	4	1	0	1	100	
100	5	0	1	1	-100	
1	1	1	0	1	100	100
1	2	1	0	1	100	
1	3	1	0	1	100	
1	4	1	0	1	100	
1	5	1	0	1	100	

Table S2. Mean value of the disaggregation indices (DI and DG) in the controls.

Index	Tire particles (TPs)	benzothiazole (BTs)
DI	0.13	0.12
DG	0.2	0.2

Chapter 4

Aggregation behavior of Terrestrial Isopods as an Indicator to assess Biotic and Abiotic Stressors on Soil Ecosystems

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Aggregation behavior of Terrestrial Isopods as an Indicator to assess Biotic and Abiotic Stressors on Soil Ecosystems

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Mandatory Keywords:	climate change, behavioral toxicology, terrestrial invertebrate toxicology, soil ecotoxicology
Additional Keywords (Optional):	
Abstract:	Shifts in abiotic and biotic factors can alter the behavior of edaphic organisms and compromise the soil habitat function. This study explores the impact of temperature, soil moisture, salinity, pH, edaphic interference, oleic acid (necromone signals), and microbial community dilution on the aggregation behavior of <Porcellionides pruinosus> as a potential new indicator of population-level stress. Aggregation patterns were quantified using two novel ecological indices, the Disaggregation Index, which measures groups formed by a single individual or at most two individuals, and the Disaggregation in Group Index, which quantifies the total number of groups formed at the end of the experiments. The disaggregation indices range from 0 (highest level of aggregation) to 1 (highest level of disaggregation). A value of 0.5 has been set as the concern threshold, i.e. the point at which more than 50% of the population is fragmented. Results reveal that most environmental factors modulate aggregation. A U-shaped disaggregation was observed for temperature and soil moisture, with population fragmentation occurring at both extremes. In contrast, salinity and biotic factors caused disaggregation following a sigmoidal curve. Only pH did not influence the aggregation behavior; on the contrary, an aggregation trend was observed at the lower and upper limits tested, showing a bimodal distribution. The cluster analysis revealed three primary trends in the disaggregation response curves as a function of stress level: parabolic, sigmoidal and bimodal. Given the need for tools to assess soil health and monitor habitat function, these findings demonstrate that terrestrial isopods' aggregation behavior is a sensitive indicator of soil changes and can be used to detect and quantify environmental stress through measurable changes in degree of aggregation. Valuable insights on laboratory conditions are also produced to standardize this bioassay.

Highlights

- Abiotic and biotic factors modulate aggregation behavior in terrestrial isopods.
- Population fragmentation observed at temperature and soil moisture (lower and upper) extremes.
- Salinity and tested biotic factors induced disaggregation in a sigmoidal-shaped response curve.
- pH did not influence the aggregation behavior of terrestrial isopods

Abstract

Shifts in abiotic and biotic factors can alter the behavior of edaphic organisms and compromise the soil habitat function. This study explores the impact of temperature, soil moisture, salinity, pH, edaphic interference, oleic acid (necromone signals), and microbial community dilution on the aggregation behavior of *Porcellionides pruinosus* as a potential new indicator of population-level stress. Aggregation patterns were quantified using two novel ecological indices, the Disaggregation Index, which measures groups formed by a single individual or at most two individuals, and the Disaggregation in Group Index, which quantifies the total number of groups formed at the end of the experiments. The disaggregation indices range from 0 (highest level of aggregation) to 1 (highest level of disaggregation). A value of 0.5 has been set as the concern threshold, i.e. the point at which more than 50% of the population is fragmented. Results reveal that most environmental factors modulate aggregation. A U-shaped disaggregation was observed for temperature and soil moisture, with population fragmentation occurring at both extremes. In contrast, salinity and biotic factors caused disaggregation following a sigmoidal curve. Only pH did not influence the aggregation behavior; on the contrary, an aggregation trend was observed at the lower and upper limits tested, showing a bimodal distribution. The cluster analysis revealed three primary trends in the disaggregation response curves as a function of stress level: parabolic, sigmoidal and bimodal. Given the need for tools to assess soil health and monitor habitat function, these findings demonstrate that terrestrial isopods' aggregation behavior is a sensitive indicator of soil changes and can be used to detect and quantify environmental stress through measurable changes in degree of aggregation. Valuable insights on laboratory conditions are also produced to standardize this bioassay.

Keywords: behavior, terrestrial isopods, climate change, soil health, soil organisms

1 Introduction

Behavioral alterations in soil organisms, induced by abiotic and biotic stressors, are considered early indicators of shifts in soil ecological conditions (Coyle et al., 2017). Variations in soil moisture gradients (Chikoski et al., 2006; Aupic-Samain et al., 2021; Frankenstein et al., 2024), temperature (Römbke et al., 2011; Benbellil-Tafoughalt, 2015; Bahrndorff et al., 2021), pH levels (van Straalen & Verhoef, 1997), and salinity (Owojori et al., 2009; Pereira et al., 2015) represent the main abiotic drivers of behavior. Biotic pressures, such as interspecific competition or predation (Migge-Kleian et al., 2006; Cazzolla Gatti et al., 2020), as well as changes in soil microbial communities (A'Bear et al., 2014; Des Marteaux et al., 2020), can also significantly influence behavioral patterns in edaphic organisms. These changes affect the communication, mobility, feeding, fitness, and social behavior of soil fauna (De Vries et al., 2012; Boiteau & MacKinley, 2013; Morgado et al., 2015; McAfee et al., 2022; Trigos-Peral et al., 2024), with potential consequences for population dynamics, reproduction, distribution, and survival. At the same time, these responses are highly species-specific, depending on species tolerance and adaptive capacity, and the choice of appropriate model organisms must be justified in terms of their ecological relevance and functional role.

Among the non-target organisms, terrestrial isopods have been proposed as a suitable model organism for behavioral testing due to their ecological relevance, as they are detritivores of the litter and play a crucial role in biogeochemical cycles (Drobne, 1997; van Gestel et al., 2018). Due to their vagile and gregarious nature, terrestrial isopods can be used to assess the impact of soil stressors on population-level aggregation patterns, whose effects have proven to be a sensitive indicator of soil quality (Federico et al., 2024; 2026).

In this context, Federico et al. (2024) introduced the concept of disaggregation effect as a novel ecotoxicological endpoint to quantify the impact of soil contaminants on the aggregation behavior of *Porcellionides pruinosus*

Aggregation operates at the population level and provides individuals with several advantages, including protection from predators, improved access to food resources and enhanced opportunities for reproduction (Hornung, 2011). Conversely, alterations of gregarious behaviour indicate fragmentation of woodlouse populations, leading to potentially decreasing litter decomposition rates and increasing the risk of dehydration (Hassall et al., 2002; Hornung, 2011; Broly et al., 2014). The proposal of defined and replicable disaggregation indices also allows for better quantification and standardization of stress effects compared to measurements based solely on counting the number of individuals physically clustered together (Cividini and Montesanto, 2018; Ďurajková et al., 2022; Delhoumi et al., 2023). From a behavioural perspective, aggregation induces a calm and stable state in terrestrial isopods, modulated both by group size and the time individuals spend together (Broly and Deneubourg, 2015). Aggregation occurs rapidly, within approximately ten minutes, starting from a minimum of ten individuals and reaching a maximum of sixty to seventy, beyond which inter-individual competition arises and the benefits, such as reduced water loss, decline (Broly et al., 2016). Therefore, through a rigorous and standardized approach, As a result, disaggregation patterns represent useful indicators of soil quality and offer a rapid, cost-efficient screening approach (Federico et al., 2026). In light of the recognition of soil degradation as the tenth planetary boundary (Kraamwinkel et al., 2021), disaggregation endpoints represent relevant and non-conventional indicators for assessing soil ecological functions, aligning with the European Commission's Soil Monitoring Law (Panagos et al., 2025). This new ecological endpoint has so far been applied only to physical, such as tire particles, and chemical pollutants (Federico et al., 2024), while the effects of other abiotic and biotic soil stressors remain to be investigated.

This study aims to assess the sensitivity to disaggregation effects in the population of *P. pruinosus*, a cosmopolitan terrestrial isopod identified as a relevant model organism in ecotoxicological testing (Loureiro et al., 2005; van Gestel et al., 2018), which has previously been used to investigate responses to a range of abiotic and biotic stressors typical of soil environments (Morgado et al., 2018). Specifically, we selected temperature, soil moisture, salinity, and pH as main soil driver of ecological regime shifts (Rengel, 2011; Corwin, 2021), while interspecific interferers with invertebrate with *Tenebrio molitor* (mealworms) and *Acheta domesticus* (crickets), exposure to oleic acid to simulate necromone signaling from conspecifics (i.e., signals associated with dead conspecifics), and varying levels of soil microbial community diversity, were selected as main and potential biotic factors involved in disaggregation effects.

These factors were selected and analyzed to address the following knowledge gaps:

- Which of these stressors induces disaggregation in terrestrial isopods?
- What are the threshold levels at which these effects occur?
- Can these environmental stressors be clustered according to their relative impact?

2 Materials and methods

2.1 Model organisms and standard soils

Individuals of *Porcellionides pruinosus* Brandt (1833) were derived from long-established, stable cultures maintained in the Laboratory of Applied Ecology and Ecotoxicology, CESAM, at the University of Aveiro (Portugal). The culture was maintained under controlled conditions (20°C temperature, 16:8 h light-dark photoperiod), sprayed with distilled water twice a week, and individuals were fed *ad libitum* with alder leaves. According to previous works (Loureiro et al., 2005; Morgado et al., 2015), during the tests, only adults with a wet weight of 14-30 mg, without sex distinction, were used, while molting animals, individuals with malformations, and females with litter in *marsupium* were excluded. Individuals were not re-used during the experiments.

The LUFA 2.2 sandy loam soil (Speyer, Germany, batch SP2.2 2123) was used in the main experiments as a standard soil. The properties of this soil include a pH = 5.5 ± 0.2 (0.01 M $CaCl_2$), Water Holding Capacity (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. To assess the effect of disaggregation induced by different pH levels, artificial soil was prepared according to OECD Guideline 207 (1984), using different percentages of ground sphagnum peat, kaolin clay, and industrial fine sand by dry weight (d.w.), and adjusting the pH with calcium carbonate.

2.2 Experimental set up

The assessment of disaggregation effects induced by several environmental stressors on individuals of *P. pruinosus* followed the same protocol reported in previous studies (Federico et al., 2024). In rectangular plastic boxes (9.5 cm × 7.3 cm × 5 cm), 50 g d.w. of standard soils (LUFA 2.2 or OECD) were added and moistened with ultrapure water until achieving 40% of WHC (at least for the control, in case of soil moisture tests). Both control and treated soils were slightly flattened to eliminate clumps, thereby reducing the thigmotaxy of terrestrial isopods (i.e., the tendency to seek physical contact with surfaces or other individuals) and encouraging them to aggregate only with conspecifics. Ten individuals of *P. pruinosus* were added to each replicate box, covered with a plastic lid. Five replicates per treatment were used for statistical analysis. The experiments were performed in thermostatic chambers at 20°C (except when temperature was the study variable and different temperature treatments were assessed), photoperiod of 16:8 h light-dark for 48 h. Organisms were never fed during the tests in order to avoid aggregation induced by food attraction. At the end of the test, each arena was gently removed from the thermostatic chamber and placed on an adjacent laboratory bench. A camera (Trust Teza 4K Ultra HD webcam, 3024 × 4032 pixels) was positioned above the arena at a fixed distance of 15 cm from the focal point, and three high-resolution color images were recorded at 5-minute intervals (without flash) for subsequent analysis of aggregation patterns.

2.3 Abiotic effect tests

Temperature experiments were conducted over a range that simulated temperate/Mediterranean climates (Morgado et al., 2015). The temperature levels selected were 5, 10, 15, 20, 25, 30, and 35°C (±1 °C). A temperature of 20°C was considered the control temperature, similar to the temperature conditions of laboratory cultures. The degree of temperature was set by increasing or decreasing the thermostatic chambers settings.

The effect of different soil moisture contents was studied covering a range that simulated the dry/wet regime conditions of soil. The moisture levels were based on WHC levels and were 10, 20, 30, 40, 60, 70, and 80% WHC_{max}. The negative control was set as 40% WHC according to reported soil toxicity testing procedures with this species (Loureiro et al., 2005). Distilled water was added to dry LUFA soil in order to achieve the different selected levels of WHC % calculated by the WHC_{max} % reported on the sheet of physical and chemical properties of the LUFA 2.2 standard soil. Any potential loss of soil moisture during the 48-hour exposure period was considered negligible in line with observations reported in previous studies (Morgado et al., 2015; González-Alcaraz et al., 2019), and was therefore not specifically monitored.

The effect of soil salinity was evaluated using NaCl as a reference substance since it represents the predominant salt in most saline environments (Owojori et al., 2008) and it is the principal additive for salting roads during winter in temperate and subarctic regions (Škarková et al., 2016). The concentrations of NaCl selected were 2.5, 3, 3.5, 4, 4.5, and 5 g/kg d.w., based on literature (Owojori and Reinecke, 2009; Škarková et al., 2016). Being a euryhaline species with extreme osmoregulatory capabilities (Wright et al., 1997), these concentrations have been considered sublethal for terrestrial isopods. NaCl was added as an aqueous solution into the LUFA 2.2 standard soil by preparing a stock solution of 46.7 mM/L in ultrapure water.

Effects on aggregation behavior induced by different soil pH values were assessed using the standard OECD soil (1984) as reference substrate, by adjusting the relative percentage dry-weight of sphagnum peat, kaolin, sand, and calcium carbonate (Table 1). All the different pH OECD soils were incubated in the thermostatic room at 20°C in the dark for two weeks before the final test. At the end of the incubation, aliquots from each soil mixture were used for the quantification of the pH using a solution of $CaCl_2$ 0.01 M (v/v %) following the OECD guideline (1984). The final pH values obtained were 4.06, 4.43, 5.15, 6.07, 7.22, 8.05, and 8.13 (± 0.1). Since the OECD soil at pH 6.07 was obtained following the standard percentages of sand, kaolin, and peat sphagnum recommended by the guidelines (OECD, 1984), it was used as the negative control soil. Moreover, the WHC_{max} % was measured for each OECD soil according to the OECD standard guidelines (OECD, 2009). The electrical conductivity and pH of the different soils, varying in salinity and pH, were analyzed at the beginning and at the end of the tests using a conductometer (WTW 3110/set meter) and a pH meter (WTW-pH 330i/set meter).

2.4 Biotic effect tests

To investigate biotic interference on aggregation behavior, we selected *Tenebrio molitor* (larvae) and *Acheta domesticus* (adults). These are model organisms widely used in ecotoxicological testing, highly reliant on chemical communication (infochemicals), and employed in this study as experimental proxies to investigate interspecific interactions among topsoil fauna (Ribeiro et al., 2018). These species may also exploit similar microhabitats to *P. pruinosus*, including moist organic layers where shelter and microbial resources are spatially limited, making them relevant potential competitors (Ghouri, 1961; Ribeiro et al., 2018). Additionally, both species are easy to keep in laboratory cultures and therefore available to use. Given that disaggregation in terrestrial isopods has been linked to the disruption of infochemical cues (Federico et al., 2024), the inclusion of these invertebrates provides an ecologically meaningful framework for examining how naturally occurring or management-mediated faunal interactions may influence social aggregation processes. Thus, their use allows us to explore biotic interference under controlled conditions while maintaining ecological realism and relevance to soil functioning.

Both species were cultured at the Laboratory of Applied Ecology and Ecotoxicology, CESAM, at the University of Aveiro (Portugal). The tests consisted of exposing terrestrial isopods to soils in which the invertebrates had been previously incubated for varying periods of time. In 50 g d.w. of standard LUFA 2.2 soil and moistened up to 40% WHC, one individual of *T. molitor* or one individual of *A. domesticus* were added separately in rectangular plastic boxes, maintaining five replicates per treatment, for different time intervals: 3 min, 7 min, 15 min, 30 min, 24 h, and 48 h (no food; 20°C temperature; photoperiod of 16:8 h light-dark). At the end of the exposure time intervals, animals were removed, and the soils were dehydrated to achieve 40% WHC. Then, ten individuals of *P. pruinosus* were added to each treatment and exposed to the soil previously incubated with invertebrates.

Oleic acid ($C_{18}H_{34}O_2$, CAS: 112-80-1, 99% purity, Sigma-Aldrich Chemical Co. and Merck) was chosen as a necromone reference substance as it has been used to simulate the presence of necromonic signals associated with predators or dead conspecifics (Yao et al., 2009; Tuf and Ďurajková, 2022). The stock solution of oleic acid at a concentration of 10 mg/mL was obtained by dissolving the liquid substance in 1.5 mL of dimethyl sulfoxide (DMSO, $(CH_3)_2SO$, CAS: 67-68-5, 99% purity, Sigma-Aldrich Chemical Co. and Merck). Subsequently, serial dilutions were carried out in ultrapure water to obtain the following increasing nominal concentrations: 0, 46.9, 93.7, 187.5, 375, 750, and 1,500 mg/kg d.w. Consequently, each serial dilution was added to 50 g d.w. of standard LUFA 2.2 soil to achieve the nominal concentrations. Solvent controls were performed using the same volume of DMSO as the highest concentration tested.

The effects of different levels of microbial community on aggregation behavior were achieved by the Dilution-to-Extinction (DtE) method (Díaz-García et al., 2021). LUFA 2.2 soils (500 g wet weight) were placed into sterilized glass vessels and autoclaved at 121 °C for 20 minutes to sterilize the soil microbial community. Sterilized soils were used to carry out 10-fold serial dilutions with non-sterile LUFA 2.2 soil, reaching a final volume of 500 g of soil (Table SM1). The final dilutions, expressed as the weight/weight (%) ratio of sterile to non-sterile soil, were 10^{-7}, 10^{-6}, 10^{-5}, 10^{-4}, 10^{-3}, 10^{-2}, and 10^{-1}. The dilution of 10^{-7} % was carried out by exposing terrestrial isopods to sterile soil, while the undiluted fresh soil was considered as a negative control (10^{0} %). The disaggregation tests on sterilized soils were performed using previously purged (organism cleared of their gut contents before the test) and non-purged isopods (organism not cleared of their gut contents before the test), following the procedure in Loureiro et al. (2006). Terrestrial isopods were purged to minimize contamination from intestinal microbes, while the non-purged individuals had not received this treatment. Soils were incubated for two weeks in thermostatic rooms (20°C, in the dark, sealed) (de Souza et al., 2017; de Souza et al., 2021). The sterilization process was confirmed by plating the soil bacterial and fungal communities in agar plates with general culture media. For this, 5 g of soil was suspended in 25 mL of sterile water, shaken at 200 rpm for 20 minutes, and then allowed to settle for 10 minutes. Then, 100 µl of that suspension were plated in culture media Tryptic Soy Agar (TSA, for bacterial community) and Potato Dextrose Agar (PDA, for fungal community), incubated for 48 h at 20ºC. After incubation, the sterilization process was validated, as the autoclaved soil samples showed no bacterial or fungal colonies on the culture plates. After two weeks of incubation, the soil was characterized by measuring WHC (OECD, 2009), pH (OECD, 1984), and conductivity. At the end of the test, the soil pH and conductivity were reassessed.

2.5 Data analysis

The disaggregation effects were measured by two ecological indices (Federico et al., 2024), the disaggregation index (DI) and the disaggregation in groups index (DG):

$$\mathrm{DI} = \frac{s+2d}{n}\ ;\ \mathrm{DG} = \frac{g}{n} \quad \text{(Eq.1)}$$

Where *s* represents the group formed by one individual, *d* the group formed by two individuals, *g* the total number of groups recorded at the end of the experiments, and *n* the number of living individuals after 48 h. For the measurement of these indices, the number of clusters formed at the end of the experiment was counted, where a “cluster” consisted of a group of individuals who touched each other with at least one part of their body. Nearby individuals, but without interaction, weren’t considered as part of the same clusters. These indices vary between 0 and 1, which represent the maximum degree of aggregation and disaggregation, respectively. Conventionally, a value of 0.5 was imposed as the threshold level above which the disaggregation affects more than 50% of the terrestrial isopods population. For the validation of the test, the mortality rate in controls should not exceed 10%, and the values of DI and DG should be lower than or equal to 0.2 (Federico et al., 2024).

From an ecological perspective, DI quantifies population fragmentation by measuring the proportion of individuals occurring as singlets or doublets, which represent the smallest and most vulnerable social units. Singlets indicate maximum fragmentation and potential isolation from mating and social benefits, whereas doublets represent the minimal condition under which reproduction may still occur. In this index, groups composed of more than two individuals are intentionally not considered, as DI was specifically designed to capture fragmentation through the smallest social units. DG measures the overall granularity of the population by relating the total number of clusters to the number of individuals. Together, these indices capture complementary aspects of aggregation behavior, allowing the detection of ecological stressors that disrupt social clustering and increase population fragmentation. Importantly, the interpretation of DI and DG does not assume that isopods under ideal conditions form a single large cluster. Terrestrial isopods naturally exhibit a dynamic balance between aggregation and dispersion, and multiple clusters may occur even in the absence of stressors. Rather than defining an absolute “optimal” configuration, the indices are intended to detect relative increases in fragmentation, reflected by a higher proportion of singlets or very small clusters, compared with the baseline spatial organization observed in control conditions. Accordingly, the validity criterion of 0.2 (i.e. at least 80% of aggregated population) was introduced to allow some flexibility in evaluating the gregariousness of the control population, acknowledging that individuals may distribute across several clusters while still maintaining a socially aggregated structure.

Indices data were tested for normality using the Shapiro-Wilk test and for homogeneity of variances using Levene’s test, while the presence of heteroscedasticity was evaluated using the Breusch–Pagan test to ensure the reliability of variance assumptions.

Generalized Additive Models (GAMs) were applied to DI and DG indices to allow modeling non-linear relationships by fitting a smooth spline function (k = 5) with a Gaussian family and identity link. Alternative model fits were assessed and compared using adjusted R^2, deviance explained, and Akaike's information criterion (AIC). Predicted responses with 95% confidence intervals were generated to visualize factors-dependent trends in DI and DG. Thin-plate regression splines and restricted maximum likelihood (REML) were used to fit the models. Models were compared using an F-test of their fits, and the alternative model was accepted when $p \leq 0.1$.

Multivariate analysis was conducted using a partitional clustering approach based on the K-means algorithm, following z-score normalization of the data to ensure comparability between variables. This method partitions the dataset into *k* non-overlapping clusters by minimizing within-cluster variance and maximizing between-cluster variance, based on Euclidean distances to cluster centroids (Jain, 2010). K-means clustering was applied to identify and group similar patterns of disaggregation behavior in *P. pruinosus* based on the relative magnitude of the impact of the tested abiotic and biotic factors. Partitional clustering was chosen over hierarchical methods due to its computational efficiency, scalability, and ability to define a set number of clusters, which aligns with the study's goal of quantitatively categorizing behavioral responses. K-means also provides clearer cluster boundaries, aiding ecological interpretation. The optimal number of clusters was determined using the Silhouette Index, with higher index values indicating greater cluster cohesion and separation (Rousseeuw, 1987). Prior to clustering, potential outliers were identified and removed to minimize distortion in cluster formation (Legendre & Legendre, 1998). To assess statistical differences among clusters, analysis of variance (ANOVA) was performed, ensuring that the assumptions outlined above were met. The data were considered statistically significant for values of $p < 0.05$. All the statistical analysis was performed using R v. 4.5.2 package (R Core Team, 2024).

3 Results

Validity criteria were met in all control tests, i.e. less than 10% of mortality and at least more than 80% of the terrestrial isopods aggregated. The majority of the treatment test experienced less than 10% of mortality, therefore the disaggregation tests were considered sublethal. However, temperature tests at 5°C and soil moisture tests at 80% of WHC met more than 50% of mortality (Table 2), therefore these point data were excluded from the statistical analysis being lethal.

3.1 Population responses induced by abiotic factors

The aggregation behavior of *P. pruinosus* was modulated by all tested abiotic factors, although the magnitude of the response varied among them (Fig. 1). The disaggregation degrees are provided in the supplementary materials (Table SM2).

Fig. 1 Disaggregation indices (DI and DG) of *Porcellionides pruinosus* exposed to different abiotic factors. GAM-predicted curves of DI (left) and DG (right) are shown for (a) temperature (°C), (b) soil moisture (%), (c) NaCl concentration (g/kg d.w.), and (d) pH. Points represent observed values; dashed black lines and dashed blue lines indicate GAM-predicted means for DG and DI, respectively. Grey and light blue shaded areas denote 95% confidence intervals for DG and DI, respectively. Index values below 0.5 indicate that more than 50% of the population maintains aggregation.

Temperature altered the aggregation behavior of terrestrial isopods following a parabolic response curve (Fig. 1a), with indices peaking at low (<15°C) and high (>25°C) temperatures and reaching a minimum around 20°C, indicating a non-monotonic response to thermal variation. For DI, the GAM results showed a significant non – linear of temperature ($F_{2.879} = 4.086$, $p = 0.0124$), with an intercept of 0.347 ± 0.046 ($p < 0.001$). The adjusted R^2 was 0.322, and the deviance explained was 39.1%, with an AIC of 7.13. For DG, temperature also had a strong effect ($F_{3.146} = 5.027$, $p = 0.00411$), with an intercept of 0.423 ± 0.037 ($p < 0.001$). The adjusted R^2 was 0.391, deviance explained 45.9%, and AIC −5.16. The DG curve similarly showed a paraboloid-like trend, with elevated values at both temperature extremes and a trough around 20°C, reflecting a sensitive, non-linear thermal response. For each index, CI (95%) were broader, reflecting greater uncertainty in predictions at extreme temperatures. Due to high mortality observed at 5°C, disaggregation indices could not be evaluated at this temperature, which was excluded from the GAM analysis. DI and DG values less than 0.2 were observed in a temperature range between 15 and 20°C, indicating a preference for aggregation of the population within this range.

Like temperature, soil moisture altered isopods' aggregation following a parabolic response curve, affecting more at the higher than at lower levels (Fig. 1b). For DI, the GAM showed significant non – linear effects of soil moisture ($F_{3.226} = 7.061$; $p = 0.00104$), with an intercept of 0.407 ± 0.0343 ($p < 0.001$). The model explained a substantial portion of variability (adjusted $R^2 = 0.45$; deviance explained = 51.1%) and exhibited good parsimony (AIC = – 9.24). The same significance was observed for DG ($F_{3.352} = 8.807$; $p = 0.000232$), with an intercept of 0.461 ± 0.0254 ($p < 0.001$), the model explaining 51.5% of the variability (adjusted $R^2 = 0.515$; deviance explained = 57.1%; AIC = −27.29). Predicted values and 95% CI were calculated across the observed soil moisture range (10–70%) to assess prediction uncertainty. The mortality rate of terrestrial isopods exposed at 80% of soil moisture has reached almost 50%. Consequently, this moisture level was deemed to be beyond sublethal and was excluded from GAM analyses. DI and DG values less than 0.2 (aggregation pattern) were shown at 40% of WHC.

The disaggregation pattern observed in NaCl followed a sigmoidal-shaped dose-response model (Fig. 1c). Regarding DI, GAM analysis revealed no significant effects ($F_{1.664} = 2.882$; $p = 0.0767$), with the intercept of 0.419 ± 0.0448 ($p < 0.001$) indicating only a weak non-linear trend. The model explained a modest portion of the variability (adjusted $R^2 = 0.121$; deviance explained = 16.4%) and had an AIC of 10.90. The predicted DI curve suggested a slight increase or decrease along the NaCl gradient, but the effect was not statistically robust, consistent with the good tolerance of *P. pruinosus* to soil salinity. Compared to DI, GAM results revealed significant non-linear effects on DG index ($F_{1.946} = 4.519$; $p = 0.0164$), with the intercept of 0.469 ± 0.0287 ($p < 0.001$). The model explained a higher proportion of variability (adjusted $R^2 = 0.22$; deviance explained = 26.6%) and exhibited better parsimony (AIC = −20.04). The predicted DG curve showed a clear trend along the NaCl gradient, with behavioral maxima and minima reflecting a non-linear response to salinity, although deviance explained remained moderate, suggesting additional factors contribute to DG. The results seem to suggest that, despite NaCl having no effects on disaggregation in singlets or doublets, they may still contribute to a fragmentation in different groups. During the test, no mortality was detected at any of the tested concentrations. The soil EC and pH values (Figure SM3) increased significantly after 48h compared to the beginning of the test (One Sample paired t-Test, $p\_EC_i = 0.00184$; $p\_EC_f = 0.00431$; $p\_pH_i < 0.0001$; $p\_pH_f < 0.0001$). However, there were no significant correlations between the increase of EC (Adj. $R^2 = 0.235$ for DI and Adj. $R^2 = 0.0235$ for DG) or pH (Adj. $R^2 = -0.0157$ for DI and Adj. $R^2 = -0.123$ for DG) and the levels of DI and DG quantified in the salinization bioassay. The observed low correlation may be due to the fact that the NaCl concentrations used in this study fall within a realistic salinity range to which these organisms are already adapted.

No disaggregation effects in terrestrial isopods were detected in the pH tests (Fig. 1d). For DI, the smooth term of GAM was not statistically significant ($F_{1.902} = 1.358$; $p = 0.256$), with the intercept of 0.375 ± 0.041 ($p < 0.001$). The model explained a small portion of variability (adjusted $R^2 = 0.0702$; deviance explained = 12.2%; AIC = 4.812). For DG, the GAM revealed similar non-significances ($F_{1.863} = 1.474$; $p = 0.252$), with the intercept of 0.433 ± 0.036 ($p < 0.001$), showing the same limited variability (adjusted $R^2 = 0.0689$; deviance explained = 12%; AIC = −4.235). Despite not being statistically robust, the predicted DI and DG curves exhibited a bell-shaped pattern of disaggregation (bimodal aggregation pattern), with slightly higher values at intermediate pH and lower values at extreme low and high pH levels. Compared to LUFA soil controls, OECD soil controls showed disaggregation indices slightly above 20%, highlighting that the gregariousness of controls depends on the type of soil used, whether artificial or natural. The pH values (Figure SM4) increased significantly after 48h compared to the beginning of the test (p_pHi < 0.0001; p_pHf < 0.0001, – One Sample paired t-Test). However, there were no significant correlations between the increase in pH and the levels of DI and DG calculated (Adj. $R^2 = -0.181$ for DI and Adj. $R^2 = -0.131$ for DG).

3.2 Population responses induced by biotic factors

All biotic factors modulated the aggregation behavior of *P. pruinosus* individuals, following a sigmoidal dose-response model (Fig. 2). The values of the indices are reported in Table SM2.

Fig. 2 Disaggregation indices (DI and DG) of *Porcellionides pruinosus* exposed to different biotic factors. GAM-predicted curves are shown for (a) *Acheta domesticus* (minutes of exposure, expressed as ln(x+1)), (b) *Tenebrio molitor* (minutes of exposure, expressed as ln(x+1)), (c) oleic acid concentration (mg/kg d.w.), and (d) different microbial community diversity of LUFA 2.2 soil (% w/w). Points represent observed values; dashed black lines and dashed blue lines indicate GAM-predicted means for DG and DI, respectively. Grey and light blue shaded areas denote 95% confidence intervals for DG and DI, respectively. Index values below 0.5 indicate that more than 50% of the population maintains aggregation.

The interference of *A. domesticus* exposure showed a positive, exposure-dependent trend (Fig. 2a). For DI, the model intercept was 0.247 ± 0.055 (t = 4.501, $p = 7.96 \times 10^{-5}$), representing the estimated mean disaggregation at zero exposure. The slope for log-transformed exposure was 0.0265 ± 0.0122 (t = 2.177, $p = 0.0367$), indicating a statistically significant increase in DI with exposure. The model explained 9.91% of the variance (adjusted R^2) with 12.6% deviance explained and an AIC = −9.65. Predicted values and 95% CI revealed a gradual increase in DI across the exposure gradient, with wider CI at higher exposure due to sparser data. Similarly, DG increased with exposure, with an intercept of 0.327 ± 0.037 (t = 8.890, $p = 2.83 \times 10^{-10}$) and a slope of 0.0234 ± 0.0082 (t = 2.863, $p = 0.00723$). The model accounted for 17.5% of the variance (adjusted R^2), with 19.9% deviance explained and an AIC = −37.71, indicating a slightly better fit than for DI. The predicted DG curve followed a similar trend, confirming a significant, albeit moderate, exposure effect.

Like crickets, the interference with *T. molitor* displayed a slight positive trend in disaggregation indices with increasing exposure (Fig. 2b). For DI, the intercept was 0.257 ± 0.064 (t = 4.009, $p = 3.28 \times 10^{-4}$), representing the estimated mean DI at zero exposure. The slope for log-transformed exposure was 0.0237 ± 0.0142 (t = 1.670, $p = 0.104$), indicating a positive but statistically non-significant effect. The model accounted for 5% of the variance (adjusted $R^2 = 0.050$) and explained 7.8% of the deviance, with an AIC = 1.067. Predicted values and 95% CI suggest a gradual increase in DI across the exposure gradient, with wider CI at higher exposure values reflecting greater uncertainty. Similarly, DG increased with exposure, with an intercept of 0.355 ± 0.049 (t = 7.169, $p = 3.25 \times 10^{-8}$) and a slope of 0.0187 ± 0.011 (t = 1.705, $p = 0.0977$). The model explained 5.31% of variance (adjusted R^2) and 8.1% of deviance, with an AIC = −16.993, indicating a slightly better model fit than for DI. Predicted DG curves mirrored those of DI, showing a positive trend with exposure, although the effect was marginally non-significant.

Both DI and DG displayed significant non-linear responses to oleic acid (Fig. 2c). For DI, the model intercept was 0.427 ± 0.031 (t = 13.69, $p = 3.7 \times 10^{-15}$), representing the baseline DI at minimal oleic acid. The smooth term for oleic acid showed significant disaggregation effects (F = 12.56, $p = 0.0012$). The model explained 25.4% of variance (adjusted $R^2 = 0.254$) and 27.6% of the deviance, with an AIC = −15.079. Predicted DI curves increased with oleic acid concentration, with 95% confidence intervals illustrating moderate uncertainty at higher concentrations. Similarly, DG exhibited a comparable response, with an intercept of 0.490 ± 0.026 ($t = 19.2$, $p < 2 \times 10^{-16}$) and high statistical significance ($F = 12.44$, $p = 0.00126$). The model explained 25.2% of variance (adjusted $R^2 = 0.252$) and 27.4% of deviance, with an AIC = −29.034.

Alterations were detected under the distinct microbial community diversity already at 10-fold dilution of the microbial community (Fig. 2d). For DI, the GAM model intercept was 0.529 ± 0.047 ($t = 11.174$, $p = 2.68 \times 10^{-14}$), with a slope was −1.252 ± 0.467 ($t = -2.679$, $p = 0.0104$), indicating a significant effect of soil microbial dilution on DI. The model explained 12.3% of variance (adjusted $R^2 = 0.123$) and 14.3% of deviance, with an AIC = 21.37. Similarly, DG increase with decrease in microbial diversity, with an intercept of 0.574 ± 0.042 ($t = 13.540$, $p < 2 \times 10^{-16}$), and a slope of −1.073 ± 0.418 ($t = -2.566$, $p = 0.0139$). The model explained 11.3% of variance (adjusted $R^2 = 0.113$) and 13.3% of deviance, with an AIC = 11.37. Considering the soil microbial community diversity level, the soil conductivity and pH values (Figure SM5) also significantly increased and decreased, respectively, after 48h compared to the beginning of the test (p_EC and $p\_pH < 0.0001$ – One Sample paired t-test). However, there were no significant correlations between the increasing EC or pH and the increasing levels of DI and DG quantified at the end of the bioassay.

3.3 Multivariate analysis

The final clusters obtained from the K-mean method are illustrated in Fig. 3.

Fig. 3 K-mean cluster analysis related to the effect of the abiotic and biotic factors tested on the disaggregation effects in individuals of *P. pruinosus*. Each data point is represented by coordinates (x, y) of the Euclidean distances from the respective cluster centers. Cluster 1 includes observations related to pH; Cluster 2 groups observations associated with NaCl, *Acheta domesticus*, *Tenebrio molitor*, oleic acid, and microbial community diversity (MCD); Cluster 3 includes observations related to temperature and soil moisture.

The optimal clustering number was $k = 3$ (Table SM6), grouping 17 to 23 observations per cluster, whose inter-cluster Euclidean distances ranged from 0.60 to 0.71 (Table 2). The mean silhouette index was positive for all clusters, indicating some degree of overlapping. The highest Euclidean distance was found between Cluster 3, which primarily assembled temperature and soil moisture data, and Cluster 1, which mainly consisted of soil pH data, while biotic factors and salinity data were mostly separated in Cluster 2 (Table 3). The ANOVA output of K-mean cluster analysis and relative F values (Table 4) shows that all tested abiotic and biotic stressors significantly affected *P. pruinosus* aggregation behavior ($p < 0.05$), confirming its high sensitivity to all the environmental disturbances.

The final partitioned cluster obtained by the K-means algorithm is consistent with the different trends in disaggregation effect observed for each environmental factor (paraboloid for temperature and soil moisture, sigmoidal shape for NaCl and biotic factors, and bimodal for pH). This partitioned analysis, therefore, contributed to visualizing and distinguishing the different disaggregation effects induced by different environmental factors, showing how cluster analysis in the study of stress ecology can contribute to understanding and comparing the effects induced by non-collinear variables.

4 Discussion

4.1 Parabolic disaggregation effects: temperature and soil moisture

Temperature and soil moisture affected aggregation in terrestrial isopods at the lower and upper limits of the tested range, showing a parabolic trend complementary to the right-shifted curves of stressors' responses in ectotherms (Huey and Berrigan, 2001). These findings suggest that the thresholds affecting aggregation and survival are aligned, reinforcing the ecological relevance of the disaggregation effect. Indeed, gregariousness (homeostatic) was observed for temperatures and soil moisture range between 15 - 20°C and 40% WHC, respectively. Such aggregation behavior reflects the temperatures that may occur of temperate or Mediterranean regions during summer nighttime hours (Morgado et al., 2015), when *P. pruinosus* perform their main activities (Hornung, 2011), as well as the moisture level required for their xerophilic nature (Quinlan & Hadley, 1983). These conditions, therefore, represent ecological optimum levels that should be maintained when testing these model organisms. In contrast, ranges outside this optimum caused terrestrial isopods to fragment, suggesting niche conformity (Müller et al., 2020).

Sensitivity to the two factors, however, was differentially shifted towards lower and upper levels, respectively. Specifically, individuals of *P. pruinosus* showed good tolerance at higher levels of temperature, in line with the tolerance range observed elsewhere (Edney, 1964), while temperatures close to 5°C induced high mortality likely due to a physiological limit condition of individuals and the absence of shelter, like rocks or food, to protect themselves. Despite their tolerance to high temperatures, terrestrial isopods did not maintain their gregarious behavior. This alteration suggests that endpoints assessed at the population level may highlight ecological risks that studies conducted on individual organisms tend to underestimate. It is hypothesized that extreme temperatures compromise isopod aggregation by reducing mobility, with high heat potentially causing numbness similar to cold coma (Morgado et al., 2015; Castañeda et al., 2004). As temperature anomalies increase worldwide, particularly in Europe, with average temperatures rising by 2-3°C compared to pre-industrial levels (García-García et al., 2023), the aggregation behavior of terrestrial isopods may be affected once species-specific tolerance thresholds are exceeded. Although some species can thrive under slightly warmer conditions, as observed along forest edge-to-interior gradients (De Smedt et al., 2016; De Smedt et al., 2018), higher temperature anomalies may disrupt aggregation patterns. Monitoring this behavioral endpoint could therefore help identify and prioritize soils that are increasingly exposed to temperature rise.

Regarding soil moisture, terrestrial isopods survival was compromised at higher moisture levels, due to their air-adapted pseudotracheae and an open "*Porcellio*-type" water-conducting system (WCS) (Spencer & Edney, 1954; Wright & Machin, 1990), making excess water potentially harmful to both respiration and WCS function. On the contrary, tolerance to low moisture levels depends on the fact that the tissues of terrestrial isopods draw on hemolymph as a source of water for cell volume regulation during periods of dehydration, showing osmoregulation during desiccation (Wright et al., 1997). Despite their tolerance, aggregation was affected even under dry soil conditions, likely due to reduced locomotion. Since climate change is expected to cause desertification in over 10–20% of drylands (Diallo, 2008), combined with extreme events like floods that cause sudden changes in soil moisture, this may threaten terrestrial isopod populations that are not tolerant of dry conditions.

4.2 Sigmoidal disaggregation effects: soil salinity and biotic factors

Even when exposed to NaCl and all the biotic factors tested, terrestrial isopods underwent niche aggregation conformity, following a sigmoidal-shaped model.

Despite the euryhalinity of *P. pruinosus*, supported by hemolymph osmoregulation through sodium and chloride accumulation in the hindgut and hepatopancreas (Wright et al., 1997), 50% of the population in sodic soils was fragmented. This highlights the importance of assessing population level, which may be underestimated when focusing on individual tolerance. Salinization endangers population stability by accelerating molting and increasing evapotranspiration. Given that soil salinization affects between 25% and 30% of irrigated land (Shaid et al., 2018), it is expected that gregariousness may be compromised in real environments. Although a complete insight into the effects of salinization on soil organisms cannot be reduced to NaCl, these preliminary studies should be used as a proxy for understanding the impact of this threat on edaphic populations and ecosystem functionality. These changes in aggregation may have direct consequences for individual fitness, as gregariousness can influence survival and reproduction under stressful conditions. Therefore, understanding the link between social behavior and fitness is crucial for interpreting population-level responses to salinization.

The results did not show alteration in aggregation patterns caused by the invertebrate interference. The transient behavioral effect induced by *A. domesticus* and *T. molitor* interference may be a result of sympatric interactions typical of cryptic environments, such as soil (Zimmer, 2003). Despite their synanthropic nature, these species exhibit divergent ecological requirements. *T. molitor* and *A. domesticus* have been shown to prefer humid conditions and high temperatures, respectively (Ribeiro et al., 2018; Kulessa et al., 2024), but these conditions are not suited to the gregarious behavior of *P. pruinosus*. Therefore, these experiments should only be considered as proof of concept for understanding the interference effects between different organisms in the topsoil. However, despite the ecological differences between the tested species, the behavioral response of *P. pruinosus* remains consistent, suggesting that the observed disaggregation phenomenon does not depend on the specific traits of the interfering species, but may represent a general response to the presence of heterospecifics in the soil. Future studies may explore these interactions in more detail. For example, these experiments have not exceeded n = 1 individual per treatment, and marked effects may be highlighted if the number of pre-exposure individuals increases.

Oleic acid has been shown to be a necromone, altering the gregarious behavior of terrestrial isopods. It acts as a repellent across phylogenetically distant taxa, such as ants (Lopez-Riquelme et al., 2006), bees (McAfee et al., 2018), cockroaches (Rollo et al., 1995), and terrestrial isopods (Yao et al., 2009). This suggests that there is a conserved evolutionary mechanism for fatty acid recognition as cues of predation or dead conspecifics. For this reason, oleic acid has been authorized in the European Union as an active ingredient in pesticides (EC, 2011). To date, the European Commission has decided not to renew its approval, leading to its withdrawal by 15 December 2024 (EC, 2024). Nevertheless, the removal of oleic acid products will require a significant time period, as Member States may grant transitional periods for the sale and use of remaining stocks (EC, 2009). Therefore, the observed effects on gregariousness provide evidence that may support the decision to withdraw oleic acid from the EU market, indicating a potential impact on non-target organisms.

Even a 10-fold dilution of the microbial community ultimately compromised the sociability of terrestrial isopods, with peaks of significance for sterile soils. Various stressors can reduce the abundance and diversity of soil microbial communities, e.g., antibiotics and pharmaceutical residues. Consequently, based on our results, it is expected that such stressors may affect gregarious behavior. The mechanisms linking these levels of disaggregation to soil microbial diversity remain unclear. However, it is well established that soil microbial communities come into contact with terrestrial isopods through the ingestion of plant litter or coprophagy of fecal pellets from conspecifics (Zimmer et al., 2003). The acquisition of certain microorganisms, especially those involved in lignocellulose degradation, may have played an important evolutionary role in the terrestrial colonization of isopods (Bredon et al., 2019; Delhoumi et al., 2020). It is expected that the dilution of the soil bacterial community may reduce the load of environmental microorganisms being ingested through the diet or alter the gastrointestinal microbial community composition. It cannot be excluded that active dispersion of isopods in sterile environments is induced precisely by the need of these organisms to seek environments more favorable for acquiring beneficial microbial communities. In this context, the "Dilution to Extinction" approach can represent a practical and ecologically relevant method to understand how such an alteration may influence the aggregation behavior in the *P. pruinosus* population. By gradually reducing microbial abundance, this method shows how the decrease in microbially derived chemical cues may affect group formation and the species' social interactions.

4.3 Bimodal aggregation patterns: soil pH

Tests conducted on different pH ranges found that these variations did not alter the gregarious behavior of terrestrial isopods. On the contrary, aggregation pattern was observed at both extremes of the pH range tested compared to the controls, indicating a case of niche regulation (Müller et al., 2020). It is known that variation in soil pH can affect the fitness and survival of soil invertebrates (van Straalen & Verhoef, 1997), but terrestrial isopods are fairly resistant to such changes. For example, some terrestrial isopod populations have been found in acidic habitats such as peat bogs (Brigić et al., 2017), and the pronounced aggregation observed in 100% sphagnum conditions may reflect the higher availability of organic matter, supporting social cohesion. Even in the absence of organic matter, as in 100% carbonate tests, terrestrial isopods regulated their sociality through the presence of fecal pellets on the surface. These pellets serve both as chemical cues that help individuals locate conspecifics and maintain aggregation, and as a source of organic matter, since isopods are coprophagous and utilize them as food. These act as an organic input in nutrient-poor soils, increasing contact between conspecifics and promoting the formation of microhabitats conducive to the development of the soil community. In both extreme conditions, enhanced gregarious behavior may represent an adaptive strategy to maintain physical fitness and reproductive success under environmental stress, highlighting the ecological value of disaggregation endpoints for assessing soil organism responses to abiotic disturbances.

As part of the development of the novel methodological approach, a comparison with LUFA soil controls yielded important results. OECD soil exhibited lower aggregation values, and this effect primarily depends on the lumpiness of artificial soils, which stimulates positive thigmotactic responses in organisms. Consequently, we recommend using natural soils, such as LUFA, instead of OECD soils to reduce the underestimation of the disaggregation degree.

4.4 Implications for soil risk assessment

The disaggregation effects in terrestrial isopods can provide a valuable addition to current soil screening and ecological risk assessment (ERA) frameworks. Traditional soil hazard assessments primarily rely on survival, or reproduction endpoints (OECD, 1994; OECD, 2009), which, although robust, may underestimate early sublethal alterations at the population level. The disaggregation indices developed in this study provide a sensitive, rapid, and non-invasive measure of soil stress at the population level, capturing ecological responses analogous to those observed in population-level avoidance tests (ISO, 2020). From a practical perspective, disaggregation tests represent an early-warning signal that can be performed under controlled laboratory conditions, both for prognostic analyses, i.e., assessing the effects of different compounds, and diagnostic analyses, i.e., applying the test to various soil samples collected from the field and prioritizing those that require further investigation (Federico et al., 2026). Incorporating aggregation behavior into soil monitoring programs provides a valuable tool for soil screening, supporting Soil Screening Level (SSL) assessments by helping to identify and prioritize critical areas at risk of depopulation and determining whether further detailed evaluations are needed. Moreover, this approach aligns with the requirements of the European Commission's Soil Monitoring Law (EC, 2025), offering an effective and compliant method for early identification of soil stress. In light of the results of this work, this tool is particularly well suited to addressing multifactorial stress scenarios, where interactions between temperature, humidity, and contaminants are expected to determine biological responses. Current results show that these factors can influence social cohesion and habitat use, suggesting that disaggregation can serve as an early warning signal for compromised soil functionality and ecosystem health. Overall, the proposed methodology represents a potential refinement of soil risk assessments, linking behavioral ecology with ecotoxicology. By quantifying disaggregation as an indicator of social stress, it can complement traditional ecotoxicological tests, improve the predictive capacity of chemical assessments as environmental factors change, and support a more integrative assessment of soil ecosystem resilience under the pressures of global change.

5 Conclusion

Marked alterations in gregarious behavior were observed in the population of *P. pruinosus* across all environmental factors, each of which influenced the population of terrestrial isopods according to different patterns that emerged from the clustering analysis. Our findings provide a relevant contribution for future soil hazard and risk assessments by demonstrating that the behavioral responses of *P. pruinosus* are not fixed, but are modulated by key abiotic and biotic factors. This knowledge can be directly integrated into soil screening and ERA frameworks (i.e. in soil monitoring models, or climate scenario simulations) by supporting the interpretation of behavioral endpoints under variable environmental conditions, reducing the uncertainty often associated with context-dependent ecotoxicological responses. In practical terms, this approach can complement current standard toxicity assays by offering a sensitive, rapid, and ethically favorable tool capable of detecting sublethal effects that may precede measurable impacts on survival or reproduction. Moreover, acknowledging the influence of multiple interacting stressors aligns with emerging regulatory efforts toward more realistic, complexity-based hazard assessment strategies.

References

A'Bear A. D., Jones T. H., Boddy L., 2014. Potential impacts of climate change on interactions among saprotrophic cord-forming fungal mycelia and grazing soil invertebrates. *Fungal Ecol*., 10, 34-43. https://doi.org/10.1016/j.funeco.2013.01.009

Aupic-Samain A., Santonja M., Chomel M., Pereira S., Quer E., Lecareu C., Limousin JM, Ourcival JM., Simioni G., Gauquelin T., Fernandez C., Baldy V., 2021. Soil biota response to experimental rainfall reduction depends on the dominant tree species in mature northern Mediterranean forests. *Soil Biol. and Biochem*., 154, 108122. https://doi.org/10.1016/j.soilbio.2020.108122

Bahrndorff S., Lauritzen J. M., Sørensen M. H., Noer N. K., Kristensen T. N., 2021. Responses of terrestrial polar arthropods to high and increasing temperatures. *JEB*, 224(7), jeb230797. https://doi.org/10.1242/jeb.230797

Benbellil-Tafoughalt S., Koene J. M., 2015. Influence of season, temperature, and photoperiod on growth of the land snail *Helix aperta*. *IRD*, 59(1), 37-43. https://doi.org/10.1080/07924259.2014.996300

Boiteau G., MacKinley P., 2013. Role of avoidance behavior in the response of *Folsomia candida* to above-freezing cooling temperatures. *Entomol. Exp. Appl*., *147*(1), 50-60. https://doi.org/10.1111/eea.12048

Blakstad, J. I., Strimbeck, R., Poveda, J., Bones, A. M., Kissen, R., 2023. Frass from yellow mealworm (*Tenebrio molitor*) as plant fertilizer and defense priming agent. *BAB*, 53, 102862. https://doi.org/10.1016/j.bcab.2023.102862

Bredon M., Herran B., Lheraud B., Bertaux J., Grève P., Moumen B., Bouchon D., 2019. Lignocellulose degradation in isopods: new insights into the adaptation to terrestrial life. *BMC genomics*, 20, 1-14. https://doi.org/10.1186/s12864-019-5825-8

Brigić A., Antonović I., Alegro A., Šegota V., Bujan J., 2017. Terrestrial isopods (Isopoda: Oniscidea) as unexpected inhabitants of extreme habitats. *Eur. J. Soil Biol*., 82, 66-71. https://doi.org/10.1016/j.ejsobi.2017.08.009

Broly, P., Devigne, L., Deneubourg, J. L., Devigne, C. 2014. Effects of group size on aggregation against desiccation in woodlice (Isopoda: O niscidea). *Physiol. Entomol*., 39(2), 165-171. https://doi.org/10.1111/phen.12060

Broly, P., Deneubourg, J. L. 2015. Behavioural contagion explains group cohesion in a social crustacean. *PLoS Comput. Biol.*, 11(6), e1004290. https://doi.org/10.1371/journal.pcbi.1004290

Broly, P., Mullier, R., Devigne, C., Deneubourg, J. L. (2016). Evidence of self-organization in a gregarious land-dwelling crustacean (Isopoda: Oniscidea). *Anim. Cogn*., 19(1), 181-192. https://doi.org/10.1007/s10071-015-0925-6

Cardé R. T., Millar J. G., 2009. Pheromones. In Encyclopedia of insects, Second Edition, 766-772. http://dx.doi.org/10.1016/B978-0-12-374144-8.X0001-X

Castañeda, L. E., Lardies, M. A., Bozinovic, F. 2004. Adaptive latitudinal shifts in the thermal physiology of a terrestrial isopod. *Evol. Ecol. Res.*, *6*(4), 579-593.

Cazzolla Gatti R., Messina G., Tiralongo F., Ursino L. A., Lombardo B. M., 2020. Learning from the environment: how predation changes the behavior of terrestrial Isopoda (Crustacea Oniscidea). *Ethol. Ecol. Evol*., 32(1), 29-45. https://doi.org/10.1080/03949370.2019.1640799

Chikoski J. M., Ferguson S. H., Meyer L., 2006. Effects of water addition on soil arthropods and soil characteristics in a precipitation-limited environment. *Acta Oecol*., 30(2), 203-211. https://doi.org/10.1016/j.actao.2006.04.005

Cividini, S., Montesanto, G. 2018. Aggregative behavior and intraspecific communication mediated by substrate-borne vibrations in terrestrial arthropods: An exploratory study in two species of woodlice. *Behav. Process*., 157, 422-430. https://doi.org/10.1016/j.beproc.2018.07.006

Corwin, D. L., 2021. Climate change impacts on soil salinity in agricultural area. *Eur. J. Soil Sci*., 72(2), 842-862. https://doi.org/10.1111/ejss.13010

Coyle D. R., Nagendra U. J., Taylor M. K., Campbell J. H., Cunard C. E., Joslin A. H., Mundepid A., Phillips C.A., Callaham Jr M. A., 2017. Soil fauna responses to natural disturbances, invasive species, and global climate change: Current state of the science and a call to action. *Soil Biol. Biochem*., 110, 116-133. http://dx.doi.org/10.1016/j.soilbio.2017.03.008

De Smedt, P., Wuyts, K., Baeten, L., De Schrijver, A., Proesmans, W., De Frenne, P., Ampoorter, E., Remy, E., Gijbels, M., Hermy, M., Bonte, D., Verheyen, K. 2016. Complementary distribution patterns of arthropod detritivores (woodlice and millipedes) along forest edge-to-interior gradients. *Insect Conserv. Divers.,* 9(5), 456–469. https://doi.org/10.1111/icad.12183

De Smedt, P., Baeten, L., Berg, M. P., Gallet-Moron, E., Brunet, J., Cousins, S. A. O., Decocq, G., Diekmann, M., Giffard, B., De Frenne, P., Hermy, M., Bonte, D., Verheyen, K. 2018. Desiccation resistance determines distribution of woodlice along forest edge-to-interior gradients. *Eur. J. Soil Sci*., 85, 1–3. https://doi.org/10.1016/j.ejsobi.2017.12.002

de Souza, A. J., de Andrade, P. A. M., de Araújo Pereira, A. P., Andreote, F. D., Tornisielo, V. L., Regitano, J. B., 2017. The depleted mineralization of the fungicide chlorothalonil derived from loss in soil microbial diversity. *Sci. Rep*., 7(1), 14646. https://www.nature.com/articles/s41598-017-14803-0

de Souza, A. J., de Araujo Pereira, A. P., Andreote, F. D., Tornisielo, V. L., Tizioto, P. C., Coutinho, L. L., Regitano, J. B., 2021. Sulfadiazine dissipation as a function of soil bacterial diversity. *Environ. Poll*., 271, 116374. https://doi.org/10.1016/j.envpol.2020.116374

De Vries F. T., Liiri M. E., Bjørnlund L., Bowker M. A., Christensen S., Setälä H. M., Bardgett R. D., 2012. Land use alters the resistance and resilience of soil food webs to drought. *Nat. Clim. Chang*., 2(4), 276-280. https://doi.org/10.1038/nclimate1368

Delhoumi M., Catania V., Zaabar W., Tolone M., Quatrini P., Achouri M. S., 2020. The gut microbiota structure of the terrestrial isopod *Porcellionides pruinosus* (Isopoda: Oniscidea). *Eur. Zool. J*., 87(1), 357-368. https://doi.org/10.1080/24750263.2020.1781269

Delhoumi, M., Bouslama, M. F., Achouri, M. S. 2023. Effects of moisture decrease on behavioural and life history traits in three species of terrestrial isopods. *Eur. Zool. J*., 90(2), 832-839. https://doi.org/10.1080/24750263.2023.2273969

Des Marteaux L. E., Kullik S. A., Habash M., Schmidt J. M., 2020. Terrestrial isopods *Porcellio scaber* and *Oniscus asellus* (Crustacea: Isopoda) increase bacterial abundance and modify microbial community structure in leaf litter microcosms: A short-term decomposition study. *Microb. Ecol*., 80(3), 690-702. https://doi.org/10.1007/s00248-020-01527-4

Diallo, H.A., 2008. United Nations Convention to Combat Desertification (UNCCD). In: Lee, C., Schaaf, T. (eds) The Future of Drylands. Springer, Dordrecht. https://doi.org/10.1007/978-1-4020-6970-3_4

Díaz-García, L., Huang, S., Spröer, C., Sierra-Ramírez, R., Bunk, B., Overmann, J., Jiménez, D. J., 2021. Dilution-to-stimulation/extinction method: a combination enrichment strategy to develop a minimal and versatile lignocellulolytic bacterial consortium. *AEM*, 87(2), e02427-20. https://doi.org/10.1128/AEM.02427-20

Drobne, D., 1997. Terrestrial isopods—a good choice for toxicity testing of pollutants in the terrestrial environment. *ETC*, 16(6), 1159-1164. https://doi.org/10.1002/etc.5620160610

Ďurajková, B., Hladký, R., Tuf, I. H. 2022. Higher temperature and substrate vibrations as stress factors for terrestrial isopods–model species matter. *ZooKeys*, 1101, 71. https://doi.org/10.3897/zookeys.1101.77549

EC, 2009. Regulation (EC) No 1107/2009 of the European Parliament and of the Council of 21 October 2009 concerning the placing of plant protection products on the market and repealing Council Directives 79/117/EEC and 91/414/EEC, 153, 1–186. ELI: http://data.europa.eu/eli/reg/2009/1107/oj

EC, 2011. Commission Implementing Regulation (EU) No. 540/2011 of 25 May 2011 implementing Regulation (EC) No. 1107/2009 of the European Parliament and of the Council as regards the list of approved active substances. Off J Eur Union, 153, 1-186. ELI: http://data.europa.eu/eli/reg_impl/2011/540/2023-08-02

EC, 2024. Commission Implementing Regulation (EU) 2024/1280 of 30 April 2024 amending Implementing Regulation (EU) No 540/2011 as regards the approval periods of the active substances dodemorph, fatty acids C8-C10 methyl esters, lauric acid, methyl octanoate, methyl decanoate, oleic acid and *Trichoderma atroviride* (formerly *T. harzianum*) strain IMI 206040 (OJ L, 2024/1280, 03.05.2024). ELI: http://data.europa.eu/eli/reg_impl/2024/1280/oj

EC, 2025. European Commission, 2025a. P9_TA(2024)0204 – Soil Monitoring and Resilience (Soil Monitoring Directive) – European Parliament legislative resolution of 10 April 2024 on the proposal for a directive of the European Parliament and of the Council on Soil Monitoring and Resilience (Soil Monitoring Law) (COM(2023)0416 – C9-0234/2023 – 2023/0232(COD)) (Ordinary legislative procedure: first reading) http://data.europa.eu/eli/C/2025/1312/oj

Edney E. B., 1964. Acclimation to temperature in terrestrial isopods: I. Lethal temperatures. *Physiol. Zool*., 37(4), 364-377. https://doi.org/10.1086/physzool.37.4.30152755

Federico L., Monti G. S., Loureiro S., Villa S., 2024. Disaggregation behavior in the terrestrial isopod *Porcellionides pruinosus* as a new ecotoxicological endpoint for assessing infochemical disrupting activity. *Ecol Indic*., 167, 112602. https://doi.org/10.1016/j.ecolind.2024.112602

Federico, L., Tatangelo, V., Pittino, F., Russo, C., Vegini, E., Citterio, S., Franzetti, A., Villa, S. 2026. The potential use of behavioural bioassays as a first-tier approach for screening urban soil biodiversity: a pilot study. *Urb. Ecosyst*., 29(1), 42. https://doi.org/10.1007/s11252-026-01908-6

Ferreira, N. G., Morgado, R. G., Amaro, A., Machado, A. L., Soares, A. M., Loureiro, S., 2016. The effects of temperature, soil moisture and UV radiation on biomarkers and energy reserves of the isopod *Porcellionides pruinosus*. Applied soil ecology, 107, 224-236. http://dx.doi.org/10.1016/j.apsoil.2016.06.007

Ferruzca-Campos, E. A., Rico-Chavez, A. K., Guevara-González, R. G., Urrestarazu, M., Cunha-Chiamolera, T. P. L., Reynoso-Camacho, R., Guzmán-Cruz, R., 2023. Biostimulant and elicitor responses to cricket frass (*Acheta domesticus*) in tomato (*Solanum lycopersicum* L.) under protected conditions. *Plants*, 12(6), 1327. https://doi.org/10.3390/plants12061327

Frankenstein D., Luu M. S., Luna-Ayala J., Willett D. S., Filgueiras C. S., 2024. Soil moisture conditions alter behavior of entomopathogenic nematodes. *J. Sci. Food Agric*., 104(7), 4383-4390. https://doi.org/10.1002/jsfa.13326

García-García A., Cuesta-Valero F. J., Miralles D. G., Mahecha M. D., Quaas J., Reichstein M., Zscheischler J., Peng J., 2023. Soil heat extremes can outpace air temperature extremes. *Nat. Clim. Chang*., 13(11), 1237-1241. https://doi.org/10.1038/s41558-023-01812-3

González-Alcaraz, M. N., Malheiro, C., Cardoso, D. N., Loureiro, S., 2019. Soil moisture influences the avoidance behavior of invertebrate species in anthropogenic metal (loid)-contaminated soils. *Environ. Poll*., 248, 546-554. https://doi.org/10.1016/j.envpol.2019.01.105

Ghouri, A. S. K., 1961. Home and distribution of the house cricket *Acheta domesticus* L. *Nature*, 192(4806), 1000-1000. https://doi.org/10.1038/1921000a0

Hassall, M., Tuck, J. M., Smith, D. W., Gilroy, J. J., Addison, R. K. 2002. Effects of spatial heterogeneity on feeding behaviour of *Porcellio scaber* (Isopoda: Oniscidea). *Eur. J. Soil Sci*., 38(1), 53-57. https://doi.org/10.1016/S1164-5563(01)01124-4

Hornung E., 2011. Evolutionary adaptation of oniscidean isopods to terrestrial life: structure, physiology and behavior. *Terr. Arthropod Rev*., 4, 95. http://dx.doi.org/10.1163/187498311X576262

Huey R. B., Berrigan D., 2001. Temperature, demography, and ectotherm fitness. *Am. Nat*., 158(2), 204-210. https://doi.org/10.1086/321314

ISO, 2020. Soil quality - Avoidance test for determining the quality of soils and effects of chemicals on behaviour - Part 1: Test with earthworms (*Eisenia fetida* and *Eisenia andrei*). ISO 7512-2:2020.

Jain, A. K., 2010. Data clustering: 50 years beyond K-means. *Pattern Recognit. Lett*., 31(8), 651-666. https://doi.org/10.1016/j.patrec.2009.09.011

Judd W. W., 1963. Studies of the Byron bog in southwestern Ontario. XIV. Observations on sowbugs *Cylisticus convexus* (Degeer) and *Tracheoniscus rathkei (*Brandt) (Isopoda: Oniscidae). *Ecology*, 44(3), 615-617. https://doi.org/10.2307/1932554

Kraamwinkel, C. T., Beaulieu, A., Dias, T., Howison, R. A. 2021. Planetary limits to soil degradation. *Commun. Earth Environ*., 2(1), 249. https://doi.org/10.1038/s43247-021-00323-3

Kulessa, A. K., Balzani, P., Soto, I., Toutain, M., Haubrock, P. J., Kouba, A., 2024. Assessing the potential phytosanitary threat of the house cricket *Acheta domesticus*. *STOTEN*, 917, 170376. https://doi.org/10.1016/j.scitotenv.2024.170376

Legendre P., Legendre L, 1998, Numerical Ecology. Elsevier Science BV, Amsterdam, 853 pp.

Lopez-Riquelme G.O., Malo E.A., Cruz-López L., Fanjul-Moles M.L., 2006. Antennal olfactory sensitivity in response to task-related odours of three castes of the ant *Atta mexicana* (hymenoptera: formicidae). *Physiol. Entomol.*, 31(4), 353-360. https://doi.org/10.1111/j.1365-3032.2006.00526.x

Loureiro, S., Sampaio, A., Brandão, A., Nogueira, A. J., Soares, A. M. 2006. Feeding behaviour of the terrestrial isopod *Porcellionides pruinosus* Brandt, 1833 (Crustacea, Isopoda) in response to changes in food quality and contamination. *STOTEN*, 369(1-3), 119-128. https://doi.org/10.1016/j.scitotenv.2006.05.023

Loureiro S., Soares A. M., Nogueira A. J. 2005. Terrestrial avoidance behaviour tests as screening tool to assess soil contamination. *Environ. Poll.*, 138, 121-131. https://doi.org/10.1016/j.envpol.2005.02.013

McAfee A., Chapman A., Iovinella I., Gallagher-Kurtzke Y., Collins T. F., Higo H., Madilao L.L., Pelosi P., Foster L. J., 2018. A death pheromone, oleic acid, triggers hygienic behavior in honey bees (*Apis mellifera* L.). *Sci. Rep.*, 8(1), 5719. https://doi.org/10.1038/s41598-018-24054-2

McAfee A., Metz B. N., Milone J. P., Foster L. J., Tarpy D. R., 2022. Drone honey bees are disproportionately sensitive to abiotic stressors despite expressing high levels of stress response proteins. *Commun. Biol.*, 5(1), 141. https://doi.org/10.1101/2021.08.28.456261

Migge-Kleian S., McLean M. A., Maerz J. C., Henegha L., 2006. The influence of invasive earthworms on indigenous fauna in ecosystems previously uninhabited by earthworms. *Biol. Invasions*, 8, 1275-1285. https://doi.org/10.1007/s10530-006-9021-9

Morgado R., Ferreira N. G., Cardoso D. N., Soares A. M., Loureiro S., 2015. Abiotic factors affect the performance of the terrestrial isopod *Porcellionides pruinosus*. *Appl. Soil Ecol.*, 95, 161-170. http://dx.doi.org/10.1016/j.apsoil.2015.06.012

Müller C., Caspers B. A., Gadau J., Kaiser S., 2020. The power of infochemicals in mediating individualized niches. *TREE*, 35(11), 981-989. https://doi.org/10.1016/j.tree.2020.07.001

OECD, 1984 Guideline for testing of chemicals no. 207. Earthworm, acute toxicity test. Adopted April 4, 1984.

OECD , 2009. OECD Guidelines for the Testing of Chemicals, Test No. 232: Collembolan Reproduction Test in Soil. OECD Publishing.

Owojori O. J., Reinecke A. J., Rozanov A. B., 2008. Effects of salinity on partitioning, uptake and toxicity of zinc in the earthworm *Eisenia fetida*. *Soil Biol. and Biochem.*, 40(9), 2385-2393. https://doi.org/10.1016/j.soilbio.2008.05.019

Owojori O. J., Reinecke A. J., 2009. Avoidance behaviour of two eco-physiologically different earthworms (*Eisenia fetida* and *Aporrectodea caliginosa*) in natural and artificial saline soils. *Chemosphere*, 75(3), 279-283. https://doi.org/10.1016/j.chemosphere.2008.12.051

Panagos, P., Jones, A., Lugato, E., Ballabio, C. 2025. A Soil Monitoring Law for Europe. *Glob. Chall.*, 9(3), 2400336. https://doi.org/10.1002/gch2.202400336

Pereira C. S., Lopes I., Sousa J. P., Chelinho S., 2015. Effects of NaCl and seawater induced salinity on survival and reproduction of three soil invertebrate species. *Chemosphere*, 135, 116-122. https://doi.org/10.1016/j.chemosphere.2015.03.094

Quinlan M. C., Hadley N. F., 1983. Water relations of the terrestrial isopods *Porcellio laevis* and *Porcellionides pruinosus* (Crustacea, Oniscoidea). J. Comp. Physiol. A, 151(2), 155-161. https://doi.org/10.1007/BF00689913

Rengel, Z., 2011. Soil pH, Soil Health and Climate Change. In: Singh, B., Cowie, A., Chan, K. (eds) Soil Health and Climate Change. Soil Biology, vol 29. Springer, Berlin, Heidelberg. https://doi.org/10.1007/978-3-642-20256-8_4

Ribeiro, N., Abelho, M., Costa, R. 2018. A review of the scientific literature for optimal conditions for mass rearing *Tenebrio molitor* (Coleoptera: Tenebrionidae). *J. Entomol. Sci.*, 53(4), 434-454. https://doi.org/10.18474/JES17-67.1

Rollo C. D., Borden J. H., Casey I. B., 1995. Endogenously produced repellent from American cockroach (Blattaria: Blattidae): function in death recognition. *Environ. Entomol.*, 24(1), 116-124. https://doi.org/10.1093/ee/24.1.116

Römbke T., Römbke J., Russell D., 2011. Effects of temperature increases on the feeding activity of two species of isopods (*Porcellio scaber*, *Porcellionides pruinosus*) in laboratory tests. *Soil Organisms*, 83(2), 211-220. https://soil-organisms.org/index.php/SO/article/view/246

Rousseeuw, P. J., 1987. Silhouettes: a graphical aid to the interpretation and validation of cluster analysis. *JCAM*, 20, 53-65. http://dx.doi.org/10.1016/0377-0427(87)90125-7

Shahid, S.A., Zaman, M., Heng, L., 2018. Soil Salinity: Historical Perspectives and a World Overview of the Problem. In: Guideline for Salinity Assessment, Mitigation and Adaptation Using Nuclear and Related Techniques. Springer, Cham. https://doi.org/10.1007/978-3-319-96190-3_2

Škarková P., Kos M., Drobne D., Vávrová M., Jemec A., 2016. Effects of food salinization on terrestrial crustaceans *Porcellio scaber*. *Appl. Soil Ecol.*, 100, 1-7. https://doi.org/10.1016/j.apsoil.2015.11.007

Spencer J. O., Edney E. B., 1954. The absorption of water by woodlice. *JEB*, 31(4), 491-496. https://doi.org/10.1242/jeb.31.4.491

Trigos-Peral G., Maák I. E., Schmid S., Chudzik P., Czaczkes T. J., Witek M., Casacci LP, Sánchez-García D., Lőrincz Á., Kochanowski M., Heinze J., 2024. Urban abiotic stressors drive changes in the foraging activity and colony growth of the black garden ant *Lasius niger*. *STOTEN*, 915, 170157. https://doi.org/10.1016/j.scitotenv.2024.170157

Tuf I. H., Ďurajková B., 2022. Antipredatory strategies of terrestrial isopods. *ZooKeys*, 1101, 109. https://doi.org/10.3897%2Fzookeys.1101.76266

Van Gestel, C. A., Loureiro, S., 2018. Terrestrial isopods as model organisms in soil ecotoxicology: a review. *ZooKeys*, (801), 127. https://doi.org/10.3897/zookeys.801.21970

van Straalen N. M., Verhoef H. A., 1997. The development of a bioindicator system for soil acidity based on arthropod pH preferences. *J. Appl. Ecol.*, 217-232. https://doi.org/10.2307/2404860

Wright J. C., Machin J., 1990. Water vapour absorption in terrestrial isopods. *JEB*, 154(1), 13-30. https://doi.org/10.1242/jeb.154.1.13

Wright J. C., O'Donnell M. J., Sazgar S., 1997. Haemolymph osmoregulation and the fate of sodium and chloride during dehydration in terrestrial isopods. *J. Insect Physiol.*, 43(9), 795-807. https://doi.org/10.1016/S0022-1910(97)00035-8

Yao M., Rosenfeld J., Attridge S., Sidhu S., Aksenov V., Rollo C. D., 2009. The ancient chemistry of avoiding risks of predation and disease. *Evol. Biol*., 36, 267-281. https://doi.org/10.1007/s11692-009-9069-4

Zimmer M., 2003. Habitat and resource use by terrestrial isopods (Isopoda, Oniscidea). In The biology of terrestrial isopods (pp. 243-261). Brill. http://dx.doi.org/10.1163/9789047412854_019

Table 1 OECD Standard Soil amended with different percentages of weight (% w/w) of sand, kaolin, sphagnum and calcium carbonate for achieving different soil pH values ($CaCl_2$ 0.01 M). The asterisk (*) identified the recommended percentages of the Standard OECD Soil (1984) and was assumed as control for the soil pH bioassay.

OECD Standard Soil				
Sand %	**Kaolin %**	**Sphagnum %**	**Calcium Carbonate %**	**pH**
80	20	0	10	8.13
70	20	0.3	10	8.05
75	20	5	0.3	7.22
70	20	10	0.3	6.07*
35	20	55	0	5.15
10	20	70	0	4.43
0	0	100	0	4.06

Table 2 Summarized values of mortality (mean ±S.D.) per each environmental stressors and tested ranges.

Environmental stressors	**Ranges**	**Mortality mean (±S.D.)**
Temperature °C	5	100
	10	4 (±5.48)
	15	4 (±5.48)
	20	0
	25	4 (±5.48)
	30	8 (±10.9)
	35	8 (±10)
Soil Moisture %	10	0
	20	0
	30	0
	40	0
	60	0
	70	4 (±0.55)
	80	46 (±1.82)
NaCl g/kg d.w.	0	0

	2.5	0
	3	0
	3.5	4 (±0.58)
	4	0
	4.5	0
	5	0
pH	4.06	0
	4.43	0
	5.15	0
	6.07	4.47 (±4.47)
	7.22	0
	8.05	0
	8.13	0
Acheta domesticus (min. or h)	0	0
	3	0
	7	0
	15	0
	30	2 (±4.47)
	24 (h)	0
	48 (h)	0
Tenebrio molitor (min. or h)	0	0
	3	0
	7	0
	15	0
	30	0
	24 (h)	2 (±4.47)

	48 (h)	0
Oleic acid mg/kg d.w.	0	0
	46,9	2 (±4.47)
	93,7	6 (±5.48)
	187,5	0
	375	4 (±5.48)
	750	0
	1500	0
Microbial Community Diversity (% w/w)	100	0
	10-1	0
	10-2	0
	10-3	0
	10-4	0
	10-5	0
	10-6	2 (±4.47)
	10-7 (not purged)	0
	10-8 (purged)	8 (±8.37)

Table 3 Number of observation and Euclidean distances between final cluster centers performing a K-mean cluster algorithm for *k* = 3. The highest number represents the most distant clusters.

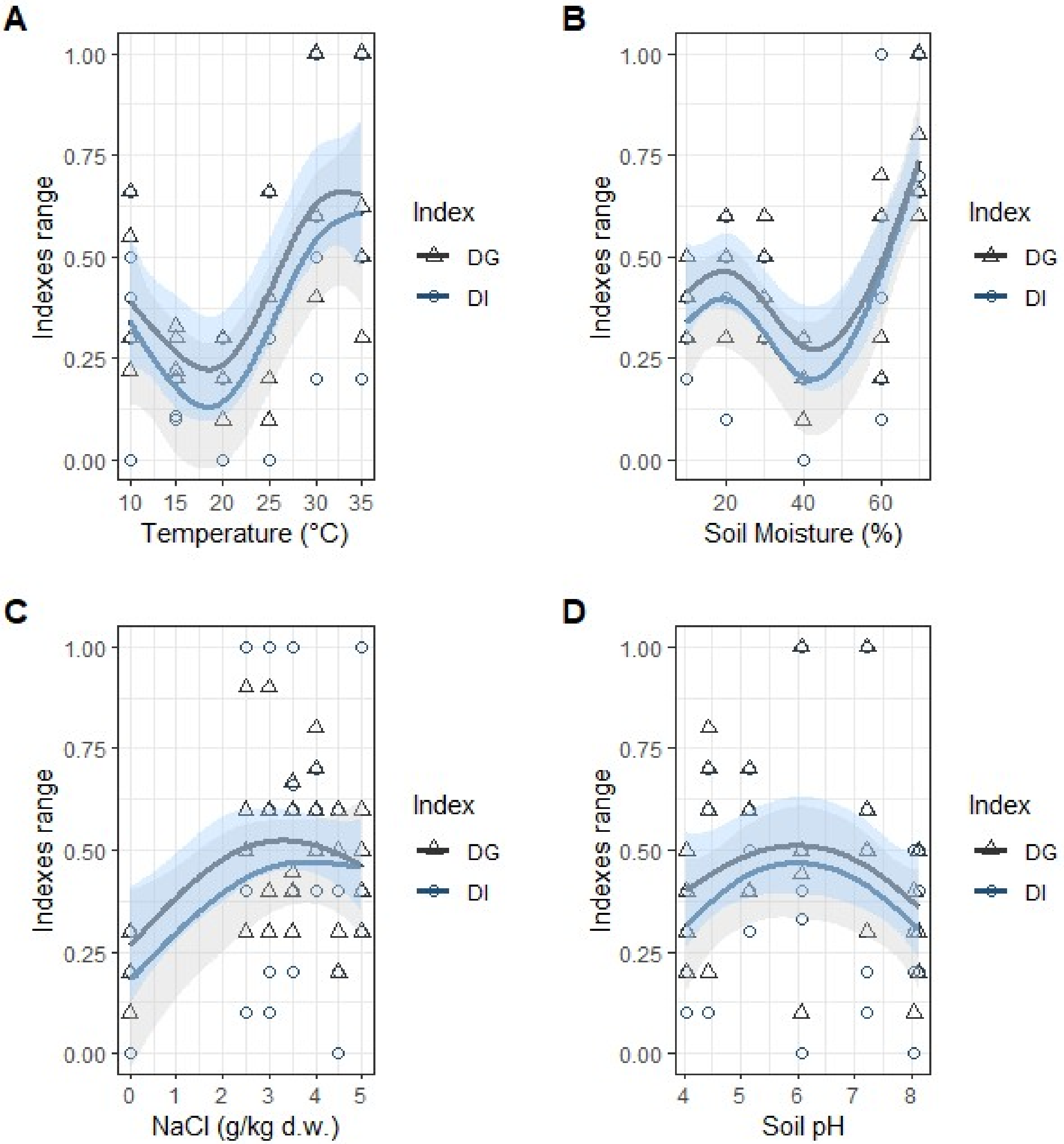
	Observation	Cluster 1	Cluster 2	Cluster 3
Cluster 1	20	0		
Cluster 2	17	0.60	0	
Cluster 3	23	0.73	0.65	0

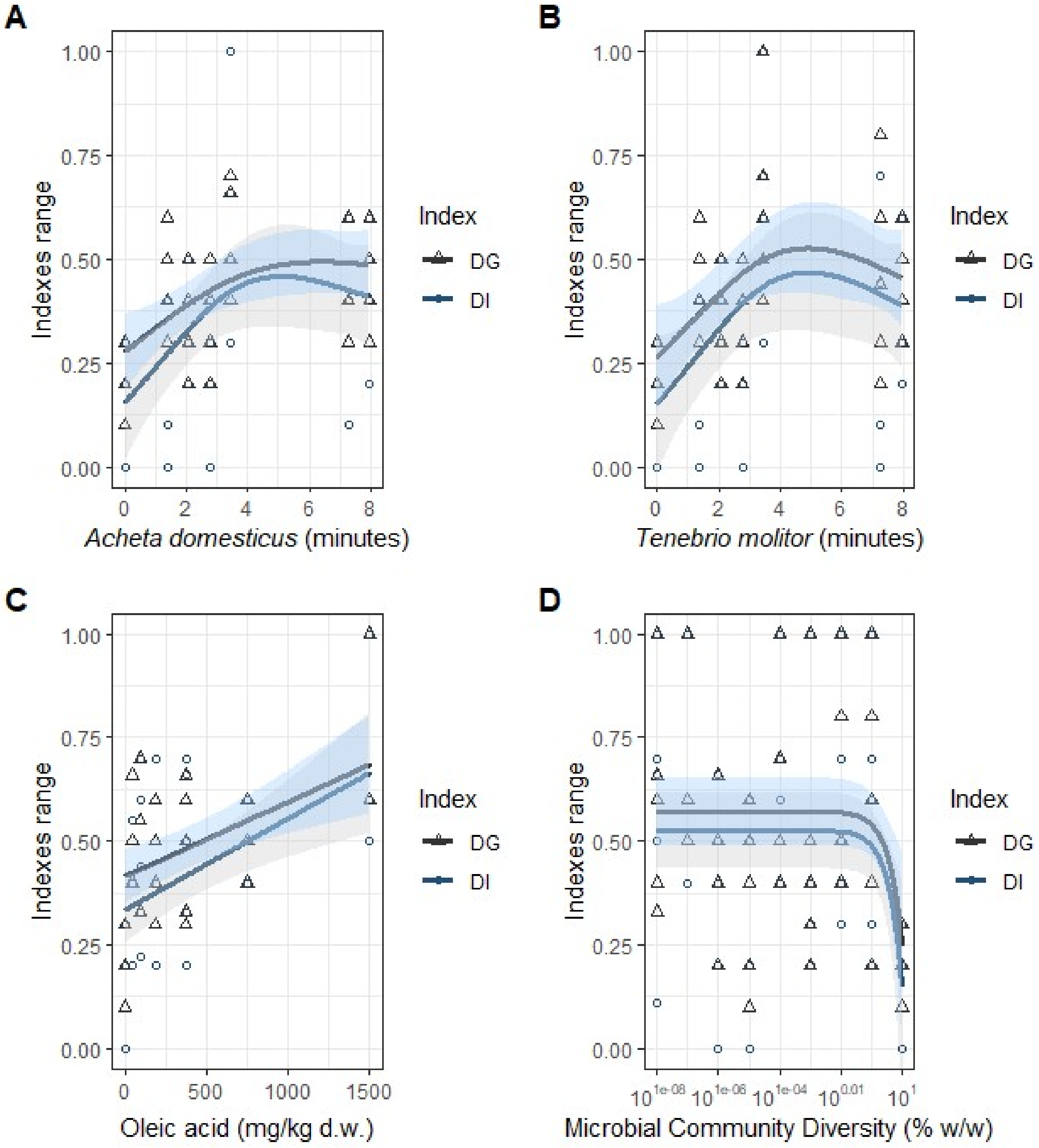
Table 4 Final cluster center output of K-means cluster analysis related to the effect of the main abiotic and biotic factors on the aggregation behavior on individuals of *Porcellionides pruinosus* separated with Euclidean distances. MCD: Microbial Community Diversity.

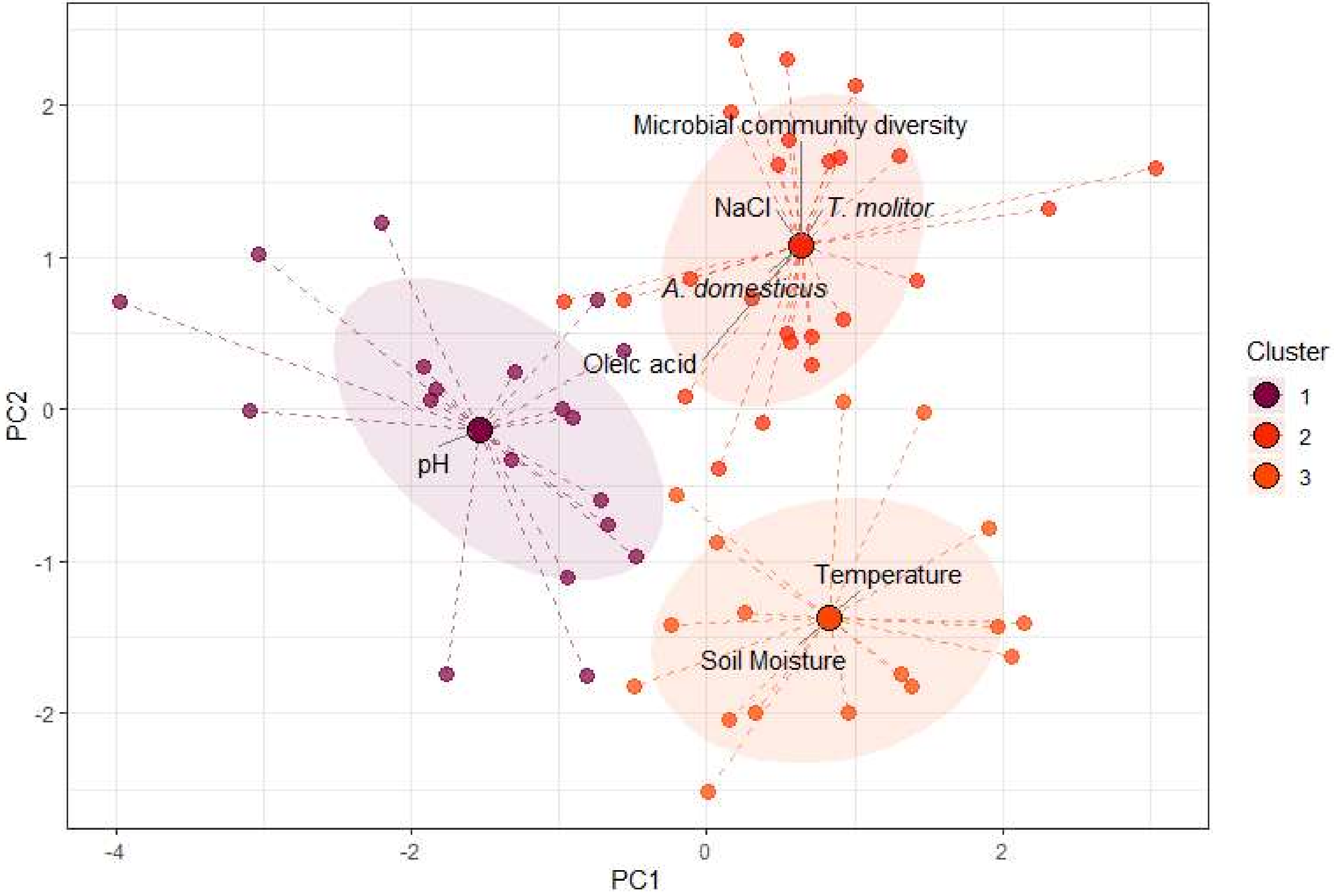
Final Cluster Center	Temperature	Moisture	NaCl	pH	*A. domesticus*	*T. molitor*	Oleic acid	MCD
Cluster 1	0.31	0.30	0.43	0.59	0.27	0.32	0.36	0.35
Cluster 2	0.22	0.33	0.62	0.51	0.52	0.54	0.54	0.75
Cluster 3	0.63	0.77	0.44	0.27	0.40	0.40	0.54	0.51

Table 5 ANOVA output of K-means cluster analysis related to the effect of the main abiotic and biotic factors on the aggregation behavior on individuals of *Porcellionides pruinosus.* MCD: Microbial Community Diversity.

Stressors		**K-mean clustering analysis - ANOVA**				
		Cluster SS	**Error DF**	**Error SS**	**F value**	**Prob > F**
Abiotic factors	Temperature	0.464	57	0.065	7.13	0.0017
	Moisture	1.503	57	0.032	46.5	< 0.0001
	NaCl	0.219	57	0.047	4.63	0.014
	pH	0.456	57	0.045	10.1	< 0.0001
Biotic factors	*A. domesticus*	0.338	57	0.020	16.7	< 0.0001
	T. molitor	0.237	57	0.038	6.22	0.0036
	Oleic acid	0.214	57	0.028	7.63	0.0012
	MCD	0.808	57	0.061	13.3	< 0.0001

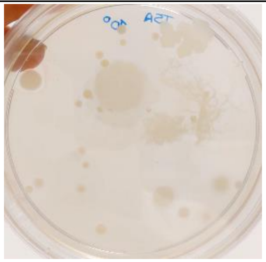
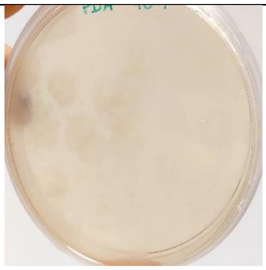
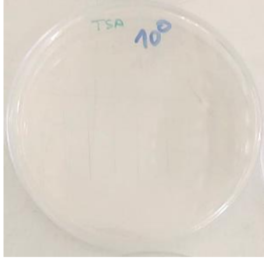
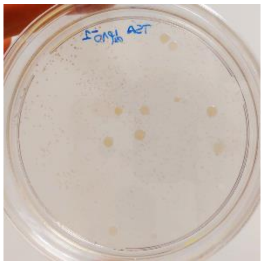
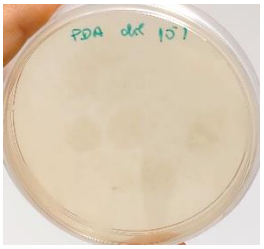

A
Indexes range
1.00
0.75
0.50
0.25
0.00
10
15
20
25
30
35
Temperature (°C)
Index
DG
DI
B
Indexes range
20
40
60
Soil Moisture (%)
Index
DG
DI
C
Indexes range
0
1
2
3
4
5
NaCl (g/kg d.w.)
Index
DG
DI
D
Indexes range
4
5
6
7
8
Soil pH
Index
DG
DI

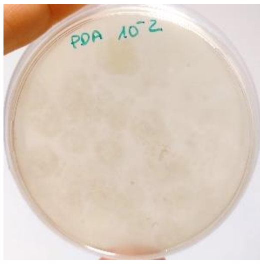
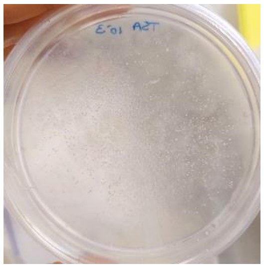
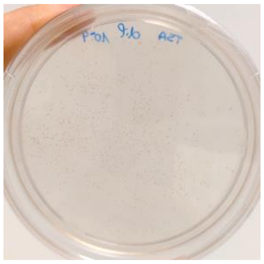
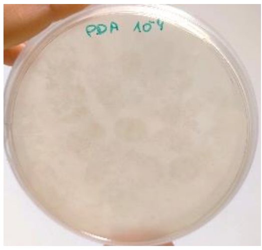
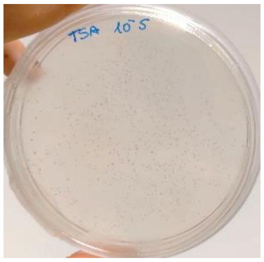
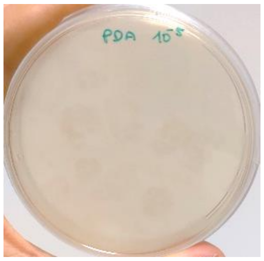
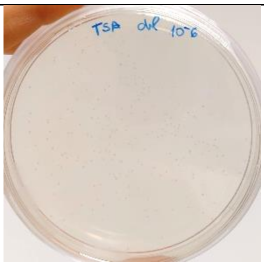
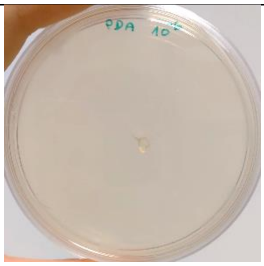

A
Indexes range
1.00
0.75
0.50
0.25
0.00
0
2
4
6
8
Acheta domesticus (minutes)
Index
DG
DI
B
Indexes range
Tenebrio molitor (minutes)
Index
DG
DI
C
Indexes range
0
500
1000
1500
Oleic acid (mg/kg d.w.)
Index
DG
DI
D
Indexes range
10^{1e-08}
10^{1e-06}
10^{1e-04}
$10^{0.01}$
10^{1}
Microbial Community Diversity (% w/w)
Index
DG
DI


Microbial community diversity
NaCl
T. molitor
A. domesticus
Oleic acid
pH
Temperature
Soil Moisture
PC1
PC2
-4
-2
0
2
-2
-1
0
1
2
Cluster
1
2
3

SUPPLEMENTARY MATERIALS

Table SM1. – Bacterial and fungal colony counts obtained after the implemented Dilution-to-Extinction methodology to obtain a gradient of diversity on the soil microbial community.

Treatment	**Bacterial colony counts** **(Colony forming unit (CFU) in Tryptic Soy Agar - TSA)**		**Fungal colony counts** **(Colony forming unit (CFU) in Potato Dextrose Agar - PDA)**	
Initial microbial community				
Fresh soil - undiluted	High diversity of bacterial colonies and morphologies.		High diversity of fungal morphologies and a visible fungal mat.	
Microbial community post-autoclave				
Autoclaved soil	No colonies.		No colonies.	
Serial dilutions plated in culture media after the equilibration time (incubation of DtE for 2 weeks)				
DtE 10^{-1} (450 g of autoclaved soil plus 50 g of undiluted soil)	Reduced number of bacterial colonies with different morphologies compared to undiluted soil.		Reduced density of fungal colonies and mat growth compared to the undiluted soil.	

DtE 10^{-2} (450 g of autoclaved soil plus 50 g of soil from DtE 10^{-1})	Reduced number of bacterial colonies with different morphologies compared to DtE 10^{-1}.		Decreased density of fungal colony and mat growth compared to DtE 10^{-1}.	
DtE 10^{-3} (450 g of autoclaved soil plus 50 g of soil from DtE 10^{-2})	Bacterial colonies with similar morphologies. Reduced number of colonies formed compared to DtE 10^{-2}.		Decreased density of fungal colony and mat growth compared to DtE 10^{-2}.	
DtE 10^{-4} (450 g of autoclaved soil plus 50 g of soil from DtE 10^{-3})	Bacterial colonies with similar morphologies. Reduced number of colonies formed compared to DtE 10^{-3}.		Decreased density of fungal colony and mat growth compared to DtE 10^{-3}.	
DtE 10^{-5} (450 g of autoclaved soil plus 50 g of soil from DtE 10^{-4})	Bacterial colonies with similar morphologies. Reduced number of colonies compared to DtE 10^{-4}.		Decreased density of fungal colony and mat growth compared to DtE 10^{-4}.	
DtE 10^{-6} (450 g of autoclaved soil plus 50 g of soil	Bacterial colonies with similar morphologies. Reduced number of colonies		Very low number of fungal colonies and mat growth compared to DtE 10^{-5}.	

from DtE 10^{-5})	compared to DtE 10^{-5}.			

Table - SM2 – Disaggregation indexes values (mean and standard deviation) in individuals of *P. pruinosus* for abiotic and biotic tested factors per each level. N.D.: No detected.

Abiotic Factors	Range	Indexes	Mean	± Standard Deviation
Temperature (°C)	5	DI	N.D.	N.D.
		DG	N.D.	N.D.
	10	DI	0,25	0,25
		DG	0,34	0,19
	15	DI	0,15	0,06
		DG	0,25	0,06
	20	DI	0,14	0,13
		DG	0,24	0,09
	25	DI	0,25	0,27
		DG	0,35	0,22
	30	DI	0,66	0,34
		DG	0,72	0,27
	35	DI	0,47	0,34
		DG	0,54	0,30
Moisture (%)	10	DI	0,26	0,17
		DG	0,38	0,13
	20	DI	0,42	0,19
		DG	0,48	0,11
	30	DI	0,36	0,09
		DG	0,42	0,13
	40	DI	0,14	0,13
		DG	0,24	0,09
	60	DI	0,46	0,36
		DG	0,48	0,22
	70	DI	0,62	0,33
		DG	0,66	0,29
	80	DI	N.D.	N.D.
		DG	N.D.	N.D.
NaCl (g/kg d.w.)	0	DI	0,14	0,13
		DG	0,24	0,09
	2.5	DI	0,48	0,33
		DG	0,56	0,22
	3	DI	0,38	0,42
		DG	0,5	0,26
	3.5	DI	0,57	0,30
		DG	0,48	0,15
	4	DI	0,54	0,11

pH		DG	0,64	0,11
	4.5	DI	0,32	0,23
		DG	0,42	0,16
	5	DI	0,46	0,31
		DG	0,44	0,11
	4.06	DI	0,22	0,13
		DG	0,32	0,13
	4.43	DI	0,42	0,30
		DG	0,5	0,28
	5.15	DI	0,5	0,16
		DG	0,5	0,14
	6.07	DI	0,45	0,36
		DG	0,51	0,32
	7.22	DI	0,48	0,36
		DG	0,54	0,29
	8.05	DI	0,26	0,18
		DG	0,3	0,12
	8.13	DI	0,3	0,14
		DG	0,36	0,13
A. domesticus (minutes)	0	DI	0,14	0,13
		DG	0,24	0,09
	3	DI	0,26	0,20
		DG	0,42	0,13
	7	DI	0,34	0,11
		DG	0,36	0,11
	15	DI	0,26	0,17
		DG	0,38	0,13
	30	DI	0,59	0,26
		DG	0,55	0,13
	1440	DI	0,38	0,18
		DG	0,52	0,16
	2880	DI	0,42	0,15
		DG	0,42	0,13
T. molitor (minutes)	0	DI	0,14	0,13
		DG	0,24	0,09
	3	DI	0,26	0,20
		DG	0,42	0,13
	7	DI	0,34	0,11
		DG	0,36	0,11
	15	DI	0,26	0,17
		DG	0,38	0,13
	30	DI	0,65	0,29
		DG	0,64	0,23

	1440	DI	0,39	0,33
		DG	0,47	0,24
	2880	DI	0,38	0,21
		DG	0,44	0,11
Oleic acid (mg/kg d.w.)	0	DI	0,14	0,13
		DG	0,24	0,09
	46.9	DI	0,39	0,12
		DG	0,51	0,09
	93.7	DI	0,46	0,20
		DG	0,52	0,19
	187.5	DI	0,38	0,20
		DG	0,46	0,11
	375	DI	0,48	0,21
		DG	0,48	0,16
	750	DI	0,34	0,19
		DG	0,42	0,18
	1500	DI	0,64	0,21
		DG	0,68	0,18
Microbial Community Diversity (%)	10^{0}	DI	0,14	0,13
		DG	0,24	0,09
	10^{-1}	DI	0,56	0,32
		DG	0,6	0,32
	10^{-2}	DI	0,58	0,28
		DG	0,64	0,25
	10^{-3}	DI	0,42	0,34
		DG	0,48	0,31
	10^{-4}	DI	0,62	0,25
		DG	0,66	0,23
	10^{-5}	DI	0,3	0,25
		DG	0,36	0,21
	10^{-6}	DI	0,35	0,26
		DG	0,39	0,20
	10^{-7} - No purgation	DI	0,76	0,33
		DG	0,82	0,25
	10^{-7} - With purgation	DI	0,59	0,33
		DG	0,6	0,26

Fig. SM3 Soil conductivity (EC) (a) and pH (b) measured in the beginning and 48h after the beginning of the salinity bioassay. EC_i and EC_f refer to the conductivity at the beginning and end of the test, respectively (expressed as dS/m) and pH_i and pH_f, the pH measured at the beginning and end of the test.

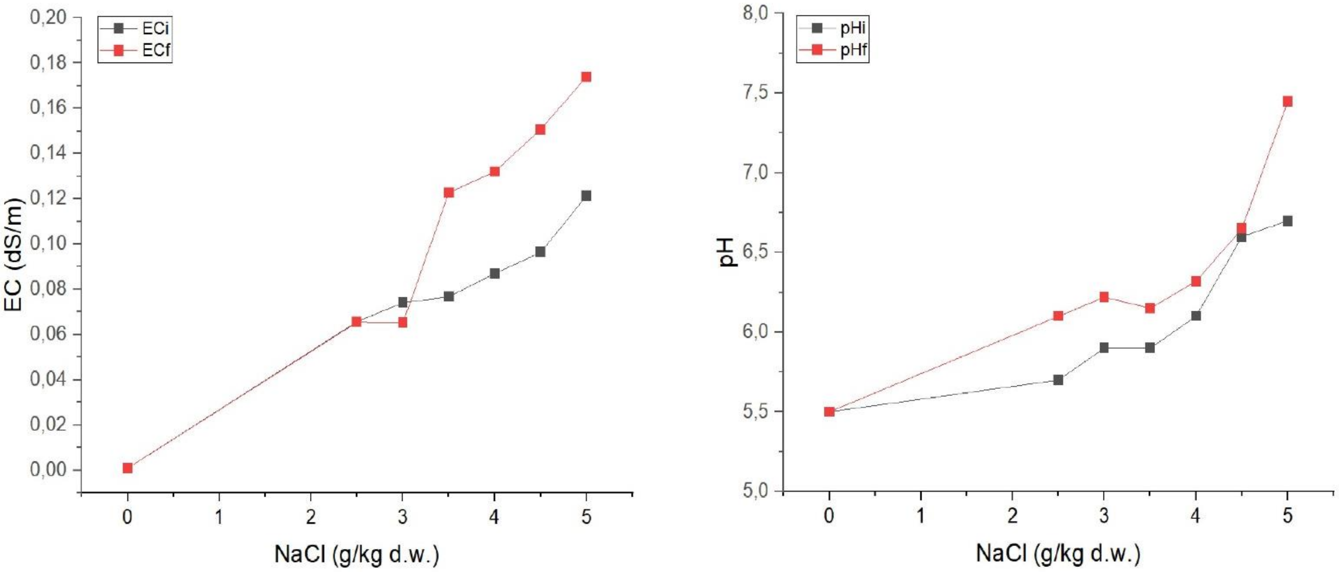


Fig. SM4 Soil pH measured in the beginning and 48h after the beginning of the pH bioassay. pHi and pHf, represent the pH measured at the beginning and end of the test.

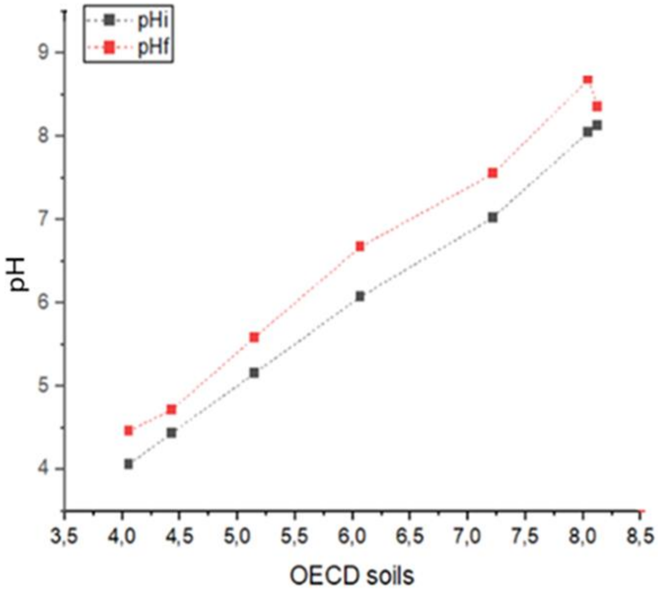


Fig. SM5 Soil conductivity (EC) (a) and pH (b) measured in the beginning and 48h after the beginning of the microbial dilution bioassay. EC_i and EC_f refer to the conductivity at the beginning and end of the test, respectively (expressed as dS/m) and pH_i and pH_f, the pH measured at the beginning and end of the test. Treatments are presented as % of the sterilized soil used, where 10^0 refers to the higher level of microbial communities in soil and 10^{-7} represents the sterilized soils.

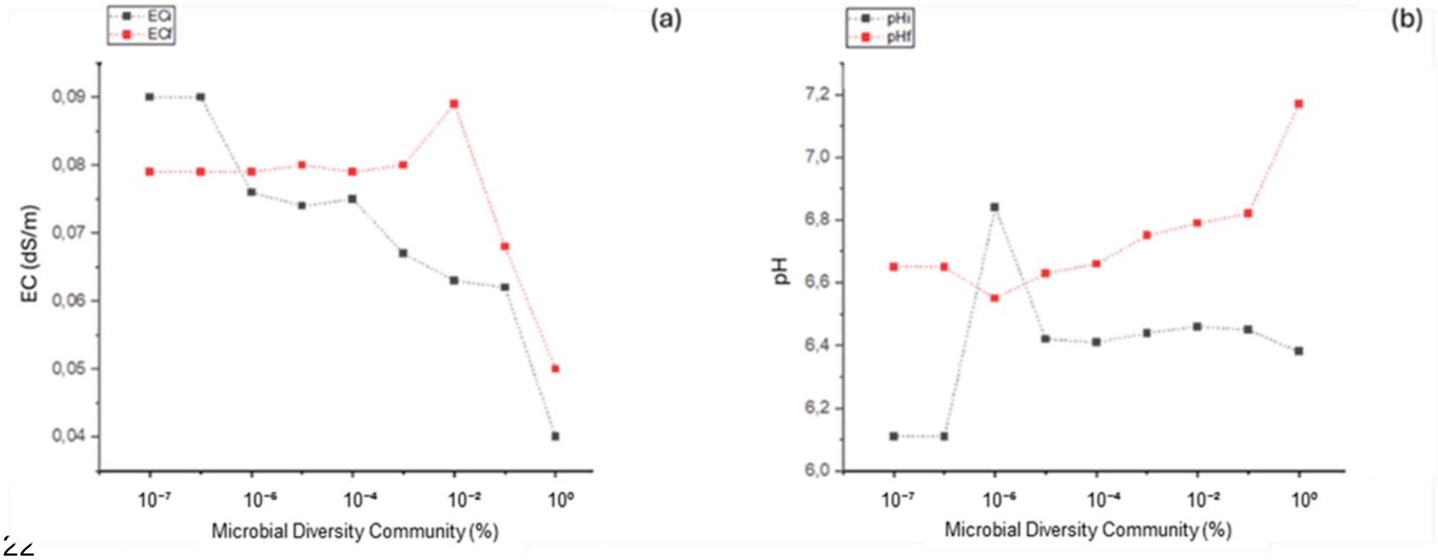


Table – SM6 – Optimal cluster number based on the Silhouette Index (SI) for the K-mean cluster analysis.

Cluster	Average Silhouette Index (SI)
3	0.185
4	0.178
5	0.169

Chapter 5

Potential of Henry's Law Constant as a Predictive Indicator of Behavioural Effect in *Porcellionides pruinosus*

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Mandatory Keywords:	soil contamination, soil ecotoxicology, soil invertebrates, terrestrial invertebrate toxicology, behavioral toxicology
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Abstract:	Behavioral alterations in terrestrial isopods emerged as early warning signals of soil pollution, but their link to specific physicochemical properties of contaminants remains unexplored. This study investigates whether specific chemical properties can predict two ecologically relevant behavioral endpoints in the gregarious isopod Porcellionides pruinosus. A combined avoidance-disaggregation bioassay was applied to a set of twenty-five substances. The substances were categorised according to the effects they induced: those that induced both avoidance and disaggregation, those that triggered only one of these effects, and those showing no detectable effects. Regression models revealed a significant positive association between disaggregation and the Henry's law constant, with a threshold value estimated at 1×10^{-3} Pa m^3 mol^{-1}, beyond which aggregation breaks down. This value can be proposed as a potential indicator in the regulatory field for predicting the impact of a substance on the social behaviour of woodlice, supporting management decisions in assessing the impact of contaminants on soil. Conversely, no predictive descriptors were found for avoidance responses, indicating that the mechanisms underlying migration remain to be elucidated. Furthermore, given the heterogeneity of behavioural responses, the combined use of avoidance and disaggregation endpoints is recommended for a more comprehensive investigation.

Abstract

Behavioral alterations in terrestrial isopods emerged as early warning signals of soil pollution, but their link to specific physicochemical properties of contaminants remains unexplored. This study investigates whether specific chemical properties can predict two ecologically relevant behavioral endpoints in the gregarious isopod *Porcellionides pruinosus.* A combined avoidance-disaggregation bioassay was applied to a set of twenty-five substances. The substances were categorised according to the effects they induced: those that induced both avoidance and disaggregation, those that triggered only one of these effects, and those showing no detectable effects. Regression models revealed a significant positive association between disaggregation and the Henry's law constant, with a threshold value estimated at 1×10^{-3} Pa m^3 mol^{-1}, beyond which aggregation breaks down. This value can be proposed as a potential indicator in the regulatory field for predicting the impact of a substance on the social behaviour of woodlice, supporting management decisions in assessing the impact of contaminants on soil. Conversely, no predictive descriptors were found for avoidance responses, indicating that the mechanisms underlying migration remain to be elucidated. Furthermore, given the heterogeneity of behavioural responses, the combined use of avoidance and disaggregation endpoints is recommended for a more comprehensive investigation.

Keywords: terrestrial isopods, Henry law constant, soil, contamination, avoidance test, disaggregation effect, robust regression.

1. Introduction

Pollution exerts an increasing pressure on the stability and integrity of soil ecosystems, making rapid and replicable risk assessment essential to predict the adverse ecotoxicological effects. To date, the risk assessment regarding the impact of contaminants in soil remains uncertain and requires further exploration (Vieira et al., 2024). These gaps reflect the greater heterogeneity and complexity of soil ecosystems compared to aquatic ecosystems (Edwards, 2002; Rodríguez-Eugenio et al., 2018). The heterogeneity of soils particularly influences the bioavailability, transport, uptake, and persistence of contaminants (Zang et al., 2017). These parameters depend in turn on the physicochemical properties of contaminants, including solubility, Henry's law constant (H) values, and partition coefficients (Tetko, 2007; Hewitt et al., 2009; Zang et al., 2017). Identifying quantitative relationships between these parameters and ecological effects on soil organisms remains a key challenge. At the same time, it is necessary to evaluate sub-lethal and ecologically relevant effects such as effects on population structures and dynamics (EC, 2025), or trophic net effects (Vighi and Villa, 2013). In light of these complexities, regulatory frameworks have begun to emphasize the importance of evaluating the impacts of chemical substances on non-target soil organisms and their behaviors, as outlined, e.g., in Regulation No. 1107/2009 of the European Commission (EC), as well as in EC Regulations No. 283/2013 and 284/2013. Indeed, behavioral endpoints are gaining attention as early and sensitive indicators of chemical stress (Ågerstrand et al., 2020); however, they remain underutilized in regulatory contexts. Among the main non-target organisms, terrestrial isopods are a suitable model organism in behavioral testing. They are representative detritivores of soil litter and are exposed to various exposure pathways, including interstitial water, soil, organic and inorganic matter, air, and food (Lokke and van Gestel, 1998; van Gestel et al., 2018). Being both vagile and gregarious organisms, woodlice can be used in a single combined avoidance and disaggregation bioassay to provide insights into how contaminants affect population density and dispersal patterns (Federico et al., 2024). Avoidance reflects active migration away from contaminated soil and can signal deteriorated habitat quality (Caro et al., 2013), whereas disaggregation reflects a breakdown in social cohesion, potentially due to the deception of chemical information signaling (Federico et al., 2024). Both endpoints are ecologically meaningful, particularly in gregarious species where chemical communication plays a role in aggregation. Despite this potential, there are still no standardized guidelines on these organisms, even though numerous studies demonstrated a greater sensitivity to soil contamination compared to other model organisms (Jones and Hopkin, 1996; Drobne, 1997; Loureiro et al., 2009; Morgado et al., 2016; Maria et al., 2024). Furthermore, the primary exposure pathways responsible for triggering these behavioral responses remain poorly characterized. In 'hard-bodied' organisms, soil pore water represents a key exposure route of pollutants (Peijnenburg et al., 2012), though direct soil contact is also critical (EFSA, 2017). This is particularly relevant for euedaphic species like springtails, but for litter organisms such as woodlice, exposure via the soil–air or water–air interface should not be neglected. In fact, as outlined by van Gestel et al. (2018), airborne chemicals may also be taken up through inhalation via ventral pleon, in addition to uptake through direct contact with contaminated soil or ingestion of food (Fig. 1).

Figure 1. The main routes of exposure for terrestrial isopods (food, contact, inhalation). Exposure through contact with solid soil or pore water is mainly absorbed through the setae of the pereopods, but contact with semiochemicals through the antennae cannot be ruled out. Inhalation of airborne substances and water vapor occurs mainly through absorption by the pleon.

For this reason, identifying physicochemical descriptors involved in behavioural onset is a key area for ecotoxicological management. However, the extent to which behavioural responses such as avoidance and disaggregation can be predicted based on the physicochemical properties of contaminants remains to be investigated. The purpose of this study was twofold:

- Identify the specific contaminants for which both avoidance and disaggregation effects occur, do not occur, or only one of the responses is triggered;
- Determine which physicochemical parameters can reliably predict the onset of disaggregation and/or avoidance behaviors.

2. Methods

2.1 Model Organisms. Individuals of *Porcellionides pruinosus* Brandt (1833) were cultured in the Laboratory of Ecotoxicology, Department of Earth and Environmental Sciences, at the University of Milano Bicocca, Italy. Cultures were maintained at 20 ± 1 °C with a photoperiod of 16:8 h light:dark and were sprayed with distilled water twice a week. They were fed *ad libitum* with alder leaves. As consolidated practice in literature (Loureiro et al., 2006; Morgado et al., 2016; van Gestel et al., 2018), only adults (wet weight of 14-30 mg) were used in the bioassays, excluding molting animals, individuals with malformations, and pregnant females.

2.2 Soil. For the toxicity bioassays, the natural LUFA 2.2 sandy loam soil (Speyer, Germany) was used as a standard soil. The properties of this soil include a pH = 5.6 ± 0.3 (0.01 M $CaCl_2$), Water Holding Capacity (WHC) = 48.9 ± 5.6 (g/100 g), C = 1.82 ± 0.5 (%), N = 0.19 ± 0.05, Cation Exchange Capacity (CEC) = 9.54 ± 1.36 meq/100 g.

2.3 Chemicals. To investigate which chemical-physical parameters played a role in the occurrence of avoidance and disaggregation effects, 24 different organic compounds belonging to different classes were selected and tested: for PPPs 1,3-dichloropropene (DCP), azadirachtin A (AZA), chlorpyrifos (CPF), dichlobenil (DCB), dimethoate (DMT), glyphosate (GLY), parathion (PAR), pendimethalin (PEN), S-metolachlor (S-MET), sulfoxaflor (SFX), terbuthylazine (TBA), triclopyr (TCP), trifluralin (TFL); for PPCP benzophenone-1 (BP-1), carbamazepine (CBZ), N,N-diethyltoluamide (DEET), fipronil (FIP), galaxolide (GAL), oxybenzone (OXB), and for PFCAs perfluorobutanoic acid (PFBA), perfluorododecanoic acid (PFDoA), perfluorooctanoic acid (PFOA), perfluoropentanoic acid (PFPeA), perfluoropropionic acid (PFPrA). Avoidance data of benzothiazole (BTZ) were collected from Federico et al. (2024) for the predictive analysis. For each substance, concentration range, physical–chemical properties, and database references from which the sources of all these compounds were obtained are reported in Supplementary Materials (Table S1). All the tested chemicals were purchased from Merck Millipore, with purities ranging from 98% to 99%.

Nominal concentrations expressed in mg/kg dry soil (d.w.) were selected based on the respective maximum solubilities (g/L) of each compound. This means that the chosen concentrations never exceeded the maximum solubility threshold of the individual contaminants, thereby avoiding the use of organic solvents that could have masked or affected the behavioral responses and the related study of physicochemical descriptors. Additional concentrations close to the maximum solubilities were performed only for those chemical contaminants that showed no significant avoidance or disaggregation effects in the chosen concentration range. Contaminated soil was prepared in 200 mL glass beakers by mixing the chemicals with LUFA 2.2 soil in an aqueous medium. For PFCA compounds, only 250 mL Nalgene HDPE containers were used to prevent adsorption onto glass (Kleiner et al., 2021). For volatile substances (DCP, GAL, PEN, TFL), contamination via an aqueous medium followed the method of An (2005), widely employed to assess the toxicity of volatile compounds (Zhiqun et al., 2017; Wang et al., 2021).

2.4 Bioassays. The avoidance and disaggregation behaviours tests were performed to evaluate the ability of edaphic organisms to avoid contaminated soil towards uncontaminated soil as requested from the International Organization for Standardization guidelines (ISO, 2020) and to determine a potential alteration on aggregation behaviour during the avoidance test (Federico et al., 2024). Glass arenas with airtight lids (9.5 × 7.3 x 5 cm) were divided into two sides using a removable plastic split and filled with 50 g d.w. of LUFA 2.2 soil per each side. One side was filled with contaminated soil, while the other part was left uncontaminated. Dual controls were set up using only uncontaminated LUFA 2.2 soil on both sides of the test boxes. In one condition, dry uncontaminated soil was treated with distilled water (control), while in the other, the soil was treated with the test chemical dissolved in a liquid solution. All controls and treated soils were gently flattened to obtain a soil surface as homogeneous and lump-free as possible, thereby minimizing thigmotactic effects from the woodlice. For each treatment, ten individuals of *P. pruinosus* were introduced into each arena, with five independent replicates per treatment. The tests were carried out in thermostatic chambers at 20 ± 1 °C, with a 16:8 h (light:dark) photoperiod, for 48 h. During the experiments, the individuals were never fed to reduce aggregation or attraction behaviour induced by food. At the end of the test, each arena was positioned beneath a camera (Trust Teza 4K Ultra HD webcam, 3840 × 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.

2.5 Statistical analysis. Avoidance response was assessed by quantifying the avoidance percentage (A %), as follows (Eq. 1):

$$A\% = \frac{n_c - n_t}{n} \cdot 100 \ (1)$$

where n_c, n_t, and n represent the number of individuals on the uncontaminated, the individuals on the contaminated soil, and the total number of individuals collected at the end of the experiment, respectively. Positive values indicate avoidance responses. If the total number of individuals in the contaminated soils is less than 20%, such soils are considered to have "limited habitat function" (ISO, 2020). Negative responses were treated as no avoidance, while no-avoidance occurs when the distribution of organisms is approximately equal between contaminated and uncontaminated soils. This distribution, including a mortality rate of less than 10%, also represents the conditions required for dual controls to validate the tests.

The disaggregation effects were measured by the disaggregation index (DI%) as follows (Eq. 2):

$$DI\ \% = \frac{s+2d}{n} \cdot 100 \ (2)$$

considering the number of singlet (s) and doublet (d) groups, and total individuals (n) recovered at the end of the test. Groups were defined as individuals in physical contact with at least one body part. Close individuals without physical interaction were not considered part of the same clusters (Federico et al., 2024). Percentage values tending towards 100% indicate an increase in the gregarious fragmentation effect in the population, and values of 50% were selected as thresholds for extrapolating effect concentrations on 50% of the population (EC50). To validate the tests, the DI values of control should not exceed 20 %.

Four standard nonlinear regression functions were selected to estimated dose–response effects (Finney, 1979): log-logistic (L), probit (P), Weibull (W), and a Brain-Cousens hormesis (B) model, also known as the biphasic model. Only two-parameter models were considered to avoid overfitting, except the Brain-Cousens hormesis model, which requires more parameters due to its ability to capture both stimulatory effects at low concentrations and inhibitory effects at higher concentrations (Table 1). Model-specific parameters include: slope (b), median effective concentration EC50 (e), upper asymptote (d), and hormetic effect (f). To select the best-fitting model, we used the Akaike and Bayesian Information Criteria (AIC and BIC), choosing the model with the lowest values as the optimal one. The goodness-of-fit of the dose-response models was assessed using the McFadden's $Pseudo\ R^2$ (McFadden, 1974) calculated as follow (Eq. 3):

$$Pseudo\ R^2 = 1 - \frac{ln\ (L_M)}{ln\ (L_0)} \ (3)$$

where $ln\ (L_M)$ is the log-likelihood of the fitted model and $ln\ (L_0)$ is the log-likelihood of the null model, i.e., intercept-only model. The resulting value, which ranges from 0 to 1, indicates the improvement in fit provided by the predictor variables, i.e., concentration, compared to the null model, with values closer to 1 signifying a stronger fit. The statistical significance of the overall dose-response relationship was tested using the Likelihood Ratio Test (LRT), comparing the deviance of the fitted model to the deviance of the null model, resulting in a χ^2 statistic. Values of $p < 0.05$ were considered statistically significant. EC50 values were estimated, along with 95% confidence intervals obtained via the delta method. Note that this approximation can yield confidence bounds extending into negative values, indicating substantial uncertainty in the estimates.

Table 1. List of regression models for the response (y = f(x)) as a function of the concentration (x) of a given contaminant, where ϕ is the cumulative distribution function of a standard normal distribution. Parameters (*b, d, e, f*) are model-specific and estimated from the data, as implemented in the drc package in R (version 3.0.1).

Regression Models	**Model equation**
Log-logistic (L)	$f(x) = (1 + exp(b(log(x) - log(e))))^{-1}$
Probit (P)	$f(x) = \phi(b(log(x) - log(e)))$
Weibull (W)	$f(x) = exp(-exp(b(log(x) - log(e))))$
Brain-Cousens hormesis (B)	$f(x) = (d + fx)/(1 + exp(b(log(x) - log(e))))$

To explore potential mechanisms underlying the observed behavioral alterations, a correlation analysis was conducted between avoidance percentages (A%) and disaggregation index (DI) values and a set of physicochemical descriptors. Based on descriptors showing strong and significant correlations, predictive models were developed to evaluate whether the physicochemical properties of contaminants could explain behavioural endpoints (avoidance and disaggregation). Specifically, regression models were fitted using two alternative response variables: (i) the maximum of the mean responses across concentration levels—computed by first averaging the five replicate avoidance values at each concentration and then selecting the highest of these means—referred to as Amax and DImax, respectively; and (ii) the 75th percentile of the A and DI values observed across all replicates for each chemical, referred to as A75 and DI75, respectively. These two metrics were chosen to capture different aspects of the upper tail of the response distribution: the mean of the most substantial replicate-level effects (Amax/DImax) and a robust, quantile-based summary (A75/DI75), which reduces the influence of outliers. All physicochemical descriptors were log-transformed to reduce skewness and to reflect the common log-scale behavior of environmental variables. Statistical significance was evaluated at the 5% significance level. All statistical analyses were carried out using R software, version 4.4.1 (R Core Team, 2024).

3. Results.

3.1 Behavioural responses

All individuals in the control groups showed a mortality rate of less than 10%, a stochastic population distribution, and a gregariousness condition greater than 80%, thereby confirming the validity and reliability of the test conditions.

The avoidance and disaggregation responses, quantified according to formulas 1 and 2, respectively, are presented in Fig. 2. The results demonstrate that exposure to several contaminants elicited significant behavioral alterations. These effects influenced either the migratory behaviour, the dispersal pattern, or both, in *P. pruinosus* populations.

Figure 2. Boxplots of behavioral responses—avoidance (blue) and disaggregation (red)—as a function of chemical concentration levels (mg/kg d.w.) in *P. pruinosus* populations. The boxes represent the interquartile range (IQR) with the median indicated by the horizontal line, while whiskers extend to the most extreme data points within 1.5 times the IQR. Response values range from -100% to 100%. Positive values indicate the expected behavioral effect (avoidance or disaggregation), while negative values represent inverse effects (attraction or aggregation). The dashed grey lines indicate the ±50% threshold, marking levels of high behavioral alteration.

For avoidance behavior (Fig. 2, blue boxplots), seven compounds (benzophenone-1, chlorpyrifos, dimethoate, PFDoA, S-metolachlor, sulfoxaflor, and triclopyr) elicited a clear dose-dependent avoidance response in more than 50% of the exposed woodlice. Hormetic effects were observed for carbamazepine, oxybenzone, and parathion, while the remaining substances failed to trigger avoidance responses, even at concentrations close to their solubility limits (Table S1).

In terms of population disaggregation (Fig. 2, red boxplots), nine chemicals (1,3-dichloropropene, chlorpyrifos, DEET, dichlobenil, galaxolide, oxybenzone, parathion, pendimethalin, and trifluralin) triggered dose-dependent fragmentation in over 50% of the tested individuals. Notably, six of these compounds (1,3-dichloropropene, DEET, dichlobenil, galaxolide, pendimethalin, and trifluralin) induced disaggregation without avoidance, suggesting that disaggregation occurred independently of the animals' ability to migrate away from the contaminated soil. No significant disaggregation was observed for the other substances, even near their maximum solubility.

Overall, the results of A% and DI% responses reveal distinct behavioural profiles among the tested chemicals, with substances that induced both avoidance and disaggregation (chlorpyrifos, benzothiazole), substances that triggered avoidance but not disaggregation (benzophenone-1, dimethoate, PFDoA, S-metolachlor, sulfoxaflor, triclopyr). substances that caused disaggregation without avoidance (1,3-dichloropropene, DEET, dichlobenil, galaxolide, PFBA, pendimethalin, trifluralin), substances that elicited hormetic responses related to avoidance, with (oxybenzone, parathion) or without (carbamazepine) disaggregation, and substances that showed no detectable behavioural effects (azadirachtin, fipronil, glyphosate, terbuthylazine, PFOA, PFPeA, PFPrA).

For all chemicals that produced measurable behavioural changes, dose–response models were fitted (Table 2 a and b). In cases where no significant behavioral response was observed for a given endpoint, no best-fitting model could be identified.

The statistical significance of the models for avoidance (Table 2.a) yielded a highly significant p-value $\leq$ 0.001, for all substances where an EC50 could be calculated.t. Furthermore, the overall goodness-of-fit for the selected models was strong. The calculated McFadden's Pseudo R^2 values, ranging from 0.63 to 0.86, indicate that the concentration variable effectively explains a large portion of the variance in the avoidance endpoint, thus demonstrating a strong fit for the selected models (W, L, B, P) to the experimental data.

The final results for the concentration-response relationship concerning the disaggregation endpoint (Table 2.b) demonstrate a statistically significant concentration-dependent effect for all tested compounds (BTZ, CPF, DCB, DCP, DEET, GAL, OXB, PAR, PEN, and TFL), yielding a highly significant p-value $\leq$ 0.001. The models exhibited a moderate to strong goodness-of-fit, with McFadden's Pseudo R^2 values ranging from 0.30 (for CPF) to 0.64 (for TFL), indicating that the selected models explain a substantial, yet lower, portion of the variance in the disaggregation response compared to the avoidance endpoint.

Table 2. Effective concentrations (EC50) expressed as mg/kg d.w. for avoidance (a) and disaggregation (b) endpoints for chemicals that induced statistically significant behavioral alterations. The lower and upper bounds of the 95% confidence interval (CI) are reported alongside the best-fitted dose-response model for each substance. Model quality indicators include the Akaike Information Criterion (AIC), the McFadden's Pseudo R^2, and the p-value from the Likelihood Ratio Test (LRT). n.d.: not detected by model.

(a)

Substance	**EC50**	**Model**	**AIC**	**Pseudo R^2**	**p-value**

BP-1	1.1 [0.71-1.47]	W	35.73	0.87	≤ 0.001
BTZ	154 [106 - 200]	L	52.82	0.86	≤ 0.001
CBZ	20.7 [4.3-37]	B	61.11	0.66	≤ 0.001
CPF	11.9 [0.44-23]	L	48	0.79	≤ 0.001
DMT	15.3 [0 -33.5]	L	52.8	0.78	≤ 0.001
OXB	3.43 [0 - 34]	B	62.7	0.7	≤ 0.001
PAR	39.5 [15.2-63.9]	B	56.1	0.64	≤ 0.001
PEN	3.44 [0-67]	B	50	0.63	≤ 0.001
PFDoA	0.02 [0-0.04]	P	46.42	0.75	≤ 0.001
SFX	13.7 [0-35.6]	W	50.9	0.72	≤ 0.001
S-MET	0.46 [0-1.26]	W	45.8	0.65	≤ 0.001
TCP	0.25 [0-0.62]	W	55	0.8	≤ 0.001

(b)

Substance	**EC50**	**Model**	**AIC**	**Pseudo R^2**	**p-value**
TZ	3,546 [0- 28,825]	L	69	0,48	≤ 0.001
CPF	0.16 [n.d.]	P	47.6	0.3	≤ 0.001
DCB	0.52 [0-2.44]	L	45.7	0.36	≤ 0.001
DCP	4.01 [0-8.97]	P	43.8	0.61	≤ 0.001
DEET	0.01 [0-0.03]	L	52.38	0.55	≤ 0.001
GAL	0.014 [0-0.07]	L	47.34	0.61	≤ 0.001
OXB	0.45 [0-1.46]	P	48.38	0.37	≤ 0.001
PAR	15.8 [1.79-29.7]	L	44.5	0.53	≤ 0.001
PEN	1.61 [0-6.8]	L	48.45	0.39	≤ 0.001
TFL	2.27 [0-4.75]	L	48.34	0.64	≤ 0.001

It is challenging to compare disaggregation EC50 values, as this parameter was recently introduced and no effect values for this endpoint have been reported elsewhere (Federico et al., 2024). Conversely, the EC50 values for avoidance responses can be compared with the results obtained in previous studies, albeit with other organisms (Table 3).

Table 3. Avoidance EC50 values, expressed as mg/kg d.w., for soil invertebrates exposed the tested chemicals. EC50 values (in mg/kg d.w**)** are reported alongside fold-differences (n×) relative to *P. pruinosus* to facilitate direct comparison of species sensitivities. Asterisk (*) denotes concentrations converted from field application rates (g a.i./ha) assuming a soil bulk density of 1.3 g/cm and a mixing depth of 15 cm. 'No avoidance' indicates that no significant behavioural response was observed at the maximum tested concentration. µg/kg range denotes values in the microgram per kilogram range. N.C.: not comfortable.

Chemical	**Species**	**EC50**	**Fold-difference**	**Reference**
CPF	*Porcellionides pruinosus*	11.8		this study
	Eisenia fetida	49	4×	Garcia-Santos & Keller-Forrer 2011
	Folsomia candida	0.45	0.04×	Santos et al. 2012
DMT	*Porcellionides pruinosus*	15.3		this study
	Porcellionides pruinosus	31.5	2×	Santos et al. 2010
	Porcellionides pruinosus	28.6	2×	Loureiro et al. 2005
	Folsomia candida	no avoidance	N.C.	Pereira et al. 2013
S-MET	*Porcellionides pruinosus*	0.46		this study
	Eisenia fetida	>40	>87×	Xu et al. 2010
TCP	*Porcellionides pruinosus*	0.25		this study
	Folsomia candida	2.65*	~1×	Voinorosky et al. 2022
	Oppia nitens	12.1*	1–5×	Voinorosky et al. 2022

PFAS	*Porcellionides pruinosus*	µg/kg range		this study
	Eisenia fetida	µg/kg range	N.C.	Melo et al. 2022
CMZ	*Porcellionides pruinosus*	hormesis		this study
	Folsomia candida	hormetis	1	Oliveira et al. 2015
GLY	*Porcellionides pruinosus*	no avoidance		this study
	Eisenia fetida	no avoidance	1	Santos et al. 2012
	Folsomia candida	no avoidance	1	Niemeyer et al. 2018

Despite the paucity of observations pertaining to *P. pruinosus* avoidance responses, there is substantial concordance among the findings of studies. As shown in Table 3, a comparison of avoidance data reveals several consistent patterns across taxa and compounds. Compared to *P. pruinosus*, individuals of *Eisenia fetida* exhibited divergent responses to chlorpyrifos, manifesting either no-choice behaviour (Santos et al., 2012) or avoidance at a concentration of 49 mg/kg d.w. (Garcia-Santos & Keller-Forrer, 2011). In contrast, chlorpyrifos induced avoidance responses in *Folsomia candida* at an EC50 of 0.45 mg/kg d.w. It is relevant to note that these studies employed chlorpyrifos-based formulations rather than the pure active ingredient. This discrepancy may therefore be attributed to the use of a high-purity compound in the present study, as opposed to commercial formulations that include co-formulants capable of enhancing behavioural responses (Klátyik et al., 2023). The EC50 of dimethoate obtained in this study (15 mg/kg d.w.) is approximately two-fold lower than previously reported values of 31.5 mg/kg d.w. (Santos et al., 2010) and 28.6 mg/kg d.w. (Loureiro et al., 2005). Unlike *P. pruinosus*, *F. candida* shows no avoidance behaviour in response to dimethoate up to the maximum tested concentration of 32 mg/kg d.w. of soil, a response that has been attributed to immobilization effects (Pereira et al., 2013). With respect to S-metolachlor, *E. fetida* has been observed to avoid contaminated soil only at concentrations exceeding 40 mg/kg d.w., whereas *P. pruinosus* exhibits avoidance at a much lower EC50 of 0.46 mg/kg d.w. (Xu et al., 2010). Although derived from experiments using contaminated litter, the sensitivity of woodlice to triclopyr was consistent with the responses observed in *F. candida* and *Oppia nitens* at application rates of 5,167 and 23,597 g a.i./ha, respectively. Assuming a soil bulk density of 1.3 g/cm³ and a mixing depth of 15 cm, these application rates correspond to approximate soil concentrations of 2.65 and 12.1 mg/kg d.w. (Voinorosky et al., 2022). A comparable sensitivity, in the µg/kg range, was also observed for PFDoA avoidance concentrations in woodlice and earthworms exposed to PFAS-contaminated soils (Melo et al., 2022). Nevertheless, direct comparisons remain challenging because that study involved complex PFAS mixtures, whereas the present experiments focused on single-compound exposures. The hormetic responses of *P. pruinosus* to carbamazepine also results comparable to *F. candida* (Oliveira et al., 2015). The lack of response exhibited by *P. pruinosus* is consistent with the responses demonstrated by *E. fetida* and *F. candida* in avoidance tests involving glyphosate (Santos et al., 2012; Niemeyer et al., 2018), but in contrast to the findings reported in the literature for the same species (Santos et al., 2010). This observed discrepancy may be attributed to the use of a high-purity active ingredient in the present study, in contrast to commercial formulations, which contain co-formulants that can enhance behavioural responses (Klátyik et al., 2023). A preliminary analysis of the existing data suggests that woodlice may exhibit a level of sensitivity to chemicals that is comparable to, or potentially even exceeds, that observed in model organisms such as earthworms and springtails, as previously suggested by Maria et al., (2024). However, the scarcity of studies addressing avoidance behaviour precludes the drawing of definitive conclusions.

3.2 Physicochemical descriptors involved in behavioral alterations.

Correlations analysis between selected physicochemical parameters (Table S1) and behavioural endpoints for avoidance or disaggregation were assessed. For disaggregation, only DI75 and DImax were significantly and positively correlated with Henry's law constant (H).

A log–log regression model was employed in both cases, as it captures power-law relationships and helps linearize the association between variables. The model was specified as follows (Eq. 3):

$$log(DI75) = \alpha + \beta log(H) + \varepsilon \, (4)$$

where ε is the model error term.

Three estimation methods were applied for comparison (Fig. 3): Ordinary Least Squares (OLS), Quantile Regression ($\tau = 0.5$), and Robust Regression using the MM-estimator. OLS relies on normality and homoscedasticity assumptions, whereas quantile and robust regression are less sensitive to their violation. The MM-estimator was used for its robustness to outliers and heteroscedasticity (Yohai, 1987; Rousseeuw and Leroy, 2006), while quantile regression was applied at DI75 ($\tau = 0.5$) to estimate median responses under asymmetric error distributions (Koenker and Bassett, 1978). All three models identified a statistically significant positive association between the log-transformed Henry's law constant and logDI75, with β = 0.02 (Table S2). From an ecological perspective, given that the regression coefficient (β = 0.02) is significant on the log-log scale, a tenfold increase in the Henry's law constant of a chemical is associated with an approximate 20% increase in DI75. This implies that more volatile or semi-volatile compounds are more likely to disrupt the chemical signals that drive aggregation in *P. pruinosus*, leading to greater fragmentation of the group. Even a modest slope, therefore, represents a significant ecological effect, as the volatility of the compounds tested covers several orders of magnitude and disaggregation intensifies steadily with increasing volatility.

Figure 3. Scatterplot of log(H) versus log(DI75), with fitted regression lines: robust regression (solid), quantile regression at $\tau = 0.5$ (twodash), and ordinary least squares (dotted). The shaded area represents the 95% confidence band for the robust regression model. Each point corresponds to a chemical substance, labeled by its acronym. According to the weights assigned by the robust MM-estimator, all observations were retained with substantial influence (i.e., all weights > 0.72), suggesting the absence of high-leverage outliers in the dataset.

To evaluate predictive performance and avoid overfitting, leave-one-out cross-validation (LOOCV) was applied to each model: Ordinary Least Squares (OLS), Quantile Regression ($\tau = 0.5$), and Robust Regression (MM-type estimator). For each left-out observation, the model was fitted on the remaining data and used to predict the excluded point. Prediction error was quantified using three metrics: Mean Absolute Error (MAE): Root Mean Squared Error (RMSE), and Pseudo-R^2. All models yielded comparable results, indicating consistent predictive accuracy across methods (Table S3). Among the tested models, quantile regression yielded the lowest MAE, indicating superior performance in minimizing typical errors. RMSE was identical across models. Pseudo-R^2 was similar for OLS and quantile regression and slightly lower for robust regression. Overall, the three models displayed very similar performance. However, in contexts with potential outliers or heteroscedasticity, robust regression remains a valuable alternative due to its resistance to outliers, albeit at the cost of a slight trade-off in overall fit.

In the context of environmental protection, it is of interest to identify a threshold value of H above which disaggregation is expected to occur in the test organism. Specifically, we estimated the value of H corresponding to a DI75 value of 0.5 by inverting the fitted robust regression function (Eq. 4), under the log-log regression model given in formula 2, to obtain the point estimate

$$\hat{H} = exp\left(\frac{log(0.5)-\hat{\alpha}}{\hat{\beta}}\right) (5)$$

where $\hat{\alpha}$ and $\hat{\beta}$ are the robust point estimates of the log-log regression model. To quantify the uncertainty around this estimate, we applied the delta method (Oehlert, 1992) to derive an approximate 95% CI for the predictor value, obtaining an estimate of H = 8x10^{-4} Pa m^3 mol^{-1} with a CI of (-1.51x10^{-3}, 3.11x10^{-3} Pa m^3 mol^{-1}). This result suggests a tentative threshold, above which adverse population-level effects are likely to occur.

Using the maximum of the mean responses across concentration levels as the response variable, all three regression models (OLS, quantile regression at $\tau = 0.5$, and robust regression) identified Henry's law constant as a statistically significant predictor (p-value < 0.01). The estimated slope was consistently around 0.03 across models, with slight variations in intercepts (Fig. 4). Notably, in this case, the robust procedure detected S-MET as an outlier. While its exclusion improves model fit, the ecological or mechanistic basis for this deviation remains to be elucidated and should be interpreted with caution.

Figure 4. Scatterplot of log(H) versus log(DImax), with fitted regression lines: robust regression (solid), quantile regression at $\tau = 0.5$ (twodash), and ordinary least squares (dotted). The shaded area represents the 95% confidence band for the robust regression model. Each point corresponds to a chemical substance, labeled by acronym.

Similarly, when using the maximum mean disaggregation response (DImax) as the outcome and setting DImax = 0.5, the estimated H value was 1.2x10^{-4} Pa m^3 mol^{-1} with a 95 % CI of (-1.41x10^{-4}, 3.80x10^{-4} Pa m^3 mol^{-1}). While the threshold estimate based on DImax is lower than that obtained using DI75, their confidence intervals partially overlap, indicating that the difference is not statistically significant. Nonetheless, both estimates consistently suggest that disaggregation effects are likely to occur when Henry's law constant values exceed 1x10^{-3} Pa m^3 mol^{-1}.

The same regression analyses were performed, using as response variables either the 75th percentile of avoidance or the maximum of the mean responses across concentration levels. However, no statistically significant descriptors were identified.

4. **Discussion**.

4.1. Avoidance responses at the population level

The avoidance results obtained from this work reinforced the proposal that terrestrial isopods are suitable biosensors for soil contamination, a proposal previously advanced by other authors (Drobne and Hopkin, 1994; Drobne, 1997). Indeed, it has been demonstrated that woodlice, specifically *P. pruinosus*, exhibit sensitivity to concentrations lower than those measured in other species, mainly *Eisenia fetida* and *Folsomia candida*. This finding attests to their suitability for use in behavioural ecotoxicity tests (Loureiro et al., 2005; van Gestel et al., 2018).

Despite this, it is still unclear what role the physicochemical properties of soil contaminants play in the onset of avoidance behaviour and whether they can serve as predictive descriptors of behavioural responses. Although this study aimed to determine descriptors that can reliably predict the occurrence of behavioural alterations, no significant descriptors emerged as predictors of avoidance responses. This suggests that avoidance may arise from mechanisms not directly linked to bulk phase partitioning, but rather to organism-specific traits or mode-of-action (MoA) dependent physiological pathways. In fact, considering also the mode of action (MoA) of chemicals, it is important to note that all neurotoxic compounds considered in this work (mainly organophosphorus insecticides) elicit a positive avoidance response; however, other chemicals with different modes of action also induce behavioral alterations (i.e., UV filters and certain herbicides). Consequently, MoA is not the only factor involved in avoidance responses and cannot explain the variability of avoidance responses. This evidence suggests that the onset of avoidance responses in woodlice requires further investigation. It is hypothesised that the mechanisms underlying these responses may be contingent, at least in part, on specific traits of this crustacean that have yet to be elucidated. Among these traits, we can include the peculiar evolution of the olfactory system in terrestrial isopods. It is known, for example, that woodlice have developed distinct tritocerebral neuropiles in the second pair of antennae, which may be involved in chemoreception in other terrestrial isopods such as *Ligia occidentalis* and *Hemilepistus reaumuri* (Seelinger, 1983; Hansson et al., 2010; Harzsch et al., 2011). These studies provide preliminary evidence that alternative chemosensory pathways may be involved in terrestrial isopods compared to their marine counterparts. Since aggregation in terrestrial isopods is mediated by pheromonal signals (Takeda, 1980), it is plausible that chemosensitivity could be maintained in these species. While we recognize that this is speculative, the purpose of this work is not to verify the biochemical processes and anatomical structures involved in the selection process. Future research could be directed at filling these knowledge gaps.

4.2 Disaggregation effects at population level

In contrast to the avoidance responses, the results of this work revealed a positive correlation between the disaggregation effects and the Henry's law constant of selected chemicals. A threshold of H equal to 1 x 10^{-3} Pa m^3 mol^{-1} has been estimated as a significant value above which gregariousness is not maintained. The model's validity has been verified by the LOOVC technique, ensuring its robustness in the quantitative prediction of the disaggregation effect. Despite the utility of the model in expeditious prediction of disaggregation in woodlice, it is acknowledged that the model was derived from a comparatively limited dataset. On the other hand, it is important to underline that the robustness of the results lies in the large number of individuals tested per concentration (50 individuals) and by the wide range of physicochemical properties covered by the selected chemicals, including an eight-order of magnitude range for solubility and a ten-order of magnitude range for H (refer to Table S1).

In general, the regression model (Eq. 3) suggests that H constant may serve as a potential descriptor, albeit one requiring validation, in the regulatory field to predict the impact of a substance on gregarious populations. We support considering sublethal effects in evaluations of environmental risk at both the individual and higher ecological levels. This finding supports the inclusion of effects at sublethal and social level in the evaluations of environmental risk, complementing approaches previously applied for social pollinators (Fisher et al., 2023). From this perspective, the use of the disaggregation bioassay in terrestrial isopods addresses the need to assess social-level alterations. At the same time, the proposal of physicochemical cut-off may serve as a strategic prognostic value above which alteration of gregariousness is already expected, to avoid over-testing.

Special attention should be given to cases in which disaggregation occurred without avoidance (groups C and D). In these groups are listed the results obtained from those molecules with an H value higher than the threshold level of 1 x 10^{-3} Pa m^3 mol^{-1}. We hypothesize a more conservative interpretation of the potential exposure mechanism: given that several of the tested compounds exhibit measurable volatility, it is plausible that woodlice may absorb a fraction of these substances from the surrounding air, possibly through the pleopodal respiratory surfaces or via antennal chemoreception. Although the subsequent distribution of airborne chemicals within the organism has not been experimentally verified, such uptake routes could, in principle, influence sensory processes involved in aggregation. This possibility, while hypothetical, suggests that airborne exposure might modulate social organization in ways that could indirectly affect measured avoidance responses. However, empirical evidence for this mechanism requires further investigation.

5. Conclusions.

This study provided pivotal insights into the responses of *Porcellionides pruinosus* exposed to various chemicals, utilizing the joint avoidance and disaggregation bioassay. The results obtained demonstrated that Terrestrial isopods show a high degree of sensitivity to sublethal concentrations of the chemicals tested, thereby supporting their use in ecotoxicological bioassays. It is important to note that neither avoidance nor disaggregation tests are exhaustive in themselves, as some compounds may induce one response rather than the other. This reinforces the recommendation to utilise these two endpoints in combination to obtain complete and comprehensive information. Furthermore, the study revealed a relationship between the constant of Henry Law and the disaggregation effects for substances with a magnitude above the cut-off of 1 x 10^{-3} Pa m^3 mol^{-1}. This cut-off is of considerable significance in the prognostic identification of chemicals that have the potential to cause disaggregation in woodlice. It is proposed to assess the effect on soil organisms of those molecules with an H above the found threshold level to reduce over testing. It is recommended that future studies be conducted to facilitate a more comprehensive understanding of the mechanisms that underpin these behavioral alterations.

Data availability

Data will be made available on request.

References

Ågerstrand et al., 2020. Emerging investigator series: use of behavioural endpoints in the regulation of chemicals. Environ. Sci.: Processes Impacts, 22, 49-65 https://doi.org/10.1039/C9EM00463G

An, Y. J. 2005. Assessing soil ecotoxicity of methyl tert-butyl ether using earthworm bioassay; closed soil microcosm test for volatile organic compounds. Environmental Pollution, 134(2), 181-186. https://doi.org/10.1016/j.envpol.2004.08.012

Caro, G., Decaëns, T., Lecarpentier, C., Mathieu, J.2013. Are dispersal behaviours of earthworms related to their functional group? Soil Biology and Biochemistry, 58, 181-187. https://doi.org/10.1016/j.soilbio.2012.11.01

Drobne, D. 1997. Terrestrial isopods—a good choice for toxicity testing of pollutants in the terrestrial environment. Environmental Toxicology and Chemistry: An International Journal, 16(6), 1159-1164. https://doi.org/10.1002/etc.5620160610

Drobne, D., Hopkin, S. P. 1994. Ecotoxicological laboratory test for assessing the effects of chemicals on terrestrial isopods. Bulletin of Environmental Contamination and Toxicology, 53(3), 390-397. https://doi.org/10.1007/BF00197231

Edwards, C. A. 2002. Assessing the effects of environmental pollutants on soil organisms, communities, processes and ecosystems. European Journal of Soil Biology, 38(3-4), 225-231. https://doi.org/10.1016/S1164-5563(02)01150-0

EFSA 2017. European Food Safety Authority (EFSA) PPR Panel (EFSA Panel on Plant Protection Products and their Residues), Ockleford C., Adriaanse P., Berny P., Brock T., Duquesne S., Grilli S., Hernandez-Jerez A.F., Bennekou S.H.,Klein M., Kuhl T., Laskowski R., Machera K., Pelkonen O., Pieper S., Stemmer M., Sundh I., Teodorovic I.,Tiktak A., Topping C.J., Wolterink G., Craig P., de Jong F., Manachini B., Sousa P., Swarowsky K., Auteri D., Arena M., Rob S., 2017. Scientific Opinion addressing the state of the science on risk assessment of plant protection products for in-soil organisms. EFSA Journal 2017;15(2):4690. https://doi.org/10.2903/j.efsa.2017.4690

European Commission, 2025. Joint Research Centre, Baccaro, M., Focks, A., Worth, A., Chinchio, E., Dubois, G., Robuchon, M., Carletti, A., Bernasconi, C. and Bopp, S., Protection of biodiversity as the ultimate goal of environmental safety assessment: how does chemical pollution affect biodiversity, Publications Office of the European Union, Luxembourg, 2025. http://dx.doi.org/10.2760/1215173

Federico, L., Monti, G. S., Loureiro, S., Villa, S. 2024. Disaggregation behavior in the terrestrial isopod *Porcellionides pruinosus* as a new ecotoxicological endpoint for assessing infochemical disrupting activity. Ecological Indicators, 167, 112602. https://doi.org/10.1016/j.ecolind.2024.112602

Finney, D. J. 1979. Bioassay and the practice of statistical inference. International Statistical Review/Revue Internationale de Statistique, 1-12. https://doi.org/10.2307/1403201

Fisher, A., Tadei, R., Berenbaum, M., Nieh, J., Siviter, H., Crall, J., Glass, J. R., Muth, F., Liao, L., Traynor, K., DesJardins, N., Nocelli, R., Simon-Delso, N., Harrison, J. F., 2023. Breaking the cycle: Reforming pesticide regulation to protect pollinators, BioScience, 73 (11), 808–813. https://doi.org/10.1093/biosci/biad088

García-Santos, G., Keller-Forrer, K. 2011. Avoidance behaviour of *Eisenia fetida* to carbofuran, chlorpyrifos, mancozeb and metamidophos in natural soils from the highlands of Colombia. Chemosphere, 84(5), 651-656. https://doi.org/10.1016/j.chemosphere.2011.03.036

Hansson, B. S., Harzsch, S., Knaden, M., Stensmyr, M. 2010. The neural and behavioral basis of chemical communication in terrestrial crustaceans. In Chemical communication in Crustaceans (pp. 149-173). New York, NY: Springer New York. http://dx.doi.org/10.1007/978-0-387-77101-4_8

Harzsch, S., Rieger, V., Krieger, J., Seefluth, F., Strausfeld, N. J., Hansson, B. S. 2011. Transition from marine to terrestrial ecologies: changes in olfactory and tritocerebral neuropils in land-living isopods. Arthropod Structure & Development, 40(3), 244-257. https://doi.org/10.1016/j.asd.2011.03.002

Hewitt, M., Cronin, M. T., Enoch, S. J., Madden, J. C., Roberts, D. W., Dearden, J. C. 2009. In silico prediction of aqueous solubility: the solubility challenge. Journal of chemical information and modeling, 49(11), 2572-2587. https://doi.org/10.1021/ci900286s

ISO 2020 - Soil quality - Avoidance test for determining the quality of soils and effects of chemicals on behaviour - Part 1: Test with earthworms (*Eisenia fetida* and *Eisenia andrei*) (ISO 17512-1:2020).

Jones, D. T., Hopkin, S. P. 1996. Reproductive allocation in the terrestrial isopods *Porcellio scaber* and *Oniscus asellus* in a metal-polluted environment. Functional Ecology, 741-750. https://doi.org/10.2307/2390509

Klátyik, S., Simon, G., Oláh, M., Mesnage, R., Antoniou, M. N., Zaller, J. G., & Székács, A. 2023. Terrestrial ecotoxicity of glyphosate, its formulations, and co-formulants: evidence from 2010–2023. Environmental Sciences Europe, 35(1), 1-29. https://doi.org/10.1186/s12302-023-00758-9

Kleiner, E. J., Sanan, T., Smith, S. J., Pressman, J. G., Abulikemu, G., Crone, B. C., Wahman, D. G. 2021. Practical implications of perfluoroalkyl substances adsorption on bottle materials: Isotherms. AWWA water science, 3(5), e1243. https://doi.org/10.1002/aws2.1243

Koenker, R., Bassett, G. 1978. Regression Quantiles. Econometrica 46, no. 1: 33–50. https://doi.org/10.2307/1913643

Lokke, H., Gestel, C. V. (Eds.) 1998. Handbook of soil invertebrate toxicity tests (pp. xxii+-281)

Loureiro, S., Soares, A. M., Nogueira, A. J. 2005. Terrestrial avoidance behaviour tests as screening tool to assess soil contamination. Environmental pollution, 138(1), 121-131. https://doi.org/10.1016/j.envpol.2005.02.013

Loureiro, S., Amorim, M. J., Campos, B., Rodrigues, S. M., Soares, A. M. 2009. Assessing joint toxicity of chemicals in *Enchytraeus albidus* (Enchytraeidae) and *Porcellionides pruinosus* (Isopoda) using avoidance behaviour as an endpoint. Environmental Pollution, 157(2), 625-636. https://doi.org/10.1016/j.envpol.2008.08.010

Maria, V. L., Santos, J., Prodana, M., Cardoso, D. N., Morgado, R. G., Amorim, M. J., Barreto, A. 2024. Toxicity mechanisms of plastic nanoparticles in three terrestrial species: antioxidant system imbalance and neurotoxicity. Nanotoxicology, 18(3), 299-313. https://doi.org/10.1080/17435390.2024.2358781

McFadden, D. (1974). Conditional Logit Analysis of Qualitative Choice Behavior. In P. Zarembka (Ed.), Frontiers in Econometrics (pp. 105–142). Academic Press.

Melo, T. M., Schauerte, M., Bluhm, A., Slaný, M., Paller, M., Bolan, N., Bosch, J., Fritzsche, A., Rinklebe, J. 2022. Ecotoxicological effects of per-and polyfluoroalkyl substances (PFAS) and of a new PFAS adsorbing organoclay to immobilize PFAS in soils on earthworms and plants. Journal of hazardous materials, 433, 128771. https://doi.org/10.1016/j.jhazmat.2022.128771

Morgado, R. G., Gomes, P. A., Ferreira, N. G., Cardoso, D. N., Santos, M. J., Soares, A. M., Loureiro, S. 2016. Toxicity interaction between chlorpyrifos, mancozeb and soil moisture to the terrestrial isopod *Porcellionides pruinosus*. Chemosphere, 144, 1845-1853. https://doi.org/10.1016/j.chemosphere.2015.10.034

Niemeyer, J. C., de Santo, F. B., Guerra, N., Ricardo Filho, A. M., Pech, T. M. 2018. Do recommended doses of glyphosate-based herbicides affect soil invertebrates? Field and laboratory screening tests to risk assessment. Chemosphere, 198, 154-160. https://doi.org/10.1016/j.chemosphere.2018.01.127

Oehlert, G. W. 1992. A Note on the Delta Method. The American Statistician, 46(1), 27–29. https://doi.org/10.1080/00031305.1992.10475842

Oliveira, M., Cardoso, D. N., Soares, A. M. V. M., Loureiro, S. 2015. Effects of short-term exposure to fluoxetine and carbamazepine to the collembolan *Folsomia candida*. Chemosphere, 120, 86-91. https://doi.org/10.1016/j.chemosphere.2014.06.038

Peijnenburg, W., Capri, E., Kula, C., Liess, M., Luttik, R., Montforts, M., Nienstedt, K., Römbke, J., Sousa, J.P., Jensen, J. 2012. Evaluation of exposure metrics for effect assessment of soil invertebrates. Critical Reviews in Environmental Science and Technology, 42(17), 1862-1893. https://doi.org/10.1080/10643389.2011.574100

Pereira, C. M., Novais, S. C., Soares, A. M., Amorim, M. J. 2013. Dimethoate affects cholinesterases in *Folsomia candida* and their locomotion—False negative results of an avoidance behaviour test. Science of the Total Environment, 443, 821-827. https://doi.org/10.1016/j.scitotenv.2012.11.044

R Core Team. 2024. *R: A language and environment for statistical computing*. R Foundation for Statistical Computing, Vienna, Austria. https://www.R-project.org/

Rodríguez-Eugenio, N., McLaughlin, M. Pennock, D. 2018. Soil Pollution: a hidden reality. Rome, FAO. 142 pp. https://openknowledge.fao.org/items/012e0992-7d74-4ee1-9ac1-204b6b32906f

Rousseeuw, P. J., Leroy, A. M. 2006. Robust Regression and Outlier Detection. Wiley Series in Probability and Statistics. John Wiley & Sons. http://dx.doi.org/10.1002/0471725382

Santos, M. J., Soares, A. M., Loureiro, S. 2010. Joint effects of three plant protection products to the terrestrial isopod *Porcellionides pruinosus* and the collembolan *Folsomia candida*. Chemosphere, 80(9), 1021-1030. https://doi.org/10.1016/j.chemosphere.2010.05.031

Santos, M. J. G., Ferreira, M. F. L., Cachada, A., Duarte, A. C., Sousa, J. P. 2012. Pesticide application to agricultural fields: effects on the reproduction and avoidance behaviour of *Folsomia candida* and *Eisenia andrei*. Ecotoxicology, 21, 2113-2122. https://doi.org/10.1007/s10646-012-0963-7

Seelinger, G. 1983. Response characteristics and specificity of chemoreceptors in *Hemilepistus reaumuri* (Crustacea, Isopoda). Journal of comparative physiology, 152, 219-229. https://doi.org/10.1007/BF00611186

Takeda, N. 1980. The aggregation pheromone of some terrestrial isopod crustaceans. Experientia, 36(11), 1296-1297. https://doi.org/10.1007/BF01969598

Tetko, I. V. 2007. The prediction of physicochemical properties. Computational toxicology: risk assessment for pharmaceutical and environmental chemicals, 240-275. 10.1002/9780470145890.ch9

Van Gestel, C. A., Loureiro, S., Zidar, P. 2018. Terrestrial isopods as model organisms in soil ecotoxicology: a review. ZooKeys, (801), 127. https://doi.org/10.3897/zookeys.801.21970

Vieira, D. C. S., Yunta, F., Baragaño, D., Evrard, O., Reiff, T., Silva, V., de la Torre, A., Zhang, C., Panagos, P., Jones, A., Wojda, P. 2024. Soil pollution in the European Union–An outlook. Environmental Science & Policy, 161, 103876. https://doi.org/10.1016/j.envsci.2024.103876

Vighi, M., Villa, S. 2013. Ecotoxicology: The challenges for the 21st century. Toxics, 1(1), 18-35. https://doi.org/10.3390/toxics1010018

Voinorosky, C. L., Standen, K. M., Stewart, K. J. 2022. Environmental Impact of Triclopyr on Habitat Quality in Boreal Rights-of-Way. Environmental Toxicology and Chemistry, 41(12), 2955-2967. https://doi.org/10.1002/etc.5475

Wang, W., Zhang, J., Wu, J., Yu, R., Zhang, Y., Sun, L., Gao, Y. 2021. Acute toxicity and ecotoxicological risk assessment of three volatile pesticide additives on the earthworm—*Eisenia fetida*. International Journal of Environmental Research and Public Health, 18(21), 11232. https://doi.org/10.3390/ijerph182111232

Xu, D., Wen, Y., Wang, K. 2010. Effect of chiral differences of metolachlor and its (S)-isomer on their toxicity to earthworms. Ecotoxicology and Environmental Safety, 73(8), 1925-1931. https://doi.org/10.1016/j.ecoenv.2010.07.035

Yohai, V. J. 1987. High breakdown-point and high efficiency robust estimates for regression. The Annals of Statistics, 15(2), 642–656. https://doi.org/10.1214/aos/1176350366

Zang, Q., Mansouri, K., Williams, A. J., Judson, R. S., Allen, D. G., Casey, W. M., Kleinstreuer, N. C. 2017. In silico prediction of physicochemical properties of environmental chemicals using molecular fingerprints and machine learning. Journal of chemical information and modeling, 57(1), 36-49. https://doi.org/10.1021/acs.jcim.6b00625

Zhiqun, T., Jian, Z., Junli, Y., Chunzi, W., Danju, Z. 2017. Allelopathic effects of volatile organic compounds from *Eucalyptus grandis* rhizosphere soil on *Eisenia fetida* assessed using avoidance bioassays, enzyme activity, and comet assays. Chemosphere, 173, 307-317. https://doi.org/10.1016/j.chemosphere.2017.01.004

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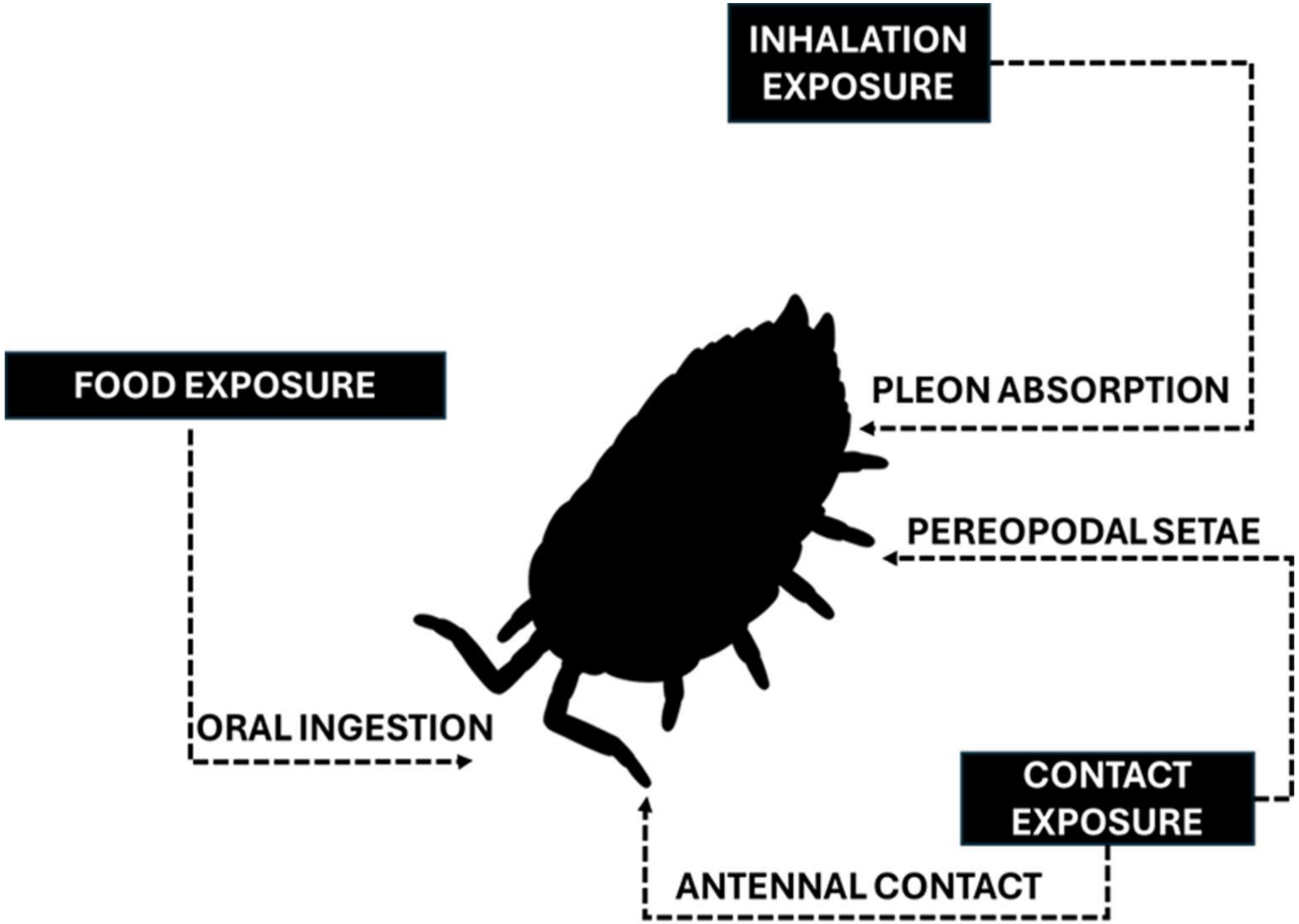
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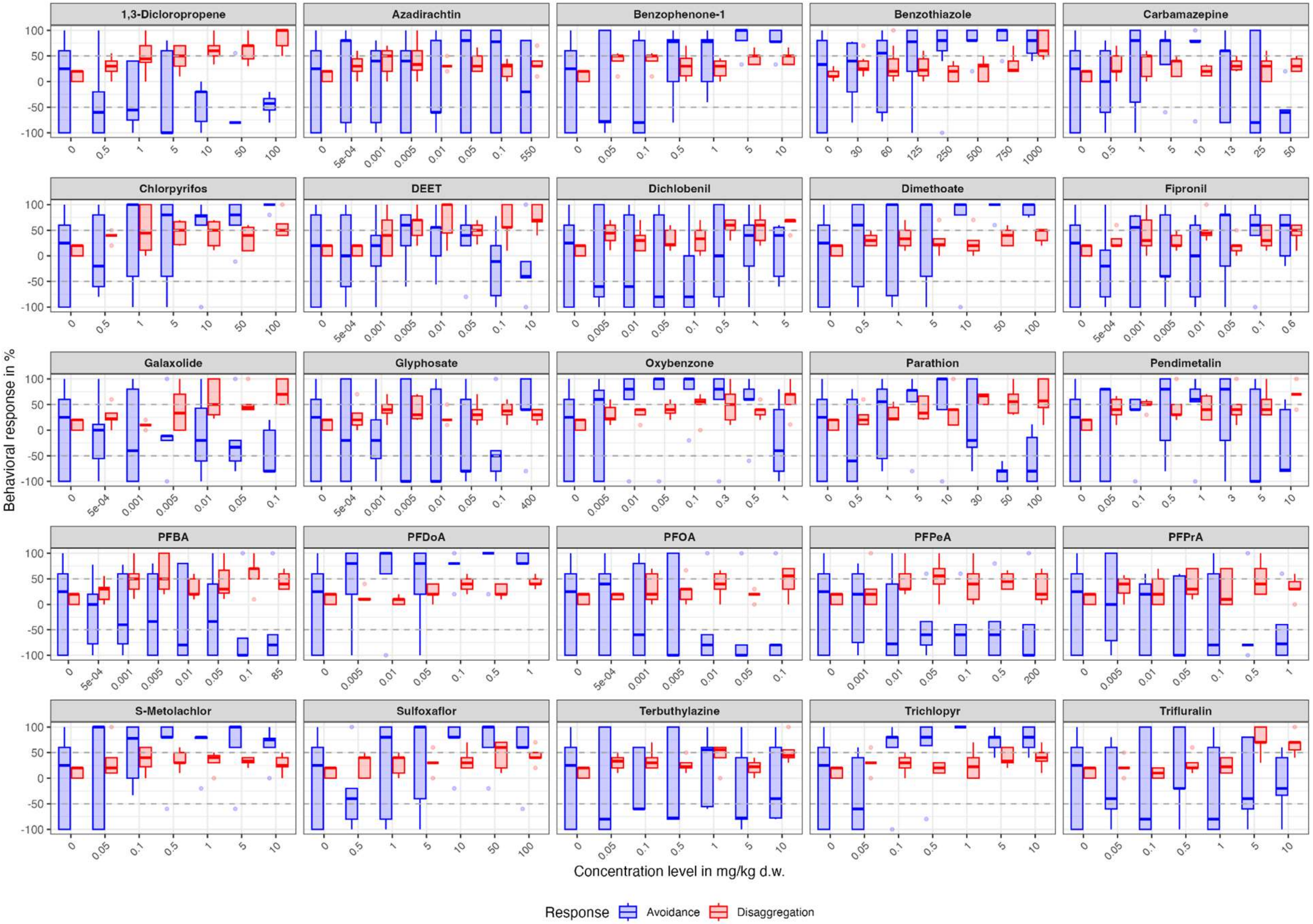
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Parathion
Pendimetalin
PFBA
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PFOA
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PFPrA
S-Metolachlor
Sulfoxaflor
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Trichlopyr
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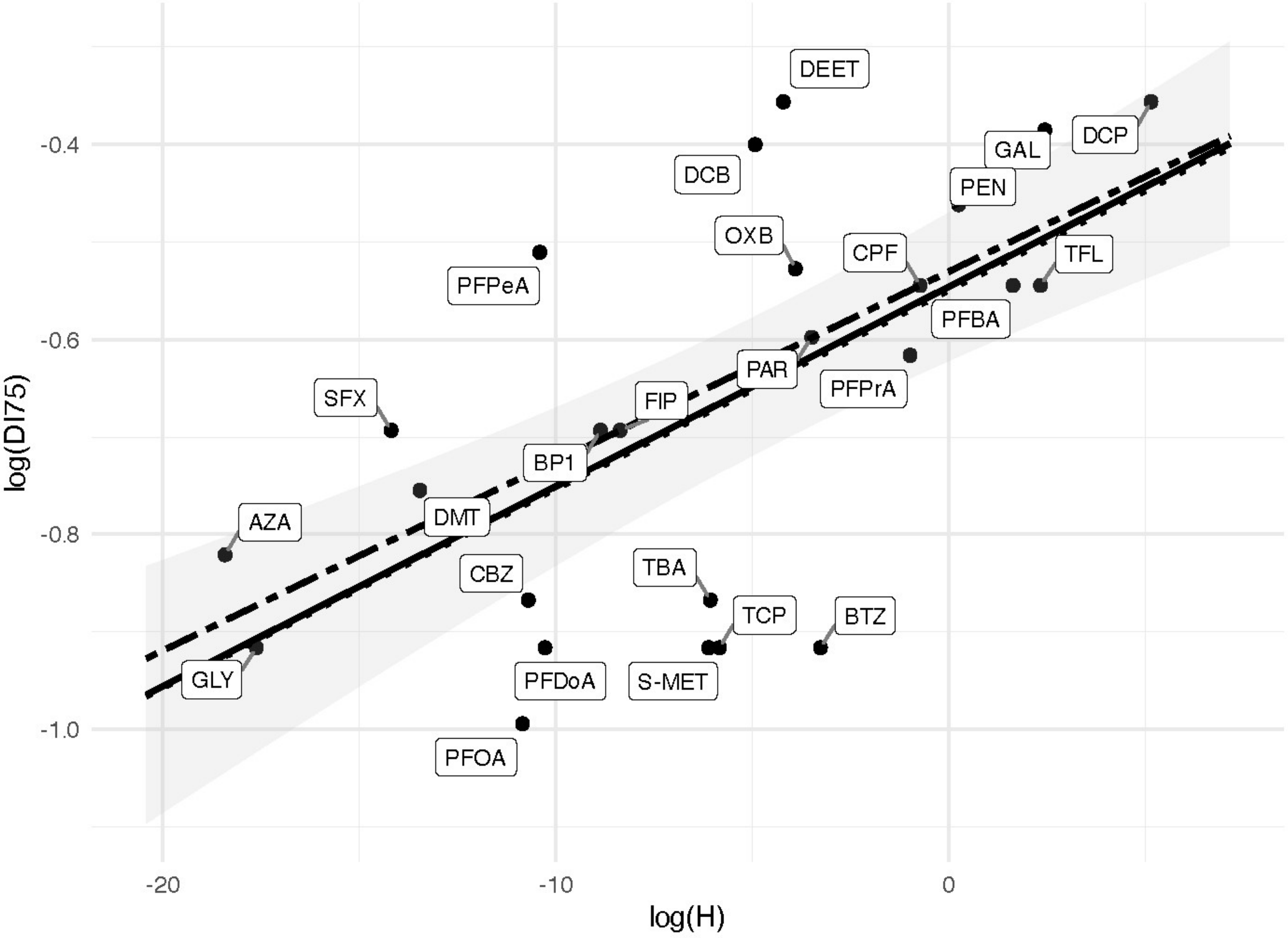
Disaggregation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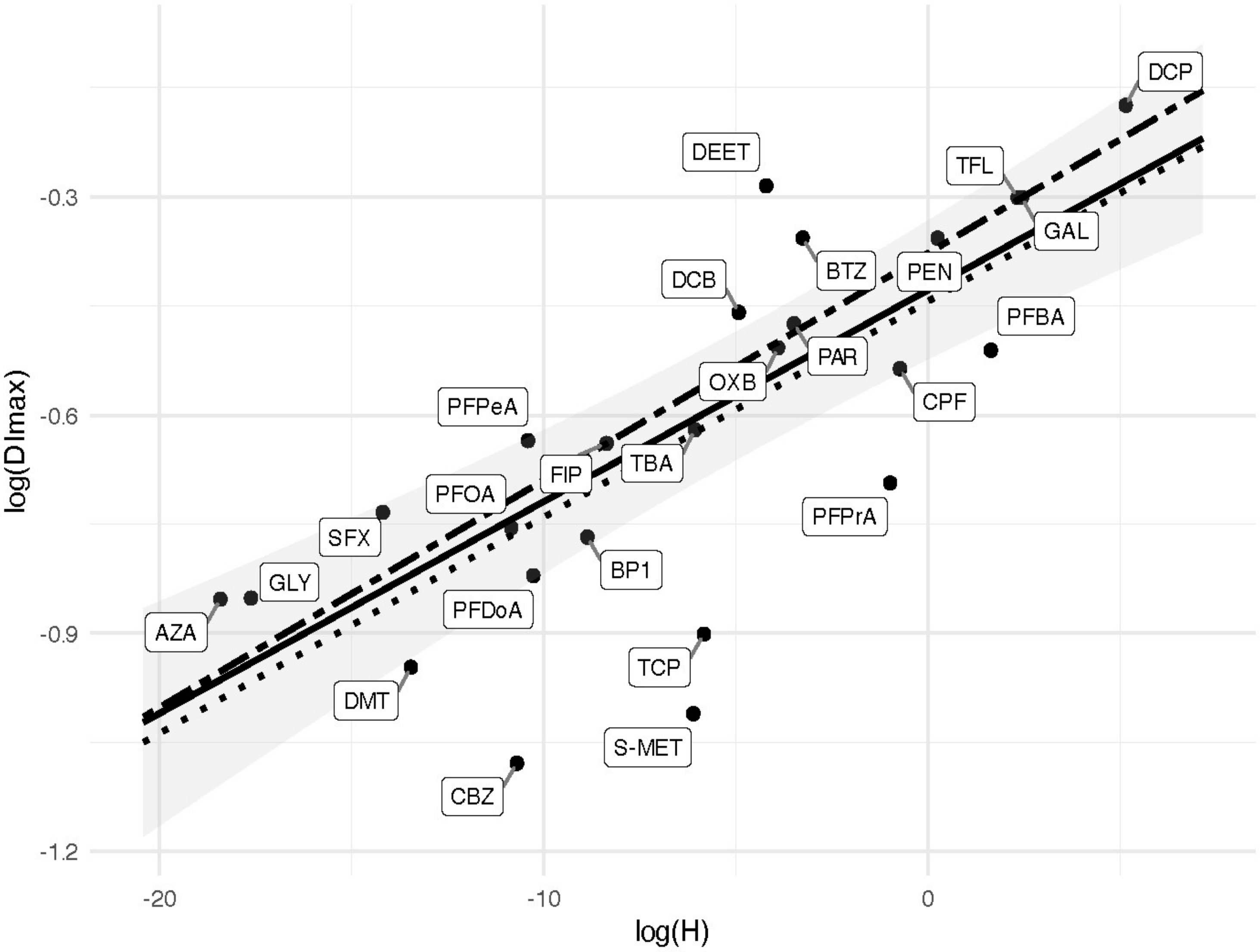
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AZA
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DMT
S-MET
CBZ
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-0.6
-0.9
-1.2
log(DImax)
-20
-10
0
log(H)

Potential of Henry's Law Constant as a Predictive Indicator of Behavioural Effect in *Porcellionides pruinosus*

Supplementary Materials

Table S1. Selected test compounds and relevant properties are classified as Plant Protection Products (PPPs), Pharmaceutical and Personal Care Products (PhPCPs), and perfluorocarboxylic acid (PFCAs). There are also reported: Chemical Abstract Service number (CAS N); concentration range tested (C); molecular weight (MW); density (D); solubility (S); vapor pressure (VP); Henry Law constant (H); logarithmic octanol-water ($\log K_{ow}$), and organic carbon-water ($\log K_{oc}$) partition coefficients.

Chemical acronym	**Class**	**CAS N**	**C** (mg/kg d.w.)	**MWs** (g mol^{-1})	**D** (g cm$^{3-1}$)	**S** (g L^{-1})	**VP** (mPa)	**H** (Pa m^3 mol^{-1})	**logKow**	**logKoc**	**REF**
AZA	PPPs	11141-17-6	0.0005-0.1	720.71	1.2	2,90E+00	3,60E-10	1,00E-08	0.99	2.26	1
BP-1	PhPCPs	131-56-6	0.05-10	182.22	1.27	2,36E-01	1,40E+01	1,40E-04	2.96	3.09	3
BTZ	PPPs	95-16-9	30-1000	135.19	1.27	4,30E+00	1,90E+02	3,79E-02	2.01	2.73	1; 2
CBZ	PhPCPs	298-46-4	0.5-50	236.27	1.25	2,00E-01	6,00E-07	2,24E-05	1.9	2.74	3
CPF	PPPs	2921-88-2	0.5-100	350.58	1.51	1,00E-03	1,43E+00	4,78E-01	4.7	3.74	1
DCB	PPPs	1194-65-6	0.005-5	172.01	1.37	3,96E-02	1,10E+00	7,20E-03	3.3	2.41	1
DCP	PPPs	542-75-6	0.5-100	111.37	1.22	2,45E+00	3,76E+06	1,70E+02	1.82	1.67	1
DEET	PhPCPs	134-62-3	0.0005-10	191.27	0.99	9,12E-01	1,10E-01	1,47E-02	2.4	1.64	1; 3
DMT	PPPs	60-51-5	0.5-100	229.26	1.31	2,59E+01	2,50E-03	1,42E-06	0.75	1.22	1; 3
FIP	PPPs	120068-37-3	0.0005-0.6	437.15	1.71	3,78E-03	2,00E-06	2,31E-04	3.75	3.72	1; 3

GAL	PhPCPs	1222-05-05	0.0005-0.1	258.4	1	1,65E-03	7,30E-02	1,14E+01	5.3	4.15	2
GLY	PPPs	1071-83-6	0.0005-0.1	169.1	1.71	1,00E+02	1,31E-05	2,21E-08	-6.28	3.15	1
OXB	PhPCPs	131-57-7	0.005-1	228.24	1.43	6,00E-03	1,00E+00	2,00E-02	3.45	2.98	2
PAR	PPPs	56-38-2	0.5-100	291.27	1.26	1,24E-02	8,90E-01	3,02E-02	3.83	3.88	1
PEN	PPPs	40487-42-1	0.05-10	281.31	1.17	3,30E-04	3,40E-03	1,27E+00	5.4	4.24	1
PFBA	PFAS	375-22-4	0.0005-0.1	214	1.65	4,50E-01	2,13E-03	5,07E+00	2.82	1.94	3
PFDoA	PFAS	307-55-1	0.005-1	614.1	1.82	7,55E-06	7,21E+01	3,43E-05	8.27	4.93	3
PFOA	PFAS	335-67-1	0.0005-0.1	414.07	1.8	2,60E-05	2,43E-07	1,93E-05	3.1	3.06	3
PFPeA	PFAS	2706-90-3	0.001-0.5	264.05	1.67	1,05E+00	6,86E-01	2,99E-05	3.36	1.98	3
PFPrA	PFAS	44864-55-3	0.005-1	163.02	1.64	2,43E+01	3,07E+00	3,70E-01	1.9	0.77	3
SFX	PPPs	946578-00-3	0.5-100	277.27	1.52	5,68E-01	1,40E-06	6,83E-07	0.8	1.61	1
S-MET	PPPs	87392-12-9	0.05-10	283.79	1.12	4,80E-01	3,70E+00	2,20E-03	3.05	2.43	1; 3

TBA	PPPs	5915-41-3	0.05-10	230.11	1.19	6,60E-03	1,52E-04	2,30E-03	3.4	0.93	1; 2
TCP	PPPs	55335-06-03	0.05-10	256.47	1.3	8,10E+00	2,00E-01	2,90E-03	-0.45	1.43	1
TFL	PPPs	1582-09-08	0.05-10	335.28	1.36	2,21E-04	9,50E+00	1,02E+01	5.27	4.2	1

References of database consulted:

1 Hertfordshire University, Database: https://sitem.herts.ac.uk/aeru/ppdb/en/

2 European Chemical Agency, ECHA, Dataset: https://echa.europa.eu/de/information-on-chemicals

3 U.S. Environmental Protection Agency, EPA, Dataset: https://comptox.epa.gov/dashboard/

Acronyms:

1,3-dichloropropene (DCP), azadirachtin A (AZA), benzophenone-1 (BP-1), oxybenzone (OXB), benzothiazole (BTZ), carbamazepine (CBZ), chlorpyrifos (CPF), dichlobenil (DCB), N,N-diethyltoluamide (DEET), dimethoate (DMT), fipronil (FIP), glyphosate (GLY), galaxolide (GAL) ,parathion (PAR), pendimethalin (PEN), perfluorobutanoic acid (PFBA), perfluorododecanoic acid (PFDoA), perfluorooctanoic acid (PFOA), perfluoropentanoic acid (PFPeA), perfluoropropionic acid (PFPrA), sulfoxaflor (SFX), S-metolachlor (S-MET), terbuthylazine (TBA), triclopyr (TCP), trifluralin (TFL).

Table S2. Association between log-transformed Henry's law constant and log-transformed disaggregation index (DI75) across regression models. Note that for robust regression, approximate inference can be conducted using t-statistics derived from the estimated coefficients and their standard errors. However, such p-values should be interpreted with caution due to the robustness adjustments.

Model	**β**	**SE**	**p-value**
Ordinary Least Squares (OLS)	0.02	0.01	< 0.001
Quantile Regression (τ = 0.5)	0.02	0.01	0.02
Robust Regression (MM)	0.02	0.01	< 0.01

Table S3. Model performance metrics (MAE, RMSE, and Pseudo-R2) obtained through leave-one-out cross-validation (LOOCV) for OLS, quantile regression (τ = 0.5), and robust regression (MM-type estimator).

Regression Model	**MAE**	**RMSE**	**Pseudo-R^2**
OLS	0.137	0.167	0.311
Quantile (τ=0.5)	0.135	0.167	0.311
Robust	0.137	0.167	0.307

Chapter 6

The potential use of behavioral bioassays as a first-tier approach for screening urban soil biodiversity: a pilot study

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RESEARCH

The potential use of behavioural bioassays as a first-tier approach for screening urban soil biodiversity: a pilot study

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Abstract

Soil health represents a key component of urban ecosystems and a priority for achieving the European Union's climate neutrality and biodiversity restoration goals. Changes in edaphic diversity are the first indicators of soil health, but require long investigation times. Therefore, rapid, multi-level, and low-impact diagnostic tools are required. Behavioural bioassays, including avoidance and disaggregation tests, serve as rapid and ecologically relevant indicators for identifying soils subject to population decline. However, the metrics of in situ biodiversity loss and the laboratory-based ecotoxicological responses are not aligned. This pilot study investigates the potential use of the behavioural endpoints as screening indicators of biodiversity in invertebrate and bacterial communities in three urban soils. Multi-species bioassays were employed using model organisms with contrasting morpho-ecological traits, i.e. soft-body (earthworms) and hard-body (collembolans, and terrestrial isopods), to evaluate soil quality gradients. The behavioural results were then compared with ecological biodiversity data concerning the soil fauna and microbial communities. The behavioural responses of model organisms consistently aligned with reductions in invertebrate biodiversity, indicating habitat population decline. These changes, however, did not emerge from microbial analysis, suggesting that links between organismal responses and microbial diversity are yet to be investigated. The results support the use of behavioural bioassays, in combination with faunal diversity assessments, as an effective first-tier screening tool for urban soil health evaluation. This multi-level framework enhances the resolution and efficiency of soil quality monitoring and supports targeted management interventions in degraded urban environments, as well as in peri-urban, agricultural, and other human-impacted landscapes.

Keywords Behavioural test · Soil biodiversity · Porcellionides pruinosus · Folsomia candida · Eisenia fetida · Urban ecosystems

Introduction

Recent years have seen a heightened focus on monitoring and remediating soil ecosystems, driven by the need to implement effective environmental strategies for ecological transition and to counteract climate change effects (Bowler et al. 2010). A significant development in this context is the adoption by the European Union of legislation to make soil health monitoring mandatory, including the proposed directive on Soil Monitoring and Resilience presented by the European Commission (EC 2025a). This directive establishes guiding principles for sustainable soil management and addresses situations where soil stressors pose unacceptable health and environmental risks. Since more than 60% of European soils are depleted, monitoring and prioritisation strategies are required to assess and achieve the agreed EU climate and biodiversity goals. Concurrently, the global community has recognised the urgency of soil ecosystem restoration, culminating in the establishment of the UN Decade on Ecosystem Restoration, which aims to encourage a coordinated and comprehensive approach to the restoration of degraded ecosystems and stem the rapid decline of biodiversity (UNEP and FAO 2020). Among the main stressors, pollution has a significant impact on soil health (Vieira

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et al. 2024), and the development of a framework for the regular assessment of soil pollution is strongly urged by the European Commission, in line with the Zero Pollution Action Plan (EC 2021).

To provide an initial snapshot of soil quality, the study of changes in edaphic communities represents a first relevant approach (Linden et al. 1994; Schloter et al. 2003; Santorufo et al. 2012). Quantifying both invertebrate and microbial biodiversity is necessary to evaluate soil health, and represents the key element of the Nature Restoration Law (EU, 2024), and the Sustainable Development Goals (SDGs) (Sachs 2012). Despite their importance, diversity analyses are primarily diagnostic and demand substantial time and costs. Therefore, it is crucial to implement rapid and cost-effective prognostic strategies to effectively support these studies.

Behavioural bioassays of edaphic organisms represent ecologically relevant indicators of changes in soil conditions (Coyle et al. 2017). Among the behavioural tests, those related to avoidance responses (ISO 2020a, 2020b) and alteration of gregariousness in social edaphic organisms (Federico et al. 2024) can be used as prognostic early-warning tools to detect effects on population decline and/or fragmentation at population level. Since these behavioural traits are indicative of limited habitat function, soils that induce such responses are expected to support reduced biodiversity, as more sensitive and vulnerable species will avoid threatened conditions. Most soil taxa exhibit limited long-distance mobility and are therefore unable to migrate across broader spatial scales (Coyle et al. 2017). Avoidance responses are expressed as active displacement away from stressed soils (Gainer et al. 2022), resulting in reduced residence time and constrained colonisation of affected patches. In contrast, disaggregation reflects a fragmentation of the gregarious behaviour of isopods, which may occur both within stressed soils and in adjacent buffer zones, where individuals redistribute locally rather than abandoning the area entirely (Federico et al. 2024). It follows that over time, such responses can result in local redistribution, population decline, and loss from the community under continued environmental stress, even in the absence of large-scale dispersal. Consequently, soils that elicit pronounced avoidance or altered social behaviours are likely to exhibit reduced local diversity, not only through migration but also through decreased activity, limited population establishment, and gradual species loss. As recently highlighted by the European Commission (EC 2025b), metrics of biodiversity loss remain poorly aligned with laboratory-based ecotoxicological endpoints. Addressing this gap, the present study moves beyond a priori assumptions by empirically linking behavioural responses to the diversity of invertebrate and bacterial communities in urban soils. The ultimate goal is to contribute to the identification of a tiered and targeted approach, balancing the speed and cost-efficiency of ecotoxicological bioassay with the complexity of biodiversity analysis, in order to guide decision-making in prioritising areas for further investigation.

Materials and methods

Study area and soil properties

Three distinct urban areas under greening strategies (designated as U2, U3, and U4) situated at the Milano Bicocca University campus (Italy, latitude: 45° 30‘49.0497’; longitude: 9° 12‘40.9114’) were considered as a case study (Fig. 1). A comprehensive description of the university square can be found in Picot (2004). Briefly, these patches boast identical extension (15 × 30 m) and share comparable vegetation species but U2 garden is distinguished by its shaded status due to its proximity to university buildings, and such distinct solar irradiance has been resulted in varied levels of student presence, since U3 and U4 areas have been noted to experience a higher influx of students compared to U2, which area remained inaccessible since the 2022 year. Soil sampling activity was conducted in April 2024. Within each green area, a 5 × 10 m grid was established to delineate different plots. Three plots (A, B, and C), representing distinct spatial zones (one central and two peripheral), were selected. Within each selected plot, six replicate samples of approximately 1 kg (10 × 10 × 10 cm) were randomly collected, excluding litter, resulting in a total of 18 samples per green area. Three replicates of each plot were designated for biological behavioural assays and physicochemical characterization, while each of the remaining three replicates was split for the assessment of soil pedofauna structural and functional diversity (QBS-ar) and for microbial diversity analyses. Soil samples were collected using a field shovel cleaned with an ethanol–water solution (70:30, v/v) prior to each sampling to prevent cross-contamination and transported to the laboratory in black plastic bags for subsequent analyses. Aliquots of sampled soils were analysed in terms of texture, water holding capacity (WHC), pH, and soil organic matter (SOM) (Supplementary Materials - SM1). These parameters were considered as the main core descriptors of soil health and habitat quality according to the Soil Monitoring and Resilience Directive (EC 2025a). Before each analysis, all samples were allowed to dry under a hood for evaporation of the water content, and then placed in an oven at 105 °C overnight for total drying.

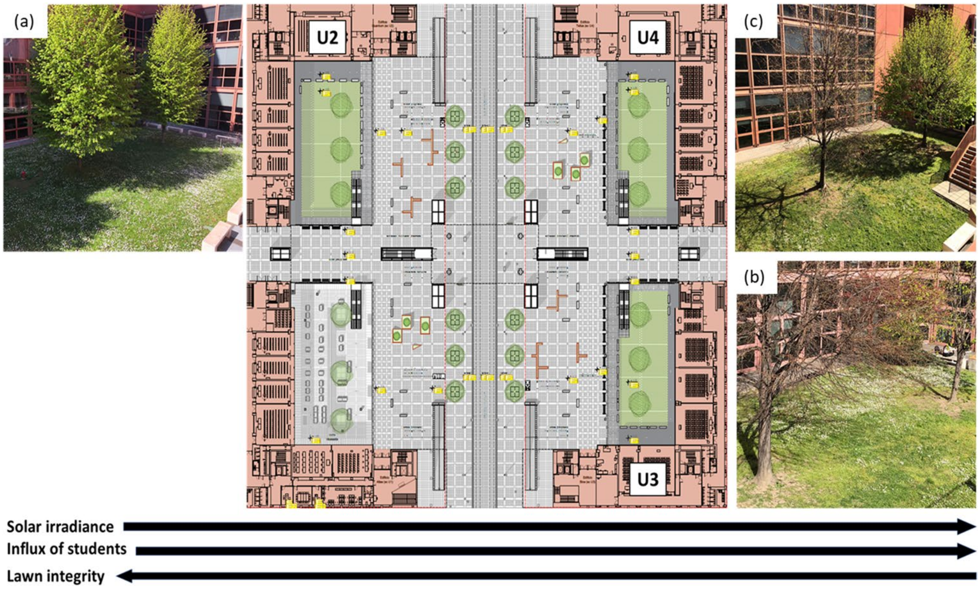


Fig. 1 Map of Piazza della Scienza with the respective green areas, designated as U2 (**a**), U3 (**b**) and U4 (**c**), located within the University of Milan Bicocca campus. The three green areas are characterised by different irradiation gradients, afflux of university community and lawn integrity (black arrows). Source of floor plan: Studio Aegis, Brescia, Italy

Photosynthetic efficiency

In each green area of the campus there are three individuals of *Tilia spp*. on which photosynthetic efficiency parameters were measured during the same sampling period, in order to confirm any differences induced by solar exposure levels. By measuring chlorophyll fluorescence efficiency, it is possible to obtain information on photosynthetic activities, since the intensity of fluorescence emission is inversely proportional to the amount of solar radiation used during photosynthetic processes (Maxwell and Johnson 2000). Leaves were taken from branches in a position of full light, at the top of the canopy, potentially subjected to a maximum photosynthetic activity (Gottardini et al. 2016). For each individual, 15 leaves were collected and chlorophyll a fluorescence was measured with the Handy-PEA fluorometer (Hansatech Instruments, Pentney, Norfolk, UK). Among the parameters measured by the instrument, Fv/Fm is the ratio of variable fluorescence (Fv) to maximum fluorescence (Fm) after dark adaptation, representing the maximum quantum yield of photosystem II. This parameter is the most widely used to investigate the photosynthetic aspects of a plant species (Sepúlveda and Johnstone 2018; Callow et al. 2018).

Behavioural bioassays

Individuals of *Porcellionides pruinosus* (Brandt, 1833), *Eisenia fetida* (Savigny, 1826), and *Folsomia candida* (Willem, 1902) were provided from the Laboratory of Ecotoxicology of Milano Bicocca University (Italy) (SM2). All the model organisms were used for performing the avoidance bioassays, following the protocol outlined in ISO guidelines (2020a, 2020b), while only terrestrial isopods were used for investigation of the avoidance and disaggregation effect (Federico et al. 2024). The soils sampled from the green areas (U2, U3 and U4) were compared with standard laboratory soil (LUFA 2.2), in order to evaluate the attractive, elusive or indifferent responses of the tested species to urban soils. Plastic boxes (170 × 120 mm) were used as test arena for earthworms and terrestrial isopods, and Petri dishes (⌀ = 100 mm) for collembolans. In one side of the container, 100, 50, and 10 g d.w. of sample soils per each green area and replicates were added, in the earthworms, terrestrial isopods, and collembola bioassays respectively. On the other side, the same relative quantity of soils was filled using the standard LUFA Speyer 2.2 soil (batch no. SP2.2 2123). The chemical and physical characteristics of this standard soil are reported in SM2. Dual controls were performed using only LUFA 2.2 soil on both sides of the box to infer the homogeneous distribution of the organisms and validate the tests. The moisture related to the experiments of terrestrial isopods, collembolans, and earthworms was maintained at WHC of 40%, 50%, and 60%, respectively. For each experiment, ten individuals of the representative species were added separately in any tests and five replicates were performed for statistical reasons. The tests were carried out in thermostatic chambers at 21 ± 2 °C, photoperiod 16:8 h light: dark for 48 h. During the test, the animals were not fed. After 48 h, plastic boxes were gently removed from the thermostatic chambers, and high-resolution colour pictures were taken and processed for the statistical analysis. Avoidance behaviour was expressed as Avoidance (A%):

$$A = \frac{C - T}{n} \times 100 \tag{1}$$

Where C = number of individuals located in the LUFA reference soil compartment, T = number of individuals located in the sampled soils, and n = the total number of live individuals at the end of the experiment. Positive values indicate avoidance behaviour, whereas negative values indicate attraction to the urban soil. Non-avoidance occurs when the distribution of organisms is approximately equal between treated and control soils. Sample soils with less than 20% of individuals were considered as having "limited habitat functions" (ISO 2020a, 2020b).

The disaggregation effects were measured by the disaggregation index (DI):

$$DI = \frac{s + 2d}{n} \times 100 \qquad (2)$$

were s = number of groups with only 1 individual, d = number of groups with only 2 individuals, and n = the number of alive individuals at the end of the experiment. The index varies between 0 and 100, which represent the maximum degree of aggregation and disaggregation, respectively, while 50 was fixed as the threshold level above which disaggregation affects 50% of the population. Further details referred to the index can be found in Federico et al. (2024).

Biodiversity analysis

Before soil arthropods extraction using the Berlese-Tullgren method, any earthworms (Lumbricinae) and snails (Gasteropoda) were removed manually and counted from each soil sample. Soil arthropods extraction was conducted within 48 h of the sampling time using a Berlese-Tullgren extractor, following the methodology outlined in Parisi et al. (2005). The extractor was basically composed by a sieve (mesh of 2 mm, ⌀ = 20 cm), resting on a plastic funnel whose end part is inserted inside a plastic container filled with 2:3 ethanol and 1:3 of glycerol. From the freshly sampled soil samples, 950 g were placed inside the sieve and placed under incandescent lamps (40–60 W) at 30 cm of distance. In this way, the soils gradually dry out and the aridity of the soil forces the fauna present in the sample to avoid towards the depth of the sieve, until they fall and are captured inside the container with the alcohol and glycerol solution. The extraction of edaphic organisms took place for 7 days. The collected organisms were therefore analysed under a stereomicroscope at low magnification and classified at order/class level according to the major taxonomic groups listed in the standard table of Parisi et al. (2005). The remaining 40 g of soil from each replicate was used for the extraction of enchytraeids and nematodes, with 20 g allocated for each extraction. For enchytraeids, soil was mixed with 96% ethanol (1:5 ratio), topped with distillate water, and stained with 10 drops of rose Bengal (Pereira et al., 2018). Nematodes were extracted for 7 days using the tray method (McSorley 2000).

For each sample, the total number of individuals (N), taxa (S), and density (ρ) per volume of soil extracted (N/m^3) were assessed. The levels of edaphic biodiversity of the soil meso and macrofauna were quantified using structural synthetic diversity indices (Table 1).

The A/C ratio structural index (Bachelier 1986) was calculated based on the most abundant group of arthropods, Acarina (A) and Collembola (C), respectively. Functional metric relating to the QBS-ar index (acronym of Soil Biological Quality based on arthropods) was calculated through the sum of the ecomorphological indices (EMI) for each arthropod detected on each soil (Parisi et al. 2005). The EMI value ranges from 1 (epigeous species) to 20 (euedaphic species). Some taxonomic groups have a single EMI value because all species within the group exhibit the same level of adaptation to soil, whereas other groups are characterized by a range of EMI values, reflecting different degrees of soil adaptation among species (Menta et al. 2018). The QBS-ar value for each green area was calculated by summing the EMI values assigned to the taxa identified in the extracted samples. When more than one EMI value was attributed to the same taxon, only the highest EMI value was considered in the QBS-ar calculation.

Molecular analysis

The diversity of bacterial communities in the soil can reflect important ecological functions and is closely linked to habitat quality and the availability of resources for soil fauna (Van Elsas et al. 2006; Hermans et al. 2017). For this reason, we investigated whether the characterization

Table 1 List of utilised structural indices, where N is the total number of individuals, S the number of taxa, $pi$the relative abundances, and H, H_1, and H_2 represent the surrogate hill's numbers for richness, Shannon-Wiener and Simpson indices, respectively

Structural Indices	References	Formula	
Richness indices	Margalef (1958)	$d = \frac{S-1}{lnN}$	(Eq. 3)
Abundance indices	Shannon-Wiener (1948)	$H = \sum_{i=1}^{s} - piln(pi)$	(Eq. 4)
	Simpson (1949)	$D = \sum_{i=1}^{s} (pi)^2$	(Eq. 5)
Absolute Effective Diversity indices (AED)	Gatti et al. (2020)	$AED = H + \frac{H_1^2}{2H_2}$	(Eq. 6)

of the soil bacterial community could reflect the responses provided by behavioural tests. Ten grams of soil from each of the three samples per plot in each green area were pooled together, homogenised, and DNA was extracted from 0.5 g of soil from each pool with the FastDNA™ SPIN Kit for Soil (MP Biomedicals). The characterisation of soil bacterial communities was achieved by sequencing the V5-V6 hypervariable regions of the 16 S rRNA, as outlined by Gandolfi et al. (2024). Amplicon sequence variants (ASVs) were then inferred with a divisive amplicon denoising (DADA2) algorithm (version 1.30.0), as described by Callahan et al. (2016). Forward reads were truncated to 180 bp and reverse reads to 150 bp, Reads containing any ambiguous base calls (Ns) were discarded, reads with an expected number of errors greater than 0.5 were removed for both forward and reverse reads and trimming was done for the first 10 bases of forward reads and the first 20 bases of reverse reads. Classification was done with the Ribosomal Database Project (RDP) 11.4 (http://rdp.cme.msu.edu/).

Data analysis

Statistical analyses were performed with R 4.2.1 (R Core Team 2022). Generalized linear models (GLMs) with a Poisson distribution and a logarithmic link function were used to model the relationship between the independent variable (green areas) and the dependent variables (soil properties, percentage of behavioural alterations in model organisms, individual counts by Order, and number of taxa), accounting for the fact that the three green areas were unreplicated. Analysis of variance (ANOVA) was conducted to assess the effect of the three green areas on Fv/Fm values. Patterns of variation in pedofauna richness of soil across the three green areas were explored using a Principal Component Analysis (PCA) to allow for the reduction of dataset dimensionality while preserving the most significant variance, enabling a refined interpretation of relationships and trends in the biodiversity data. Eigenvalues greater than or equal to 1.0 (Keiser criterion) were considered significant for the extraction of the principal components. To investigate bacterial community diversity, cluster analyses were performed on the Hellinger-transformed ASV table. GLMs with a Poisson distribution corrected for overdispersion were performed on the most abundant classified genera to see their variation according to the area. The ASV table rarefied at 2820 sequences was used to obtain a Venn diagram to investigate the shared ASVs among different areas and to calculate alpha-diversity indices, i.e. Gini index, Shannon index, and the number of ASVs, Chao index was calculated on a non-rarefied dataset (Gini 1912; Shannon 1948; Chao 1984). Differences in the alpha diversity indices according to the sampling areas were further investigated with GLM with a Gaussian distribution. The data were considered statistically significant for values of $p < 0.05$.

Results

Green areas characterizations

Properties of sampled soils (Table SM3) were evaluated to contextualise any differences in environmental conditions that might have influenced behavioural responses or species composition. The three soils displayed the same texture. The soil pH values of the three green areas were near-neutral, ranging from 6.6 to 7.1. On the contrary, U4 showed the lowest level of WHC percentage compared to the soils from U2 ($p < 0.05$), and U3 ($p < 0.001$), the last of which showed the highest WHC_{max} level detected. The SOM % content was significantly higher in U3 soils compared to U2 ($p < 0.01$), and U4 ($p < 0.001$) soils.

Regarding the photosynthetic efficiency in the three areas, the results revealed a statistically significant difference between the groups ($p = 0.016$), and the comparison showed that the Fv/Fm value of U3 was significantly higher than that of U2 (< 0.05, Tukey *post hoc* test). No significant differences were found between U4 and the other groups.

Behavioural bioassays

Within each green area, the three plots (A, B, and C) and their replicates exhibited minimal variability in avoidance responses, with a standard error (SE) lower than 12, 13, and 14% for woodlice, springtails and earthworms, respectively.

Referring to the green areas (U2, U3, and U4), distinct responses emerged (SM4). The avoidance results suggested a no-choice response for all the model organisms tested exposed to U2 soils (Fig. 3a and b, and 3c), and the disaggregation bioassay showed that more than 70% of the terrestrial isopods were aggregated in groups in the condition with U2 soil (Fig. 3d). These results suggest a good quality for the U2 soils in terms of habitat function.

U3 soils elicited attraction in more than 40% of collembolans ($p < 0.05$), and over 70% of terrestrial isopods ($p < 0.0001$) compared to the LUFA soil controls (Figs. 2c and 3b), whereas earthworms displayed no statistically significant preference, despite more than 40% of the individuals being found in U3 soils. Attraction phenomenon could be induced by specific soil properties, but can even be attributed to immobilization or locomotor alterations (Oliveira et al. 2015). In contrast to this hypothesis, the outcomes of *P. pruinosus* showed a higher degree of aggregation (Fig. 3d), suggesting an active behaviour

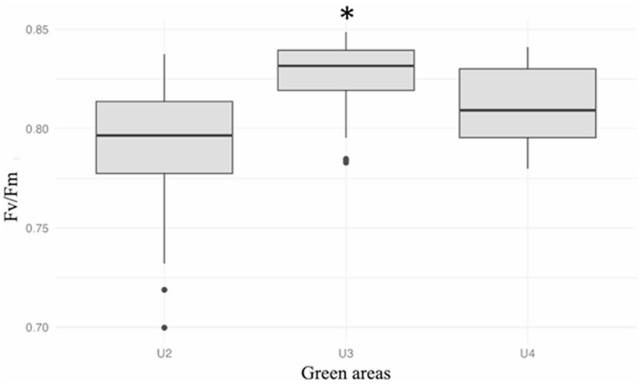


Fig. 2 Box charts referred to the ratio of variable fluorescence (Fv) to maximum fluorescence (Fm) of the *Tilia spp*. leaves from U2, U3, and U4 green areas. Data are expressed as mean (±standard error) ($n=5$ replicates per site). Asterisks denote significant differences to control (Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05)

instead of a lack of locomotion, suggesting that the observed attraction is driven by soil characteristics, such as high organic matter content identified in soil analyses.

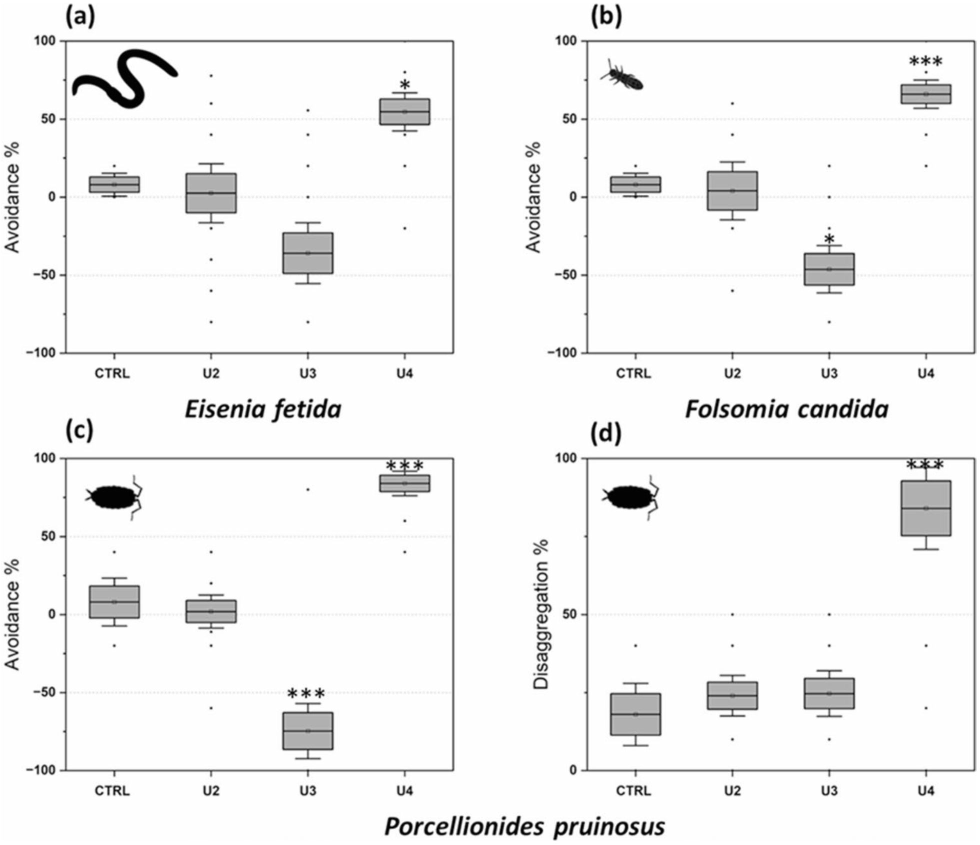
In contrast, U4 soils significantly induced a strong avoidance behaviour (Fig. 3) in more than 50% of earthworms ($p<0.05$), more than 60% of springtails ($p<0.0001$), and more than 80% of terrestrial isopods ($p<0.0001$). U4 soils also triggered a strong disaggregation effect on the terrestrial isopod population, showing a disaggregation index (DI) mean of 84% ± 5.2% SE ($p<0.0001$) (Fig. 3d). The behavioural tests were conducted under controlled laboratory conditions, so it is likely that the limited habitat function observed in U4 soils is due to specific characteristics of these soils. Federico et al. (2024) demonstrated that soil contaminants can induce infochemical disruption; similarly, the effects observed in U4 soils may reflect contaminant presence.

In essence, avoidance responses are ranked in sensitivity as *P. pruinosus* > *F. candida* > *E. fetida*, underlying a different degree of susceptibility of these model organisms. The heightened sensitivity of terrestrial isopods aligns with literature reports of soil contaminant impacts (Maria et al. 2024). Furthermore, the employment of disaggregation indices has enhanced comprehension of soil quality in the case of attractive responses, as emerged from U3 soils. Therefore, the combination of the two ecological endpoints in a single bioassay are a promising tool in the framework of the environmental safety assessment.

Edaphic invertebrates diversity

A total of 1,395 individuals, representing 20 distinct taxa, were extracted from soil collected from the university green areas, showing significant differences in richness and providing valuable insights into the sub-optimal quality of green

Fig. 3 Box charts referred to the Avoidance percentage (A %) responses in *E. fetida* (**a**), *F. candida* (**b**), and *P. pruinosus* (**c**) populations, with the relative disaggregation index percentage (**d**), exposed to U2, U3, and U4 soils. Data are expressed as mean (±standard error) ($n=5$ replicates per site). Grey dot lines present the threshold level of ±50%. Asterisks denote significant differences to control (Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05)



spaces (Table SM5). Almost all taxa, except Symphyla, were found in U2, while the taxonomic richness in U3 and U4 was significantly reduced compared to U2 ($p < 0.0001$). GLMs results showed variable responses across the three urban areas. The abundance of Acarina ($p < 0.0001$) and Isopoda ($p < 0.0001$) was significantly lower in both U3 and U4 compared to U2. The number of Hymenoptera ($p < 0.0001$) and Enchytreideae ($p < 0.001$) was significantly different only between U2 and U4. Additionally, Diplura ($p < 0.0001$) and Pauropoda ($p < 0.05$) showed significant differences between U2 and U3. The PCA identified five principal components (PCs), which together explained 74.13% of the total variance in the multifactorial analysis (Table SM6), while 49.8% was explained by the first two components (PC1 and PC2) (Fig. 4). The PC1 accounted for 33.9% of total variance with positive weak loading of Acarina (0.35), Chilopoda (0.36), Protura (0.37) and Isopoda (0.36). In contrast, PC2 accounted for 15.9% of total variance, with positive weak loading of Collembola (0.38), Lumbricidae (0.34), Diplura (0.35), and Symphila (0.32), while Hymenoptera (−0.43) and Gasteropoda (−0.3) showed negative weak loading.

These results emphasise a clear separation of the three urban soil communities, in line with the prediction of previously conducted behavioural tests. Specifically, U2 soils, which did not show restricted habitat functionality, displayed a greater edaphic composition, with a high percentage of species sensitive to changes in soil conditions such as Acarina (32.3%), Protura (7.8%), detected exclusively in this green area, and Isopoda (6.7%) (Toth et al. 2023). Regarding U3 soils, behavioural tests had previously indicated an attraction due to an enrichment of organic matter, and these results were subsequently confirmed by both the analysis of soil characteristics and diversity analyses. These analyses revealed a community dominated by species sensitive to moisture loss and organic matter content (Lapied et al. 2009), including Collembola (43.1%), Diplura (14.6%), Lumbricinae (4.1%) and Symphila (0.8%), the latter occurring only in U3 green areas. Finally, the results of the behavioural tests in U4 soils outlined a limited habitat function, consistent with the low edaphic composition and with the community partitioning identified through PCA based on taxa abundances. Importantly, the PCA also reflected the reduced contribution of epiedaphic taxa, indicating an overall impoverishment of soil biodiversity, an aspect that would not be captured by indices such as QBS-ar focusing exclusively on edaphic adaptations.

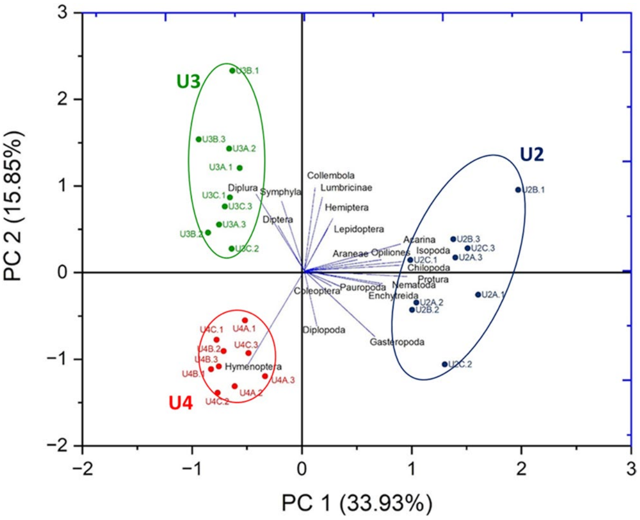


Fig. 4 Principal Component Analysis (PCA) related to the number of individuals per each taxon identified per each urban soil area ($n = 18$ replicates per green area), specifically U2 (blue dots), U3 (green dots), and U4 (red dots)

Bacterial communities' diversity

Following high-throughput sequencing, a total of 6,838 ASVs were detected per green area (Figure SM7). The taxonomic composition of the bacterial communities across the three urban green spaces indicated that the most abundant classified genera overall in ascending order were: *Microlunatus* sp. (3.88%), *Nocardioides* sp. (2.26%), *Gaiella* sp. (1.72%), *Agromyces* sp. (2.52%), *Mycobacterium* sp. (0.84%), *Microvirga* sp (0.63%), *Flavobacterium* sp. (0.6%) and *Pedomicrobium* sp. (0.62%). In particular, *Gaiella* sp. ($F_{2,6}$ = 22.50, P_{FDR} = 0.035), and *Pedomicrobium* sp. ($F_{2,6}$ = 22.43, P_{FDR} = 0.035) emerged from the GLMs as more abundant in U2 and U4 soils, respectively (Fig. 5).

Pedomicrobium sp. has been identified in soil samples as a heterotrophic bacterium that can oxidise manganese and iron (Rosenberg et al. 2014). Also, it's reported to be a metal-tolerant species and present in Cr-contaminated sites (Sheik et al. 2012; Araujo et al. 2023), in microcosm experiments it showed hydrocarbon-clastic capacities (de la Cueva et al. 2016), and was significantly predominant in soils contaminated with low-density polyester microplastics (LDPE-MPs) up to 7% w/w (Rong et al. 2021).

Gaiella sp. has been identified as a strict chemoorganotroph that can also be encountered in soil (Albuquerque and da Costa 2014). Its presence also seems to be positively correlated with the presence of polycyclic aromatic hydrocarbons (PAHs) in soils, but information on the ecological response of this bacterium to PAH contamination is still limited (Zhang et al. 2023).

The Venn diagram (Fig. 6a) revealed that site U3 harboured the highest proportion of unique ASVs (22%), suggesting a more distinct microbial community structure, potentially driven by site-specific environmental conditions or human impact, as a consequence of a higher influx of

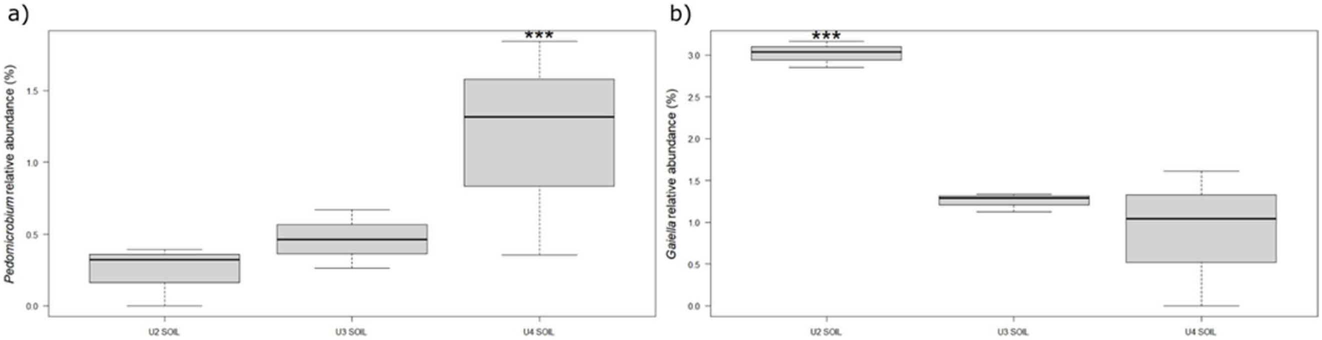


Fig. 5 Boxplot showing the statistical significance of the relative amplicon read abundances of (**a**) *Pedomicrobium sp.* and (**b**) *Gaiella sp.* in the different sampling areas ($n = 18$ replicates per green area). Asterisks denote significant differences to control (Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05)

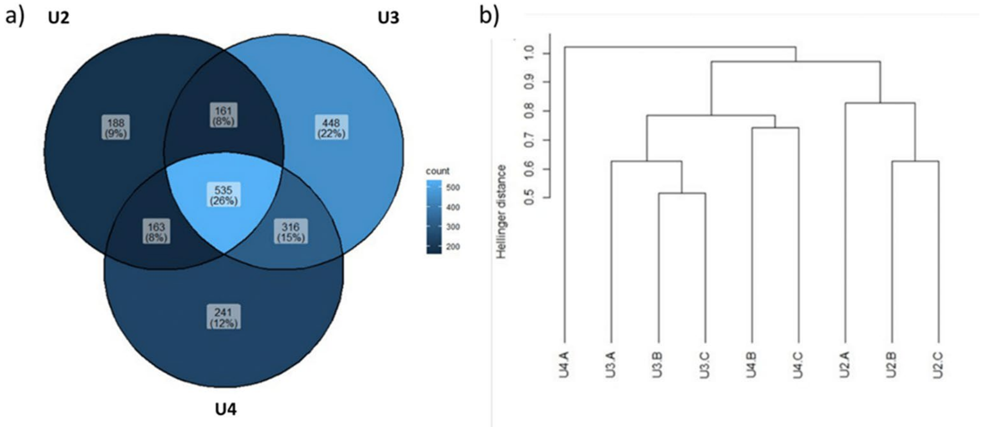


Fig. 6 (**a**) Venn diagram of similar shared ASVs for the three different areas ($n = 18$ replicates per green area). (**b**) Hierarchical cluster analysis on the Hellinger-transformed ASV table of the bacterial communities

Table 2 Pedofauna diversity refers to the structural (d-Margaled, D-Simpson, H-Shannon, AED, A/C ratio, Gini, Chao, number of ASV) and functional indices (QBS-ar) of soils from U2, U3, and U4 green areas. Values are expressed as mean ± standard error ($n = 18$ replicates per green are)

Sites	Group	d-Margalef	D-Simpson	H-Shannon	AED	A/C ratio	QBS-ar
U2	Pedofauna	3.3 (±0.10)	0.19 (±0.02)	2.1 (±0.10)	25.6 (±1.02)	1.40 (±0.52)	142 (±6.7)
U3	Pedofauna	2.2 (±0.23)	0.26 (±0.01)	1.7 (±0.05)	15.6 (±1.51)	0.49 (±0.01)	115 (±17.3)
U4	Pedofauna	2.1 (±0.42)	0.23 (±0.01)	1.8 (±0.06)	14.9 (±2.38)	0.48 (±0.04)	97 (±4.01)

students in these green areas. In contrast, U4 and U2 presented lower proportions of unique ASVs, at 12% and 9%, respectively. The overlap in community composition was minimal between U2 and the other sites (8% shared ASVs), whereas U3 and U4 exhibited a greater degree of similarity (15% shared ASVs). Importantly, 26% of ASVs were shared among all three sites, indicating a core microbiome potentially reflective of common ecological functions or environmental baselines across urban green areas.

Cluster analysis on the Hellinger-transformed ASV table of the bacterial communities (Fig. 6b) further supported this spatial differentiation, with samples from U3 and U4 predominantly clustering by site. Notably, U4A samples formed a distinct subgroup with a high heterogeneity when comparing all the replicates, separate from the remaining samples, while U2 samples diverged early from the other clusters (Fig. 6b). These patterns indicate a higher degree of similarity in both ASV richness and relative abundance between the U3 and U4 sites. This observation aligns with the Venn diagram results, which showed a higher proportion of shared ASVs between U3 and U4 (15%), reinforcing the potential use of shared microbial taxa as indicators of ecological similarity and site conditions.

Ecological indices

The utilisation of ecological indices (Table 2) facilitated the integration of diversity data, confirming a significant decline in edaphic invertebrates in U3 and U4 compared to U2 areas, whereas no significant differences were detected between U3 and U4 areas (Table SM8).

All structural and functional indices showed the highest level of invertebrate species, more widely distributed and belonging to edaphic groups with ecomorphological

Table 3 Bacterial diversity refers to the structural (H-Shannon, Gini, Chao, number of ASV) indices of soils from U2, U3, and U4 green areas. Values are expressed as mean ± standard error ($n = 18$ replicates per green area)

Sites	Group	H-Shannon	Gini	Chao	ASV
U2	Bacteria	5.75 (±0.26)	0.87 (±0.03)	778.59 (±268.44)	563 (±47.41)
U3	Bacteria	6.17 (±0.05)	0.8 (±0.01)	1487.3 (±204.65)	846.33 (±43.24)
U4	Bacteria	5.8 (±0.37)	0.85 (±0.05)	971.69 (±370.4)	623.33 (±192.3)

adaptations relevant to U2 soils. Conversely, the soils of U3 and U4 showed a worse condition in terms of diversity, with a dominance in generalist and tolerant edaphic species. These outcomes were corroborated and consistent with the results previously detected by behavioural tests. U4 soils, which had limited habitat function, showed the lowest diversity, and U3 soils also showed reduced diversity compared to U2 soils, despite displaying a higher quantified organic matter content. The results outlined from U3 also demonstrated that conducting avoidance or disaggregation tests alone would not be exhaustive for understanding alterations in soil diversity, as aggregation behaviour, a positive condition of ecological quality, occurred in U3 soils with low edaphic diversity, while avoidance tests itself for these soils would not have clarified whether it was movement inhibition or spontaneous attraction. It is therefore suggested that the combination of the two endpoints is essential for a correct environmental safety assessment of soil quality.

Conversely to the invertebrate diversity, analysis of alpha diversity indices revealed no statistical difference between the sampling areas in terms of bacterial communities (Table 3).

Discussion

The habitat function of urban soils was assessed through a combined approach between behavioural responses of soil fauna and biodiversity analyses, providing a comprehensive framework to evaluate ecosystem quality. This multilevel strategy enabled us to identify ecological indicators that reflect key aspects of soil community structure and potential habitat suitability, highlighting the added value of behavioural bioassays as early-warning tools. y. Our findings confirmed that behavioural responses of soil organisms, particularly in avoidance and disaggregation bioassays, serve as indicator tools for assessing soil quality, offering a rapid and cost-effective screening method. Unlike traditional ecotoxicological endpoints, behavioural responses integrate multiple environmental constraints and reflect the capacity of soils to support organismal activity, persistence, and ecological interactions. In this context, avoidance and disaggregation emerged as complementary indicators of habitat quality. Avoidance behaviour reflected the perception of unfavourable edaphic conditions and was expressed as active displacement away from stressed soils, resulting in reduced residence time and limited colonisation. Disaggregation responses, by contrast, revealed more subtle functional impairments through the fragmentation of social behaviour, particularly in gregarious taxa such as terrestrial isopods. Importantly, disaggregation did not necessarily imply complete abandonment of the soil, but rather a local redistribution of individuals, occurring both within stressed soils and in adjacent buffer zones, under suboptimal conditions. The choice of model organisms, including both soft-bodied (e.g., earthworms) and hard-bodied invertebrates (e.g., woodlice and springtails), was essential to capture a wide range of ecological sensitivities and behavioural responses. This selection allowed for a broader assessment of edaphic conditions, as these taxa differ in physiological tolerance, mobility, and habitat requirements (Menta and Remelli 2020). The inclusion of all three organism types within the broad soft- and hard-bodied categories was justified by the observed differences in responses, which were complementary rather than redundant. Notably, terrestrial isopods displayed the most distinct and sensitive behavioural patterns, likely due to their gregarious nature, which amplifies responses to environmental cues and enhances detectability of habitat quality differences. The utilisation of terrestrial isopods also facilitated the observation of variations in population structure through the disaggregation endpoints, which are not captured by conventional avoidance test (Federico et al. 2024). Consequently, these model organisms may represent a rapid assessment tool for soil quality to complement standard ecotoxicological assays.

Analysis of biodiversity provided quantitative confirmation of soil habitat functionality, as predicted by the behavioural tools. This consistency was corroborated by the study of invertebrate diversity, where the U2 soils were found to be those with no limited habitat functions and with the greatest diversity. The study of bacterial communities revealed no significant variations of the alpha diversity indexes. Although high bacterial alpha diversity is indicative of a healthy soil (Van Elsas et al. 2006; Hermans et al. 2017), it is expected that diversity indices do not show marked differences, as microbial communities may not follow the same basic rules of ecology as many edaphic organisms in response to a disturbance (Fierer et al. 2011; Lear et al. 2011). Microbial communities respond rapidly to environmental changes (Rutigliano et al. 2023), and show broad tolerance e for a range of stresses, such as temperature, pH, heavy metal and radionuclide concentrations (Satyanarayana et al. 2005), allowing high levels of diversity even under extreme environmental conditions. These results suggest that behavioural bioassays may be more sensitive than microbial diversity metrics in detecting local-scale functional habitat degradation.

Conversely, invertebrate communities may be more directly affected by even small variations in edaphic and physico-chemical factors than bacterial communities, such as variations in water retention and pH, as well as the presence of chemical or physical contaminants (Menta and Remelli 2020). Specifically, sampled soils from U2 areas showed a high percentage of species sensitive to changes in soil conditions such as of Acarina, Protura, and Isopoda compared to the other green areas. Mites and Proturans are generally sensitive to mechanical stresses induced by trampling (Maraun et al. 2003), and deforestation (Toth et al. 2023). As a consequence of U2 isolation, the reduction of human disturbance may facilitate the increase in abundance of mites and proturans. Isopoda represent synanthropic species successfully adapted to urban soils (Vilisics et al. 2012; Hornung et al. 2018; Szlavecz et al. 2018), and their significant decrease in abundances in disturbed habitats, such as U3 and U4 soils, highlights their potential role as indicators of soil quality, as supported by previous studies (Paoletti and Hassall 1999; van Gestel et al. 2018). These outcomes suggest that the presence and abundance of Acarina, Protura and Isopoda could be indicative of the soil's ecological integrity, further emphasizing the importance of habitat preservation and management in urban green spaces.

As for the U2 soils, also for the U3 and U4 soils, the results of the behavioural tests were found to be consistent with those relating to invertebrate diversity, as well as with the percentage levels of WHC and SOM detected, the latter likely reflecting inputs from bars and restoration areas near the university refectory or the effects of soil compaction from foot traffic. In fact, U3 soils exhibited a higher abundance of species sensitive to soil moisture loss, such as collembolans (Hopkin 1997), or symphylans (Edwards 1961), and species sensitive to organic matter content, such as diplopoda, and earthworms (Lapied et al. 2009; Huerta et al. 2013). These results indicate that the abundance of springtails, symphylans, and earthworms may be indicators of soils with higher moisture and organic matter content. The high percentage of SOM likely induced attractive behaviour in all populations of model organisms tested in behavioural bioassays, as well as increasing the gregarious behaviour of terrestrial isopods. It is interesting to note that the SOM increases in U3 was higher despite a decline in faunal diversity. This may be an example of the "enrichment paradox" (Rosenzweig 1971), where an increase in productivity does not correspond to an increase in fauna diversity. However, the effect of additional organic matter on soil biodiversity depends on its quality and structure, and the present study does not allow for verification of mechanistic explanations of this effect, which would require more detailed studies on interactions between populations.

Conversely, U4 soils exhibited the most restricted levels of both structural and functional diversity. It is noteworthy that behavioural assessments proved to be more discerning indicators of habitat functional limitations in comparison to bacterial analyses. These soils showed a high abundance of Hymenoptera, specifically of ants (Formicidae). The higher abundance of ants can be attributed to their competitive behaviour for food and territory (Trainello, 1989; Duma 2003). Cakir (2019) showed that ants reduce and replace Collembola and Protura in arid and semi-arid environments. Furthermore, ants modify soil texture, increasing porosity and reducing water retention capacity (Cammeraat et al. 2002; Frouz and Jilková 2008), which affects edaphic species requiring higher moisture, such as Collembola. Furthermore, the family Formicidae consists of vagile species, and this could be a possible explanation for their low sensitivity to current soil disturbances compared to other taxa (Remelli et al. 2024). The results suggest that an increasing abundance of ants may be an indicator of low diversity. At the same time, it cannot be excluded that their abundance may depend on an induced recruitment effect induced by an alteration of the ecological conditions of the U4 soils, such as the presence of contaminants or fertilisers.

In light of these findings, it is necessary to recognize the spatial and temporal limitations of our biodiversity assessments. Sample size and representativeness are essential parameters for reliable analysis and that the implementation of seasonally repeated sampling is crucial to improve the resolution of biodiversity dynamics and trends, especially in an explicit spatial context (Hillebrand et al. 2018). Specifically, arthropod communities are affected by seasonal variations in both highly and slightly polluted soils, exhibiting distinct responses to the same site when sampled at different times (Santorufo et al. 2014). Despite these constraints, the aim of this study was to undertake a first attempt at proposing a framework that links behavioural early-warning responses to biodiversity loss. This endeavour was undertaken in order to address the priorities of the European Commission (EC 2025b), which highlights the current misalignment between biodiversity loss metrics and laboratory-based ecotoxicological assessments. We acknowledge and support the need for future studies incorporating a larger number of samples and seasonal sampling, which will be essential to strengthen the validity and robustness of these preliminary results.

Conclusion

This work represents a first attempt to use behavioural responses as a tool to support soil biodiversity assessment within a multilevel framework. By integrating dispersal traits of different hard- and soft-bodied edaphic organisms, along with fragmentation traits of gregarious species such as terrestrial isopods, it is possible to identify soils potentially subject to invertebrate diversity loss. This approach allows for the prioritization of economic and management interventions in a targeted and efficient manner for assessing urban ecosystems. Future studies will need to validate this framework across different environmental contexts (i.e. agriculture, forest, wetland, and prairie soils), at larger spatial scales and over multiple sampling periods, to consolidate its applicability and robustness for urban soil health assessment.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s11252-026-01908-6.

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Author contributions Lorenzo Federico (Conceptualization, Visualization, Data curation, Formal analysis, Investigation, Writing – original draft, Writing – review and editing), Valeria Tatangelo (Data curation, Investigation), Francesca Pittino (Data curation, Formal analysis), Claudia Russo (Investigation), Emanuele Vegini (Investigation), Sandra Citterio (Supervision, Resources, Funding acquisition, Writing – review and editing), Andrea Franzetti (Supervision, Resources, Writing – review and editing), Sara Villa (Conceptualization, Resources, Validation, Data curation, Supervision, Project administration, Writing – original draft, Writing – reviewer & editing).

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Data availability The data that support the findings of this study are openly available in ZENODO at https://zenodo.org/records/17367638.

Declarations

Supplementary Information Below is the link to the electronic supplementary material.

Competing interests The authors declare no competing interests.

References

Albuquerque L, da Costa MS (2014) The family Gaiellaceae. In: Rosenberg E, DeLong EF, Lory S, Stackebrandt E, Thompson F (eds) The prokaryotes. Springer, Berlin, Heidelberg. https://doi.org/10.1007/978-3-642-30138-4_394

Araujo ASF, Miranda ARL, de Araujo Pereira AP, de Melo WJ, Melo VMM, Ventura SH, Brito Junior ES, de Valente Meiros E, Araujo FF, Mendes LW (2023) Microbial communities in the rhizosphere of maize and cowpea respond differently to chromium contamination. Chemosphere 313:137417. https://doi.org/10.1016/j.chemosphere.2022.137417

Bachelier G (1986) La vie animale dans le sol. O.R.S.T.O.M., Paris, 171–196

Bowler DE, Buyung-Ali L, Knight TM, Pullin AS (2010) Urban greening to cool towns and cities: a systematic review of the empirical evidence. Landsc Urban Plan 97(3):147–155. https://doi.org/10.1016/j.landurbplan.2010.05.006

Çakır M (2019) The negative effect of wood ants (*Formica rufa*) on microarthropod density and soil biological quality in a semi-arid pine forest. Pedobiologia 77:150593. https://doi.org/10.1016/j.pedobi.2019.150593

Callahan BJ, McMurdie PJ, Rosen MJ, Han AW, Johnson AJA, Holmes SP (2016) DADA2: high-resolution sample inference from Illumina amplicon data. Nat Methods 13:581–583. https://doi.org/10.1038/nmeth.3869

Callow D, May P, Johnstone DM (2018) Tree vitality assessment in urban landscapes. Forests 9(5):279. https://doi.org/10.3390/f9050279

Cammeraat LH, Willott SJ, Compton SG, Incoll LD (2002) The effects of ants’ nests on the physical, chemical and hydrological properties of a rangeland soil in semi-arid Spain. Geoderma 105(1–2):1–20. https://doi.org/10.1016/S0016-7061(01)00085-4

Chao A (1984) Non-parametric estimation of the number of classes in a population. Scand J Stat 11:265–270. https://doi.org/10.2307/4615964

Coyle DR, Nagendra UJ, Taylor MK, Campbell JH, Cunard CE, Joslin AH, Mundepid A, Phillips CA, Callaham MA Jr (2017) Soil fauna responses to natural disturbances, invasive species, and global climate change: current state of the science and a call to action. Soil Biol Biochem 110:116–133. https://doi.org/10.1016/j.soilbio.2017.03.008

de la Cueva SC, Rodríguez CH, Cruz NOS, Contreras JAR, Miranda JL (2016) Changes in bacterial populations during bioremediation of soil contaminated with petroleum hydrocarbons. WASP 227:1–12. https://doi.org/10.1007/s11270-016-2789-z

Duma I (2003) The impact of red wood ants *Formica Rufa* on the distribution of invertebrate fauna from the forest's floor. AWUT-SerBio 1011:121–130. https://www.jstor.org/stable/4223297

EC (2025b) European Commission: Joint Research Centre, Baccaro, M., Focks, A., Worth, A., Chinchio, E., Dubois, G., Robuchon, M., Carletti, A., Bernasconi, C. and Bopp, S., Protection of biodiversity as the ultimate goal of environmental safety assessment: how does chemical pollution affect biodiversity, Publications Office of the European Union, Luxembourg, 2025. https://doi.org/10.2760/1215173

EC (2021) European Commission, 2021. Communication from the Commission to the European Parliament, the Council, the European Economic and Social Committee and the Committee of the Regions Pathway to a Healthy Planet for All EU Action Plan: Towards Zero Pollution for Air, Water and Soil. https://eur-lex.europa.eu/legal-content/EN/TXT/?uri=celex:52021DC0400

EC, Commission E (2025a) 2025a. P9_TA(2024)0204 – Soil Monitoring and Resilience (Soil Monitoring Directive) – European Parliament legislative resolution of 10 April 2024 on the proposal for a directive of the European Parliament and of the Council on Soil Monitoring and Resilience (Soil Monitoring Law) (COM(2023)0416 – C9-0234/2023–2023/0232(COD)) (Ordinary legislative procedure: first reading) http://data.europa.eu/eli/C/2025/1312/oj

Edwards CA (1961) The ecology of symphyla part III. Factors controlling soil distributions. Entomol Exp Appl 4(4):239–256. https://doi.org/10.1111/j.1570-7458.1961.tb02139.x

EU, European, Parliament, Council of the European Union, 2024. Regulation (EU) 2024/1991 of the European Parliament and of the Council of 24 June 2024 on nature restoration and amending Regulation (EU) 2022/869 (Text with EEA relevance). Off J Eur, Union (2024) Brussels (BE). http://data.europa.eu/eli/reg/2024/1991/oj

Federico L, Monti GS, Loureiro S, Villa S (2024) Disaggregation behavior in the terrestrial isopod *Porcellionides pruinosus* as a new ecotoxicological endpoint for assessing infochemical disrupting activity. Ecol Indic 167:112602. https://doi.org/10.1016/j.ecolind.2024.112602

Fierer N, McCain CM, Meir P, Zimmerman M, Rapp JM, Silman MR, Knight R (2011) Microbes do not follow the elevational diversity patterns of plants and animals. Ecology 92:797–804. https://doi.org/10.1890/10-1170.1

Frouz J, Jilková V (2008) The effect of ants on soil properties and processes (Hymenoptera: Formicidae). Myrmecol News 11(11):191–199

Gainer A, Owojori OJ, Maboeta M (2022) Use of soil invertebrate avoidance tests as an emerging tool in soil ecotoxicology. Rev Environ Contam Toxicol 260(1):2. https://doi.org/10.1007/s44169-021-00004-4

Gandolfi I, Canedoli C, Rosatelli A, Covino S, Cappelletti D, Sebastiani B, Tatangelo V, Corengia T, Pittino F, Padoa-Schioppa E, Báez-Matus X, Hernández L, Seeger M, Saati-Santamaría Z, García-Fraile P, López-Mondéjar R, Ambrosini R, Papacchini M, Franzetti A (2024) Microbiomes of urban trees: unveiling contributions to atmospheric pollution mitigation. Front Microbiol 15:1470376. https://doi.org/10.3389/fmicb.2024.1470376

Gatti RC, Amoroso N, Monaco A (2020) Estimating and comparing biodiversity with a single universal metric. Ecol Modell 424:109020. https://doi.org/10.1016/j.ecolmodel.2020.109020

Gini CW (1912) Variability and mutability, contribution to the study of statistical distributions and relations. Studi Economico-Giuridici della R. Università de Cagliari

Gottardini E, Cristofolini F, Cristofori A, Pollastrini M, Ferretti M (2016) Misura della fluorescenza della clorofilla A, contenuto di clorofilla e tratti fogliari: campionamento, raccolta e misurazioni: guida per studi in campo. IT. http://hdl.handle.net/10449/38027

Hermans SM, Buckley HL, Case BS, Curran-Cournane F, Taylor M, Lear G (2017) Bacteria as emerging indicators of soil condition. Appl Environ Microbiol 83(1):e02826-16. https://doi.org/10.1128/AEM.02826-16

Hillebrand H, Blasius B, Borer ET, Chase JM, Downing JA, Eriksson BK, Filstrup CT, Harpole WS, Hodapp D, Larsen S, Lewandowska AM, Seabloom EW, de Van Waal DB, Ryabov AB (2018) Biodiversity change is uncoupled from species richness trends: consequences for conservation and monitoring. J Appl Ecol 55(1):169–184. https://doi.org/10.1111/1365-2664.12959

Hopkin SP (1997) Biology of the springtails:(Insecta: Collembola). OUP Oxford

Hornung E, Kásler A, Tóth Z (2018) The role of urban forest patches in maintaining isopod diversity (Oniscidea). ZooKeys 801:371. https://doi.org/10.3897/zookeys.801.22829

Huerta E, Brunet D, Velazquez E, Lavelle P (2013) Identifying earthworm's organic matter signatures by near infrared spectroscopy in different land-use systems in Tabasco, Mexico. Appl Soil Ecol 69:49–55. https://doi.org/10.1016/j.apsoil.2013.01.015

ISO (2020b) Soil quality - Avoidance test for determining the quality of soils and effects of chemicals on behaviour - Part 2: Test with collembolans (*Folsomia candida*). ISO 7512-2:2020

ISO (2020a) Soil quality - Avoidance test for determining the quality of soils and effects of chemicals on behaviour - Part 1: Test with earthworms (*Eisenia fetida* and *Eisenia andrei*). ISO 7512-2:2020

Lapied E, Nahmani J, Rousseau GX (2009) Influence of texture and amendments on soil properties and earthworm communities. Appl Soil Ecol 43(2–3):241–249. https://doi.org/10.1016/j.apsoil.2009.08.004

Lear G, Dopheide A, Ancion P, Lewis GD (2011) A comparison of bacterial, ciliate and macroinvertebrate indicators of stream ecological health. Aquat Ecol 45(4):517–527. https://doi.org/10.1007/s10452-011-9372-x

Linden DR, Hendrix PF, Coleman DC, van Vliet PC (1994) Faunal indicators of soil quality. Defining soil quality for a sustainable environment, vol 35. pp 91–106. https://doi.org/10.2136/sssaspecpub35.c6

Maraun M, Salamon JA, Schneider K, Schaefer M, Scheu S (2003) Oribatid mite and collembolan diversity, density and community structure in a moder beech forest (*Fagus sylvatica*): effects of mechanical perturbations. Soil Biol Biochem 35(10):1387–1394. https://doi.org/10.1016/S0038-0717(03)00218-9

Margalef R (1958) Temporal succession and spatial heterogeneity in phytoplankton. Perspectives in Marine Biology, edited by A. A. Buzzati-Traverso, Berkeley: University of California Press. https://doi.org/10.1525/9780520350281-024

Maria VL, Santos J, Prodana M, Cardoso DN, Morgado RG, Amorim MJ, Barreto A (2024) Toxicity mechanisms of plastic nanoparticles in three terrestrial species: antioxidant system imbalance and neurotoxicity. Nanotoxicology 18(3):299–313. https://doi.org/10.1080/17435390.2024.2358781

Maxwell K, Johnson GN (2000) Chlorophyll fluorescence—a practical guide. J Exp Bot 51(345):659–668. https://doi.org/10.1093/jexbot/51.345.659

McSorley R (2000) Sampling and extraction techniques for nematodes. Techniques in nematode ecology. eds T Wheeler, T Forge, E Caswell-Chen. Hyattsville, MD: Society of Nematologists

Menta C, Remelli S (2020) Soil health and arthropods: from complex system to worthwhile investigation. Insects 11:54. https://doi.org/10.3390/insects11101005

Menta C, Conti FD, Pinto S, Bodini A (2018) Soil biological quality index (QBS-ar): 15 years of application at global scale. Ecol Indic 85:773–780. https://doi.org/10.1016/j.ecolind.2017.11.030

Oliveira M, Cardoso DN, Soares AMVM, Loureiro S (2015) Effects of short-term exposure to fluoxetine and carbamazepine to the collembolan *Folsomia candida*. Chemosphere 120:86–91. https://doi.org/10.1016/j.chemosphere.2014.06.038

OriginLab Pro 2021b software. url: www.OriginLab.com/2021b

Paoletti MG, Hassall M (1999) Woodlice (Isopoda: Oniscidea): their potential for assessing sustainability and use as bioindicators. Agric Ecosyst Environ 74(1–3):157–165. https://doi.org/10.1016/S0167-8809(99)00035-3

Parisi V, Menta C, Gardi C, Jacomini C, Mozzanica E (2005) Microarthropod communities as a tool to assess soil quality and biodiversity: a new approach in Italy. Agric Ecosyst Environ 105(1–2):323–333. https://doi.org/10.1016/j.agee.2004.02.002

Pereira CS, Lopes I, Abrantes I, Sousa JP, Chelinho S (2019) Salinization effects on coastal ecosystems: a terrestrial model ecosystem approach. Philos Trans R Soc Lond B Biol Sci 374(1764):20180251. https://doi.org/10.1098/rstb.2018.0251

Picot X (2004) Thermal comfort in urban spaces: impact of vegetation growth: case study: Piazza Della Scienza, Milan, Italy. Energy Build 36(4):329–334. https://doi.org/10.1016/j.enbuild.2004.01.044

R Core Team (2022) R foundation for statistical computing. Chin J Hepatol 30:9–20

Remelli S, Danise T, Galli L, Menta C (2024) Soil arthropods in bioindication and ecotoxicological approach: the case of the extreme environment Mefite (Ansanto Valley, Southern Italy). Heliyon. https://doi.org/10.1016/j.heliyon.2024.e36342

Rong L, Zhao L, Zhao L, Cheng Z, Yao Y, Yuan C, Wang L, Sun H (2021) LDPE microplastics affect soil microbial communities and nitrogen cycling. Sci Total Environ 773:145640. https://doi.org/10.1016/j.scitotenv.2021.145640

Rosenberg E, DeLong EF, Lory S, Stackebrandt E, Thompson F (2014) The Prokaryotes – Alphaproteobacteria and Betaproteobacteria. Springer Berlin, Heidelberg. https://doi.org/10.1007/978-3-642-30138-4_394

Rosenzweig ML (1971) Paradox of enrichment: destabilization of exploitation ecosystems in ecological time. Science 171(3969):385–387. https://doi.org/10.1126/science.171.3969.385

Rutigliano FA, Marzaioli R, Grilli E, Coppola E, Castaldi S (2023) Microbial, physical and chemical indicators together reveal soil health changes related to land cover types in the Southern European sites under desertification risk. Pedobiologia 99:150894. https://doi.org/10.1016/j.pedobi.2023.150894

Sachs JD (2012) From millennium development goals to sustainable development goals. Lancet 379(9832):2206–2211. https://doi.org/10.1016/S0140-6736(12)60685-0

Santorufo L, Van Gestel CA, Rocco A, Maisto G (2012) Soil invertebrates as bioindicators of urban soil quality. Environ Poll 161:57–63. https://doi.org/10.1016/j.envpol.2011.09.042

Santorufo L, Van Gestel CA, Maisto G (2014) Sampling season affects conclusions on soil arthropod community structure responses to metal pollution in mediterranean urban soils. Geoderma 226:47–53. https://doi.org/10.1016/j.geoderma.2014.02.001

Satyanarayana T, Raghukumar C, Shivaji S (2005) Extremophilic microbes: diversity and perspectives. Curr Sci 89:78–90. https://www.jstor.org/stable/24110434

Schloter M, Dilly O, Munch JC (2003) Indicators for evaluating soil quality. Agric Ecosyst Environ 98(1–3):255–262. https://doi.org/10.1016/S0167-8809(03)00085-9

Sepúlveda P, Johnstone M, D (2018) A novel way of assessing plant vitality in urban trees. Forests 10(1):2. https://doi.org/10.3390/f10010002

Shannon CE (1948) A mathematical theory of communication. BSTJ 27(3):379–423. https://doi.org/10.1002/j.1538-7305.1948.tb01338.x

Sheik CS, Mitchell TW, Rizvi FZ, Rehman Y, Faisal M, Hasnain S, McInerney MJ, Krumholz LR (2012) Exposure of soil microbial communities to chromium and arsenic alters their diversity and structure. PLoS ONE 7(6):e40059. https://doi.org/10.1371/journal.pone.0040059

Simpson EH (1949) Measurement of diversity. Nature 163(4148):688–688. https://doi.org/10.1038/163688a0

Szlavecz K, Vilisics F, Tóth Z, Hornung E (2018) Terrestrial isopods in urban environments: an overview. ZooKeys 801:97. https://doi.org/10.3897/zookeys.801.29580

Tóth Z, Dombos M, Hornung E (2023) Urban soil quality deteriorates even with low heavy metal levels: an arthropod-based multi-indices approach. Ecol Appl 33(4):e2848. https://doi.org/10.1002/eap.2848

Traniello JF (1989) Foraging strategies of ants. Annu Rev Entomol 34(1):191–210. https://doi.org/10.1146/annurev.en.34.010189.001203

UNEP and FAO (2020) Strategy of the UN decade on ecosystem Restoration. Nairobi and Rome. Draft 6 February 2020

Van Elsas JD, Trevors JT, Jansson JK, Nannipieri P (2006) Modern soil microbiology. CRC. https://doi.org/10.1201/9781420015201

van Gestel CA, Loureiro S, Zidar P (2018) Terrestrial isopods as model organisms in soil ecotoxicology: a review. ZooKeys 801:127. https://doi.org/10.3897/zookeys.801.21970

Vieira DCS, Yunta F, Baragaño D, Evrard O, Reiff T, Silva V, De La Torre A, Zhang C, Panagos P, Jones A, Wojda P (2024) Soil pollution in the European Union–an outlook. Environ Sci Policy 161:103876. https://doi.org/10.1016/j.envsci.2024.103876

Vilisics F, Bogyó D, Sattler T, Moretti M (2012) Occurrence and assemblage composition of millipedes (Myriapoda, Diplopoda) and terrestrial isopods (Crustacea, Isopoda, Oniscidea) in urban areas of Switzerland. ZooKeys 176:199. https://doi.org/10.3897/zookeys.176.2153

Zhang Q, Gao M, Sun X, Wang Y, Yuan C, Sun H (2023) Nationwide distribution of polycyclic aromatic hydrocarbons in soil of China and the association with bacterial community. J Environ Sci 128:1–11. https://doi.org/10.1016/j.jes.2022.07.026

Supplementary Materials

SM1 - Soil Properties Analysis

Texture: the soil texture was determined using the sedimentation cylinder technique in accordance with Stokes's Law (ISO, 2009). in 100 mL cylinders, 50 g of dry weight (d.w.) soil were weighted and distilled water was added to achieve a final volume of 50 mL. The two fractions were mixed by shaking the cylinder. Once emulsified, the soil was expected to settle for 2 days, in order to measure (cm) the layers of clay, silt and sand percentage. The results were used to determine the soil type using a soil texture triangle based on the United States Department of Agriculture (USDA) classification.
Water Holding Capacity (WHC): the determination of soil WHC was quantified following the Annex E reported in ISO 11268-2 guidelines (2023a). 20 g d.w. of the sampled soils were first weighed, and then placed on perforated vials with filter paper at the bottom. The vials were then placed in a water bath in a plastic container, with the water level not higher than the soil surface. The samples were left to moisten for 2 hours and then allowed to drain for 1 hour. Finally, the wet samples were weighed and the WHC was calculated according to the formula:

$$\mathrm{WHC} = [(S - T - D)/D] \times 100$$

Where S = water-saturated substrate + tare, T = tare vials, D = dry mass of substrate.
pH: the determination of soil pH was determined following the ISO guidelines (ISO, 2005). 20 g d.w. of soil samples were placed into vials, to which 20 mL of a 0.01 M calcium chloride ($CaCl_2$) solution was added. The resulting suspension was then agitated for 15 minutes using an agitator, after which it was left to settle for a period of 2 hours. Following this, the pH of the liquid phase was measured using a pH Multi-parameter portable meter (WTW, ProfiLine Multi 3320 model Germany).
Soil Organic Matter (SOM): The SOM % was estimated using the loss-on-ignition (LOI) technique (Cambardella et al., 2000). 2 g d.w. soil samples were heated at 500 °C for 6 hours in a muffle. The percentage of SOM was determined as the subtraction of incinerated soil dry weight from the dried soil weight, normalizing the value to the initial dry weight of soil.

SM2 - Animal cultures and LUFA 2.2 soil

Terrestrial isopod: Woodlice breeding was carried out following methods previously established in various lines of research (Loureiro et al., 2005; Morgado et al., 2015; Federico et al., 2024). *Porcellionides pruinosus* (Brandt, 1833) culture was maintained in the laboratory at controlled conditions (20 ± 2°C of temperature, photoperiod of 16:8 h light-dark) and were fed *ad libitum* with alder leaves (*Alnus glutinosa*). The cultures were sprayed with ultra-pure water twice a week and food was provided. Only adults with a wet weight of 14-30 mg, without sex distinction, were used during the tests. Moulting animals, individuals with malformations, and pregnant females were excluded.

Earthworms: Earthworm breeding was carried out as reported in Annex D of ISO guidelines (2023a). *Eisenia fetida* (Savigny, 1826) were collected from a vermicompost (50% sphagnum peat and 50% cattle dung) at controlled conditions of 20 ± 2°C of temperature, and 16:8 h light-dark. Earthworm cocoons were periodically separated from the culture to which earthworm cohorts synchronized for tests. Only adult worms with well-developed clitellae and fresh weight between 250-300 mg were random selected for the behavioural tests

Collembolans: Collembolans breeding was carried out as reported in Annex A of ISO guidelines (2023b). *Folsomia candida* (Willem, 1902) were cultured in glass Petri-dishes (⌀ = 8.5 cm) on a moist substrate of plaster of Paris and activated charcoal (8:1), and maintained at temperature of 20 ± 2°C, photoperiod regime of 16:8h light:dark and fed weekly with dried baker's yeast (*Saccharomyces cerevisae*). Collembola eggs were periodically separated from the culture to obtain synchronized individuals for tests. Only adults 20 days after hatching were selected.

LUFA 2.2 soil: the properties of this soil include a 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.

SM3 - Table 2. Mean (± standard deviation) values referred to the sand (%), silt (%), clay (%), texture, pH, Water Holding Capacity (%), and Soil Organic Matter (%) of the three urban soils (U2, U3, and U4) from the University of Milano Bicocca, Italy.

Sites	Sand %	Silt %	Clay %	pH	WHC %	SOM %
U2	85.61 (± 1.3)	9.50 (± 0.45)	3.95 (± 0.13)	6.75 (± 0.54)	49.1 (± 3.6)	5.59 (± 0.36)
U3	88.13 (± 0.97)	9.53 (± 0.06)	2.9 (± 0.26)	7.11 (± 0.07)	51.6 (± 3.12)	6.11 (± 0.84)
U4	86.9 (± 0.70)	9.25 (± 0.31)	3.26 (± 0.16)	6.89 (± 0.49)	44.9 (± 2.78)	5.42 (± 0.83)

SM4 - Statistical results of generalized linear models (GLMs) for the behavioural response of invertebrate model organisms across different green areas. For each model organism, the p-values indicate the significance of differences between green areas (U2, U3, and U4) (Signif. codes: 0 '*' 0.001 '**' 0.01 '*' 0.05). Model fit statistics, including estimate, standard error (SE.), z value, Confident Interval (CI), and Akaike Information Criterion (AIC) are also presented.**

Model organisms	Behavioural endpoint	Samples	Estimate	SE	z value	p value	Signif.	CI	AIC
E. fetida	Avoidance	(Intercept)	1.23	1.14	1.08	0.28		[-5.53-4.18]	n.d.
		U2	2.37	1.30	1.82	0.068			
		U3	2.06	1.3	1.58	0.11			
		U4	2.82	1.3	2.17	0.036	*		
F. candida	Avoidance	(Intercept)	1.39	0.41	3.38	0.00072	***	[-5.52-4.61]	524
		U2	0.71	0.48	1.48	0.14			
		U3	1.89	0.91	2.08	0.037	*		
		U4	2.71	0.45	5.97	2.39e-9	***		
P. pruinosus	Avoidance	(Intercept)	2.17	0.33	6.52	6.92e-11	***	[-4.48-6.15]	514
		U2	0.55	0.37	1.47	0.14			

	U3	1.97	0.38	5.13	2.97e-7	***		
	U4	2.23	0.36	6.13	8.92e-10	***		
Disaggregation	(Intercept)	2.71	0.21	12.9	< 2e-16	***	[-7.64-7.74]	991
	U2	0.48	0.26	1.85	0.065			
	U3	0.41	0.24	1.69	0.09	.		
	U4	1.68	0.24	7.14	9.19 e-13	***		

SM5 - Statistical results of generalized linear models (GLMs) for the comparison of taxa and invertebrate groups across different green areas. For each taxon or invertebrate group, the p-values indicate the significance of differences between green areas (U2, U3, and U4) (Signif. codes: 0 '*' 0.001 '**' 0.01 '*' 0.05). Model fit statistics, including dispersion, null deviance (Null Dev.), residual deviance (Res. Dev.), and Akaike Information Criterion (AIC), are also presented. The number of interactions (N. of Inter.) refers to the number of pairwise comparisons conducted within each model.**

Variables	Areas	p value	Dispersion	Null Dev.	Res. Dev.	AIC	N. of Inter.
Taxa	U2	intercept	1	22.64	5.18	124.3	4
	U3	0.00051***					
	U4	0.00039***					
Acarina	U2	intercept	1	104.11	6.28	127.06	4
	U3	6.66e-12***					
	U4	< 2e-16***					
Araneae	U2	intercept	1	16.86	10.88	26.88	18
	U3	0.215					
	U4	0.997					
Chilopoda	U2	intercept	1	55.47	7.13	37.12	19
	U3	0.998					
	U4	0.998					
Coleoptera	U2	intercept	1	22.3	21.08	74.51	5

	U3	0.533					
	U4	0.280					
Collembola	U2	intercept	1	16.93	9.25	140.25	4
	U3	0.4354					
	U4	0.0546 *					
Diplopoda	U2	intercept	1	32.28	30.17	76.11	5
	U3	0.166					
	U4	0.670					
Diplura	U2	intercept	1	75.26	30.74	99.4	5
	U3	0.384e-7***					
	U4	0.244					
Diptera	U2	intercept	1	28.42	23.86	64.96	5
	U3	0.0571*					
	U4	0.5299					
Enchytreideae	U2	intercept	1	62.28	26.39	62.82	17
	U3	0.99485					
	U4	0.00239**					
Gasteropoda	U2	intercept	1	35.12	7.33	54.87	18
	U3	0.996750					
	U4	0.111245					
Hemiptera	U2	intercept	1	32.53	28.41	78.68	6
	U3	0.5645					
	U4	0.0578*					
Hymenoptera	U2	intercept	1	161.66	19.07	97.73	5
	U3	0.683					
	U4	1.3e-13***					

Isopoda	U2	intercept	1	57.12	12.54	51.47	6
	U3	0.000311***					
	U4	0.0001059***					
Lepidoptera	U2	intercept	1	21.67	21.4	40.01	6
	U3	0.6569					
	U4	0.6569					
Lumbricinae	U2	intercept	1	31.3	23.52	72.69	5
	U3	0.3387					
	U4	0.0832					
Nematoda	U2	intercept	1	28.07	14.33	38.18	17
	U3	0.9951					
	U4	0.0544*					
Opiliones	U2	intercept	1	16.86	5.88	21.88	19
	U3	0.998					
	U4	0.998					
Pauropoda	U2	intercept	1	31.12	24.20	75.64	5
	U3	0.01615*					
	U4	0.26059					
Protura	U2	intercept	1	112.47	4.80	42.48	19
	U3	0.998					
	U4	0.998					
Symphila	U2	intercept	1	15.96	9.36	19.98	18

SM6- Extracted eigenvectors and eigenvalues from the Principal Component Analysis (PCA) of soils from U2, U3, and U4 green areas. Bold values: eigenvector positive and negative weak loading of first (PC1) and second (PC2) principal components.

Taxa	**Extracted Eigenvectors**				
	PC1	**PC2**	**PC3**	**PC4**	**PC5**
Acarina	**0,35**	0,12	0,05	-0,08	-0,02
Araneae	0,19	0,05	0,12	-0,14	0,54

Chilopoda	**0,36**	0,03	-0,08	-0,07	0,03
Coleoptera	0,10	-0,07	0,22	-0,56	0,24
Collembola	0,04	**0,38**	0,37	0,06	-0,04
Diplopoda	0,05	-0,25	0,53	0,11	-0,09
Diplura	-0,17	**0,35**	0,24	0,04	0,11
Diptera	-0,09	0,21	0,24	0,39	0,34
Hemiptera	0,10	0,24	-0,16	-0,39	-0,31
Enchytreida	0,29	-0,07	-0,11	0,18	0,27
Gasteropoda	0,26	**-0,30**	-0,01	0,22	-0,01
Hymenoptera	-0,20	**-0,43**	-0,13	0,01	0,14
Isopoda	**0,36**	0,04	-0,04	-0,01	-0,01
Lepidoptera	0,09	0,19	-0,42	0,13	-0,01
Lumbricinae	0,07	**0,34**	-0,17	-0,07	0,26
Nematoda	0,28	-0,06	0,22	0,21	-0,11
Opiliones	0,28	0,05	0,10	0,16	-0,44
Pauropoda	0,13	-0,07	-0,25	0,26	0,23
Protura	**0,37**	-0,03	0,04	-0,04	0,03
Symphyla	-0,08	**0,32**	-0,11	0,30	-0,07
Eigenvalue	6,79	3,17	1,95	1,71	1,20
Variances %	33.93%	15.85%	9.77%	8.56%	6.02%
Cumulative Variances %	33.93%	49.78%	59.55%	68.11%	74.13%

SM7 – Bacterial communities' genera abundances of the three green sampling areas (U2, U3, and U4).

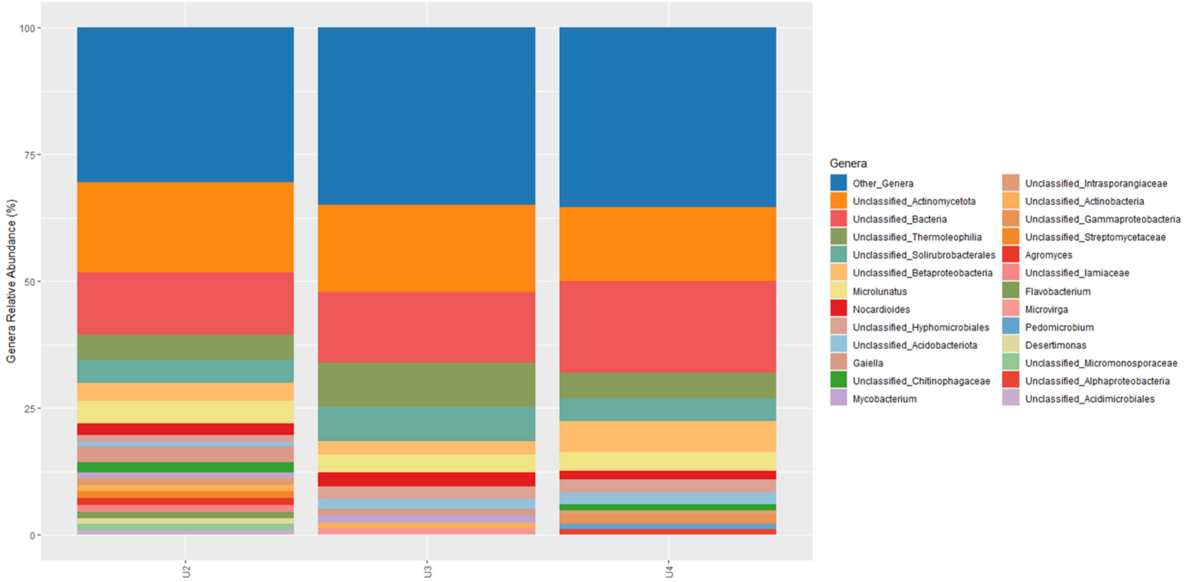

Figure 5. Barplot showing genera relative abundances of the three sampling areas. Genera with a relative abundance <1% were grouped in "Other Genera".

SM8 - Statistical results of generalized linear models (GLMs) for the comparison of diversity indices across different green areas. The p-values indicate the significance of differences between green areas (U2, U3, and U4) (Signif. codes: 0 '*' 0.001 '**' 0.01 '*' 0.05). Model fit statistics, including dispersion, null deviance (Null Dev.), residual deviance (Res. Dev.), and Akaike Information Criterion (AIC), are also presented. The number of interactions (N. of Inter.) refers to the number of pairwise comparisons conducted within each model.**

Variables	Areas	p value	Dispersion	Null Dev.	Res. Dev.	AIC	N. of Inter.
d-Margalef	U2	intercept	1	3.33	0.48	7.22	2
	U3	0.0028**					
	U4	0.0017**					
D-Simpson	U2	intercept	1	0.0078	0.0014	45.96	2
	U3	0.0017**					
	U4	0.029*					
H-Shannon	U2	intercept	1	0.36	0.034	16.73	2
	U3	0.00041***					
	U4	0.001**					
Absolute Effective Diversity (AED)	U2	intercept	1	230.83	18.01	39.78	2
	U3	0.00041***					
	U4	0.00029**					
A/C ratio	U2	intercept	1	2.21	0.54	8.14	2
	U3	0.0097**					
	U4	0.0092**					
QBS-ar	U2	intercept	1	5174.9	2090.7	82.57	2
	U3	0.123					
	U4	0.025*					

References

1. Cambardella, C. A., Gajda, A. M., Doran, J. W., Wienhold, B. J., Kettler, T. A. 2000. Matter by Weight Loss-on-Ignition. Assessment methods for soil carbon. Adv. Soil Sci. CRC Press, Boca Raton. Pp. 349–359.
2. Federico, L., Monti, G. S., Loureiro, S., Villa, S. 2024. Disaggregation behavior in the terrestrial isopod *Porcellionides pruinosus* as a new ecotoxicological endpoint for assessing infochemical disrupting activity. *Ecol. Indic.*, 167, 112602. https://doi.org/10.1016/j.ecolind.2024.112602
3. ISO 2009. ISO 11277:2009. Soil quality — Determination of particle size distribution in mineral soil material — Method by sieving and sedimentation
4. ISO 2023a. ISO guidelines 11268-2:2023 - Soil quality—Effects of pollutants on earthworms—Part 2:Determination of effects on reproduction of *Eisenia fetida*/*Eisenia andrei* and other earthworm species.
5. ISO 2023b. ISO guidelines 11267:2023. Soil quality - Inhibition of reproduction of Collembola (*Folsomia candida*) by soil contaminants.
6. ISO 2005. ISO.guidelines 10390: 2005. Soil Quality–Determination of pH.
7. Loureiro, S., Soares, A. M., Nogueira, A. J. 2005. Terrestrial avoidance behaviour tests as screening tool to assess soil contamination. *Environ. Pollut.*, 138(1), 121-131. https://doi.org/10.1016/j.envpol.2005.02.013
8. Morgado, R., Ferreira, N. G., Cardoso, D. N., Soares, A. M., Loureiro, S. 2015. Abiotic factors affect the performance of the terrestrial isopod *Porcellionides pruinosus*. *Appl. Soil Ecol.*, 95, 161-170. https://doi.org/10.1016/j.apsoil.2015.06.012
9. Parisi, V., Menta, C., Gardi, C., Jacomini, C., Mozzanica, E. 2005. Microarthropod communities as a tool to assess soil quality and biodiversity: a new approach in Italy. *Agric. Ecosyst. Environ.*, 105(1-2), 323-333. https://doi.org/10.1016/j.agee.2004.02.002

Chapter 7

Ecological Facilitation of Terrestrial Isopods indirectly Modulate Collembolan Behaviour exposed to Biochars

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Biochar,

Under review

Biochar

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Ecological Facilitation of Terrestrial Isopods indirectly Modulate Collembolan Behaviour exposed to Biochars

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Abstract

Biochar is applied to soils under EU regulations to enhance fertility and sequester carbon, but its impacts on soil fauna remain debated, with studies reporting adverse effects. This work assessed the behavioural responses of collembolans (*Folsomia candida*) and terrestrial isopods (*Porcellionides pruinosus*) to soils amended with different samples of biochar derived from two different feedstocks, *Ricinus communis* and *Brassica juncea*, both pyrolyzed at 400°C and 600°C. The four biochars were tested at 1% and 10% w/w. *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, highlighting the joint influence of feedstock and pyrolysis conditions. Avoidance in collembolans strongly correlated with increased soil pH and conductivity, suggesting alkaline and osmotic stress. For isopods, these parameters explained behavioural responses only moderately, implying additional factors. Furthermore, we examined whether terrestrial isopods 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 terrestrial isopods, suggesting facilitation likely mediated by bioturbation, microbial conditioning, or faecal deposition. These findings suggest that the observed effects were shaped by a combination of factors, including the model organism, biochar feedstock type, application rate, pyrolysis temperature, and interspecific interactions. We suggest incorporating facilitation processes into ecotoxicological assessments of biochar to better understand natural mitigation mechanisms.

Keywords: Biochar; Ecological facilitation; Soil fauna; Behavioural ecotoxicology; Species interactions

Highlights

- 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.

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 CO_2 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 2024), 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, in order 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., 2026). 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, terrestrial isopods 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, terrestrial isopods 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 (⌀= 8.5 cm) on a moist substrate of Calcium Sulphate Hemihydrate, also known as plaster of Paris ($CaSO_4 \cdot 1/2\ H_2O$), 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. Terrestrial isopods 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 *Brassica juncea* were by-products of the processing of the original feedstock, while *Ricinus communis* stems, branches and leaves were collected from sites subjected to phytoremediation treatments. The two parental feedstocks were dessicated at 80°C for 48h and then pyrolysed for 1 hour 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 $cm^3\ min^{-1}$ for 20 minutes 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 Hibler 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 hours, 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 hours (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 x 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 specifically chosen because they provide sensitive indicators of soil quality in terms of habitat function. Soils that cause avoidance or reduced aggregation behaviour typically reflect a decline in habitat suitability, which may experience reduced species richness and functional diversity due to the loss of suitable microhabitats.

Collembolan adults (9-12 days after hatching) were selected for the bioassays, while terrestrial isopods' adults with a wet weight of 14–30 mg were used, regardless of sex. Moulting animals, abnormal individuals, pregnant females and individuals lacking antennae were excluded. Glass boxes (9.5 x 7.3 x 5 cm) were used as test arena for terrestrial isopods and collembolans. LUFA 2.2 Soil Speyer was used for the ecotoxicological bioassays. The properties included pH = 5.5 ± 0.2 (0.01M $CaCl_2$), 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 biochar at 1 % and 10 % w/w, representing realistic and worst-case application scenarios, respectively. These correspond approximately to field application rates of 22 and 225 t/ha, assuming a bulk density of 1.5 g/cm^3 and incorporation to 15 cm depth (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 (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 terrestrial isopod bioassays. This technique is necessary to reduce the tigmotaxis of woodlice and induce them to aggregate only with conspecifics. 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 of the dishes was counted following the ISO (2020), while the arenas of terrestrial isopods were gently positioned beneath a camera (Trust Teza 4K Ultra HD webcam, 3840 × 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 terrestrial isopod 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 behavioral 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 (2021) and ISO 11265 (2025), respectively.

2.4 *Statistical analysis*

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

$$A\,(\%) = \frac{C - T}{N}\ x\ 100 \quad \text{(eq. 1)}$$

Where *C* = number of individuals moved to unamended soils, *T* = number of individuals which moved towards the biochar-amended soils, and *N* = 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):

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

were *s* = number of groups with 1 individual, *d* = number of groups with 2 individuals, and *n* = 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).

Avoidance behavioural data were analysed using generalized linear models (GLMs) with a Poisson distribution and a log-link function to account for non-normal response distributions. Zero values were retained in the analysis for negative data. Overall model significance was assessed via chi-square (χ^2) deviance tests, with statistical significance set at $p < 0.05$. For disaggregation responses, GLMs with a Gaussian distribution and identity link were fitted to continuous, approximately normally distributed data. Overall significance for these models was evaluated using analysis of variance (ANOVA) with F-tests. To examine the effect of collembolan recruitment in response to the presence of terrestrial isopods, a Mann–Whitney U test was performed. Differences in soil pH and electrical conductivity were assessed using ANOVA. Paired t-tests were applied to evaluate changes in these parameters between pre- and post-mitigation conditions following terrestrial isopod activity. All statistical analyses were conducted in R version 4.2.1 (R Core Team 2022).

3. Results

3.1 *Behavioural test results*

All behavioural tests performed with individuals of *Folsomia candida* and *Porcellionides pruinosus* showed stochastic population distribution and no mortality in controls.

Exposure to soils treated with biochar from *R. communis* and *B. juncea* resulted in consistent elusive responses by *F. candida* at concentration of 10% w/w ($\chi^2 = 103.9$, $p < 0.0001$), regardless of the pyrolysis degree, suggesting a higher sensitivity to the concentration of biochar (Fig. 1a).

In contrast, the behaviour of *P. pruinosus* (Fig. 1b) was influenced predominantly by biochar type and pyrolysis temperature ($\chi^2 = 1137.4$, $p < 0.0001$). Individuals were significantly attracted to both soils amended with *B. juncea* biochar pyrolyzed at 400 °C ($p < 0.0001$), but avoided those containing the same biochar pyrolyzed at 600 °C ($p < 0.0001$). No significant behavioural responses of terrestrial isopods were observed in soils treated with *R. communis* biochar ($p > 0.05$).

Figure 1. Avoidance test results for *Folsomia candida* 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 (± standard error). Different letters indicate significant differences among treatments (post hoc test, $p < 0.05$).

Terrestrial isopods exhibited a dose-dependent disaggregation behaviour across all biochar types (Fig. 2), with statistically significant effects observed only at the highest concentration of 10% w/w (*B. juncea*: F = 0.814, $p = 0.011$; *R. communis*: F = 1.12, $p = 0.013$).

The disaggregation observed in the *R. communis* bioassay may be influenced by biochar granularity, which appears to trigger a positive thigmotactic response in the isopods, drawing them toward the biochar particles. This hypothesis is further supported by the lower p-values observed for *B. juncea* biochar at 400°C ($p = 0.0011$) and 600°C ($p = 0.0012$) compared to *R. communis* biochar at 400°C ($p = 0.0046$) and 600°C ($p = 0.0088$). In contrast, the alteration of gregariousness in the *B. juncea* bioassay is more likely attributable to intrinsic biochar contamination rather than a thigmotactic effect, as no isopods were recorded in the treated soil at 10% w/w and 600°C. These results suggest that soils amended with *B. juncea* biochar are more repellent to terrestrial isopods, with behavioral responses influenced by pyrolysis temperature in the case of avoidance behaviors, and by both pyrolysis temperature and concentration in the case of gregariousness alterations.

Figure 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, $p < 0.05$).

The reintroduction of new collembolans in the terrestrial isopod-bioassays showed a different pattern of behavioral responses after 48 hours of exposure. Notably, no significant avoidance behavior was observed in any of the treatments (Fig. 3), suggesting that terrestrial isopods may function as niche-regulating organisms. This active role of terrestrial isopods may depend on the deposition of fecal pellets or bioturbation resulting in a selective microhabitat for maintaining homeostasis in collembolans.

As a result, the effects induced by biochar appear to vary according to successional changes in soil species composition. In our study, the presence of litter-dwelling species like terrestrial isopods modified the susceptibility of the biochar-amended soil compared to single-species exposure scenarios.

Figure 3. Avoidance test results for *Folsomia candida* after the 48 h colonization of *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 (± standard error). Different letters indicate significant differences among treatments (post hoc test, $p < 0.05$).

To assess the impact of terrestrial isopods activity on collembolan behavior, avoidance responses were compared between pre- and post-incubation substrates using the Mann–Whitney U test. For *R. communis* biochar, median avoidance shifted from –20% (interquartile range [IQR]: –20 to 20%) in the absence of isopods to –10% (IQR: –40 to 11%) following incubation. The difference was statistically significant (U = 548.5, p = 0.018), indicating a measurable influence of terrestrial isopod activity on collembolan distribution. In contrast, *B. juncea* biochar exhibited median avoidance values of –20% (IQR: –20 to 20%) without isopods and 0% (IQR: –35 to 30%) after incubation. The difference did not reach significance (U = 513, p = 0.074), suggesting no detectable effect of isopods on collembolan behavior in this substrate. These results indicate a species-specific modulation of collembolan avoidance by terrestrial isopods activity, highlighting an ecological facilitation effect depending on the type of biochar.

3.2 *Changes of pH and conductivity in soils amended with biochar*

The pH and electrical conductivity (EC) of LUFA soils amended with biochar varied significantly after 48 h of exposure as a function of feedstock type, application rate, and pyrolysis temperature (Table 1). Soils amended with *B. juncea* biochar exhibited significant increases in both pH (F = 14.7, p < 0.0001) and EC (F = 7.16, p = 0.0055). A stronger response was observed in soils treated with *R. communis* biochar, which induced marked changes in pH (F = 95.8, p < 0.0001) and EC (F = 70.5, p < 0.0001), indicating a higher capacity of this feedstock to alter soil chemical properties compared to *B. juncea* biochar. The magnitude of the pH response was consistently greater than that of EC, suggesting that alkalinity-driven processes dominated the short-term soil–biochar interactions. These biochar-induced alterations persisted following biological mitigation by terrestrial isopods, confirming the structural stability of the biochar effects. In post-mitigation soils, *B. juncea* biochar remained associated with significant variation in pH (F = 86.4, p < 0.0001) and EC (F = 20.8, p < 0.0001). In contrast, soils amended with *R. communis* biochar displayed a strong pH response (F = 260.3, p < 0.0001), while the effect on EC was reduced, although still significant (F = 11.1, p = 0.001), suggesting a partial buffering of soluble ions following faunal activity.

Direct comparisons between pre- and post-colonization soils revealed that terrestrial isopods primarily influenced EC rather than pH. Significant differences were detected only for conductivity, with a markedly stronger reduction in *B. juncea* amended soils (t = −29.1, p < 0.0001) compared to those amended with *R. communis* biochar (t = −4.08, p = 0.001). This indicates that isopod-mediated processes preferentially modulated ion mobility and redistribution, while the biochar-driven alkalinisation remained largely unaffected.

Collectively, all biochar types increased soil pH and EC; however, the EC response was strongly concentration-dependent, with significant increases observed exclusively at the highest application rate (10% w/w). These findings demonstrate that feedstock identity and dosage are key determinants of short-term soil chemical shifts, while biological activity can selectively mitigate biochar-induced changes in soil conductivity without substantially altering pH.

Samples	**Dose**	**Pyrolysis**	**Pre-ecological facilitation**		**Post-ecological facilitation**	
	%	**°C**	**pH**	**EC μS cm^{-1}**	**pH**	**EC μS cm^{-1}**
Control			6.08 (± 0.12) a	212.6 (± 9.05) a	6.14 (± 0.03) a	226.1 (± 3.64) a
B. juncea	1	400	6.61 (± 0.04) b	208 (± 2.08) bc	6.96 (± 0.06) b	226.3 (± 3.64) bc
		600	6.75 (± 0.14) b	200.7 (± 3.88) bd	6.85 (± 0.04) b	248 (± 11.8) bd
	10	400	6.96 (± 0.03) b	371.3 (± 31.1) be	7.04 (± 0.03) b	304.8 (± 3.8) be
		600	6.74 (± 0.02) b	333.3 (± 59.1) bf	6.96 (± 0.03) b	291.4 (± 1.55) bf
R. communis	1	400	7.64 (± 0.12) bc	255.7 (± 13) bc	7.77 (± 0.12) bc	255.83 (± 19.9) a

	600	7.69 (± 0.21) bd	545.7 (± 100) bd	7.93 (± 0.03) bd	580 (± 19.7) a
10	400	8.53 (± 0.04) be	1,888 (± 123.6) be	8.11 (± 0.06) be	1,006.6 (± 5.9) b
	600	9.41 (± 0.07) bf	2,797.3 (± 261.3) bf	9.01 (± 0.04) bf	2,077.2 (± 512) b

Table 1. Soil conductivity (EC, expressed as $\mu S\ cm^{-1}$) and pH measured at the beginning of the collembolan test (pre-ecological facilitation) and 48h after the behavioural bioassay of collembola reintroduced in terrestrial isopod tests (post-ecological facilitation) for *B. juncea* and *R. communis* biochar. Different letters indicate significant differences among treatments (post hoc test, $p < 0.05$).

Pearson correlation analysis revealed consistent and robust associations between soil chemical parameters and biological responses, with distinct patterns depending on biochar feedstock and mitigation conditions (Fig. 4). Across all treatments, soil pH and electrical conductivity (EC) were strongly and positively correlated, indicating a tightly coupled response of soil alkalinity and ionic strength following biochar amendment.

In soils amended with *B. juncea* biochar, avoidance behaviour of *F. candida* showed only weak associations with soil pH and EC, both prior (r_pH = 0.32; r_EC = 0.27) and after terrestrial isopod colonisation (r_pH = 0.23; r_EC = 0.25). In contrast, soils amended with *R. communis* biochar exhibited stronger positive correlations between *F. candida* avoidance and both pH (r = 0.72) and EC (r = 0.76) levels. These results suggest that higher pH and EC in the *R. communis* amended soils may have contributed to increased repellence in collembolans, likely due to shifts in soil chemistry or osmotic stress, in line with other studies (Bastos et al. 2022). Following the introduction of terrestrial isopods, these correlations were moderately attenuated, with reduced associations for both pH (r = 0.45) and EC (r = 0.53). These results indicate that the introduction of terrestrial isopods altered soil properties, influencing collembolan perception of habitat quality and potentially reducing avoidance behavior in previously repellent substrates.

In contrast, no strong correlations were observed between avoidance responses of *P. pruinosus* and soil pH or EC in soils amended with either *B. juncea* or *R. communis* biochar. Disaggregation behaviour was rather moderately correlated with pH (r = 0.49) and strongly correlated with EC (r = 0.69) in *B. juncea* biochar, suggesting an influence of osmotic stress on gregariousness. A similar, though less pronounced, pattern was observed in *R. communis* biochar, with moderate correlations for pH (r = 0.58) and EC (r = 0.46).

Overall, the correlation patterns indicate that biochar increases in pH and EC are closely interrelated and constitute the primary drivers of the behavioural responses recorded. Avoidance behaviour in collembolans appears to be more sensitive to chemical shifts in the soil, particularly under *R. communis* amendment, and can be partially mitigated by the presence of terrestrial isopods. By contrast, social behaviour of terrestrial isopods appears more closely linked to changes in EC, highlighting a differential sensitivity of soil fauna to biochar-mediated chemical alterations.

Figure 4. Pearson correlation matrices illustrating relationships between soil pH and electrical conductivity (EC) and behavioral responses of soil arthropods under biochar amendment. Panels (a - b) show correlations between initial and final soil pH (pHi, pHf) and EC (ECi, ECf) and *F. candida* avoidance behavior before (*F1*) and after (*F2*) ecological facilitation by terrestrial isopods in soils amended with *B. juncea* (a) and *R. communis* (b) biochars. Panels (c - d) show correlations between soil physicochemical parameters and avoidance (*P1*) and disaggregation (*P2*) behaviors of *P. pruinosus* in *B. juncea* (c) and *R. communis* (d) amended soils.

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 mesofauna species, *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 conductivity (EC) 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 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 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 toxic 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. Results in the *F. candida* bioassay showed effects 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* 400 °C were additionally detected traces of steroid derivatives. 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 behavior 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. Pearson correlation analysis revealed a strong association between soil electrical conductivity (EC) in *B. juncea*-amended soils and the disaggregation index, but this relationship alone does not seem to fully explain this effect. In fact, despite higher EC, soils treated with *R. communis* showed moderate correlations with soil electrical conductivity, suggesting that this parameter may not be the only factor involved. Rather, the phenomenon appears to be primarily determined by biochar composition. 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) but not for KCl-amended soils with similar EC (0.74–1.67 dS/m) (Škarková et al. 2016).

The decrease in soil pH and EC 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, where correlations between avoidance and soil properties changed direction. Such results point to biochar transformation or detoxification processes mediated by isopods and associated microbes, ultimately influencing collembolan habitat preferences and indicating complex trophic and microbial interactions (Scheu 2002; Frouz 2018). The role of isopod 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 U-test 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.

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 investigated the effects of two types of biochar derived from *Brassica juncea* and *Ricinus communis* biomasses, pyrolyzed at 400°C and 600°C, on the behaviour responses of *Folsomia candida* and *Porcellionides pruinosus*. Biochar samples were applied at 1% and 10% w/w to assess potential alterations in structure and dynamics of population of both species. The findings demonstrated that biochar effects on soil fauna were modulated by feedstock type, pyrolysis temperature, application rate, and species-specific sensitivity. *F. candida* exhibited a concentration dependent sensitivity to both biochar types, whereas *P. pruinosus* demonstrated attraction to *Brassica* sp. biochar produced at 400°C, but avoided the biochar at 600°C, regardless of concentration. Furthermore, terrestrial isopods exhibited modified aggregation behaviour in response to biochar doses. Of particular significance was the observation that the presence of *P. pruinosus* modulated the behavioural responses of *F. candida*, thus suggesting a potential ecological facilitation effect that is likely to be mediated by isopod-driven bioturbation and faecal pellet deposition. These findings emphasise the necessity of incorporating interspecific interactions in ecological assessments, as they may elucidate natural mitigation processes and recolonization processes.

Credit authorship contribution statement

Lorenzo Federico: Conceptualization, Methodology, Resources, Data curation, Formal analysis, Investigation, Writing – original draft. **Asia Rosatelli**: Conceptualization, Investigation, Writing – reviewer & editing. **Niccolò Lamanna**: Investigation, Writing – reviewer & editing. **Heiko Lange**: Resources, Writing – reviewer & editing. **Termopili 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, Writing – reviewer & editing.

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References

Bastos, A. C., Verheijen, F. G. A., Amaro, A., Prodana, M., Cardoso, D. N., Morgado, R. G., Santos, M.J., Soares, A.M.V.M., Keizer, J.J., Loureiro, S. 2022. Screening the habitat function of biochar-amended vineyard soils at field plot-scale, based on invertebrate avoidance behaviour. *Appl. Soil Ecol.*, 177, 104526. https://doi.org/10.1016/j.apsoil.2022.104526

Bastow, J. L. 2011. Facilitation and predation structure a grassland detrital food web: the responses of soil nematodes to isopod processing of litter. *J. Anim. Ecol.*, 80(5), 947-957. https://doi.org/10.1111/j.1365-2656.2011.01853.x

Cao, X., Ma, L., Gao, B., Harris, W. 2009. Dairy-manure derived biochar effectively sorbs lead and atrazine. *Environ. Sci. Technol*., 43(9), 3285-3291. https://doi.org/10.1021/es803092k

Conti, F.D., Visioli, G., Malcevschi, A., Menta, C., 2018. Safety assessment of gasification biochars using *Folsomia candida* (Collembola) ecotoxicological bioassays. *Environ. Sci. Pollut. Res*. 25, 6668–6679. https://doi.org/10.1007/s11356-017-0806-4

Glaser, B., Lehmann, J., Zech, W. 2002. Ameliorating physical and chemical properties of highly weathered soils in the tropics with charcoal–a review. *Biol. Fertil. Soils*, 35, 219-230. https://doi.org/10.1007/s00374-002-0466-4

Godlewska, P., Ok, Y. S., Oleszczuk, P. 2021. The dark side of black gold: Ecotoxicological aspects of biochar and biochar-amended soils. *J. Hazard. Mater*., 403, 123833. https://doi.org/10.1016/j.jhazmat.2020.123833

EU/2024/3012 - Regulation (EU) 2024/3012 of the European Parliament and of the Council of 27 November 2024 establishing a Union certification framework for permanent carbon removals, carbon farming and carbon storage in products. http://data.europa.eu/eli/reg/2024/3012/oj

Federico, L., Monti, G. S., Loureiro, S., Villa, S. 2024. Disaggregation behavior in the terrestrial isopod *Porcellionides pruinosus* as a new ecotoxicological endpoint for assessing infochemical disrupting activity. *Ecol. Indic.*, 167, 112602. https://doi.org/10.1016/j.ecolind.2024.112602

Federico, L., Tatangelo, V., Pittino, F., Russo, C., Vegini, E., Citterio, S., Franzetti, A., Villa, S. 2026. The potential use of behavioural bioassays as a first-tier approach for screening urban soil biodiversity: a pilot study. *Urb. Ecosyst.* https://doi.org/10.1007/s11252-026-01908-6

Hilber, I., Blum, F., Leifeld, J., Schmidt, H.P., Bucheli, T.D. 2012. Quantitative Determination of PAHs in Biochar: A Prerequisite to Ensure Its Quality and Safe Application. *J. Agric. Food Chem.*, 60, 3042–3050, https://doi.org/10.1021/jf205278v

Hopkin, S. P. 1997. Biology of the springtails:(Insecta: Collembola). OUP Oxford. https://doi.org/10.1093/oso/9780198540847.001.0001

ISO 2020. Soil quality - Avoidance test for determining the quality of soils and effects of chemicals on behaviour - Part 2: Test with collembolans (*Folsomia candida*). ISO 7512-2:2020.

ISO 2021. Soil, treated biowaste and sludge – Determination of pH. ISO 10390:2021.

ISO 2025. Environmental solid matrices — Determination of the specific electrical conductivity. ISO 11265:2025

Lehmann, J., Rillig, M. C., Thies, J., Masiello, C. A., Hockaday, W. C., Crowley, D. 2011. Biochar effects on soil biota–a review. *Soil Biol. Biochem.*, 43(9), 1812-1836. https://doi.org/10.1016/j.soilbio.2011.04.022

Lehmann, J., Joseph, S. (Eds.) 2024. Biochar for environmental management: science, technology and implementation. *Taylor & Francis*. https://doi.org/10.4324/9780203762264

Li, D., Hockaday, W. C., Masiello, C. A., Alvarez, P. J. 2011. Earthworm avoidance of biochar can be mitigated by wetting. *Soil Biol. Biochem.*, 43(8), 1732-1737. https://doi.org/10.1016/j.soilbio.2011.04.019

Liesch, A. M., Weyers, S. L., Gaskin, J. W., Das, K. C. 2010. Impact of two different biochars on earthworm growth and survival. *Ann. Environ. Sci.*, 4, 1-9. http://hdl.handle.net/2047/d20000234

Madej, J., Hilber, I., Bucheli, T.D., Oleszczuk, P. 2016. Biochars with Low Polycyclic Aromatic Hydrocarbon Concentrations Achievable by Pyrolysis under High Carrier Gas Flows Irrespective of Oxygen Content or Feedstock. *J. Anal. Appl. Pyrolysis*, 122, 365–369. https://doi.org/10.1016/j.jaap.2016.09.005

Madžarić, S., Kos, M., Drobne, D., Hočevar, M., Kokalj, A. J. 2018. Integration of behavioral tests and biochemical biomarkers of terrestrial isopod *Porcellio scaber* (Isopoda, Crustacea) is a promising methodology for testing environmental safety of chars. *Environ. Pollut.*, 234, 804-811. https://doi.org/10.1016/j.envpol.2017.12.024

Marks, E. A., Mattana, S., Alcañiz, J. M., Domene, X. 2014. Biochars provoke diverse soil mesofauna reproductive responses in laboratory bioassays. *Eur. J. Soil Biol*., 60, 104-111. https://doi.org/10.1016/j.ejsobi.2013.12.002

Mayer, P., Hilber, I., Gouliarmou, V., Hale, S.E., Cornelissen, G., Bucheli, T.D. 2016. How to Determine the Environmental Exposure of PAHs Originating from Biochar. *Environ. Sci. Technol*., 50, 1941–1948, https://doi.org/10.1021/acs.est.5b05603

Onay, O. 2007. Influence of pyrolysis temperature and heating rate on the production of bio-oil and char from safflower seed by pyrolysis, using a well-swept fixed-bed reactor. *Fuel Process. Technol*., 88(5), 523–531. https://doi.org/10.1016/J.FUPROC.2007.01.001

Parmentier, T., Taiti, S., De Smedt, P. 2025. Ant-isopod interactions: spanning predation, facilitation, and myrmecophily. *Eur. J. Soil Biol.*, 126, 103753. https://doi.org/10.1016/j.ejsobi.2025.103753

Pieper, S., Weigmann, G. 2008. Interactions between isopods and collembolans modulate the mobilization and transport of nutrients from urban soils. *Appl. Soil Ecol*., 39(2), 109-126. https://doi.org/10.1016/j.apsoil.2007.11.012

Prodana, M., Bastos, A. C., Amaro, A., Cardoso, D., Morgado, R., Machado, A. L., Verheijen, F.G.A., Keizer, J.J., Loureiro, S. 2019. Biomonitoring tools for biochar and biochar-compost amended soil under viticulture: Looking at exposure and effects. *Appl. Soil Ecol*., 137, 120-128. https://doi.org/10.1016/j.apsoil.2019.01.007

R Core Team 2024. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. https://www.R-project.org /

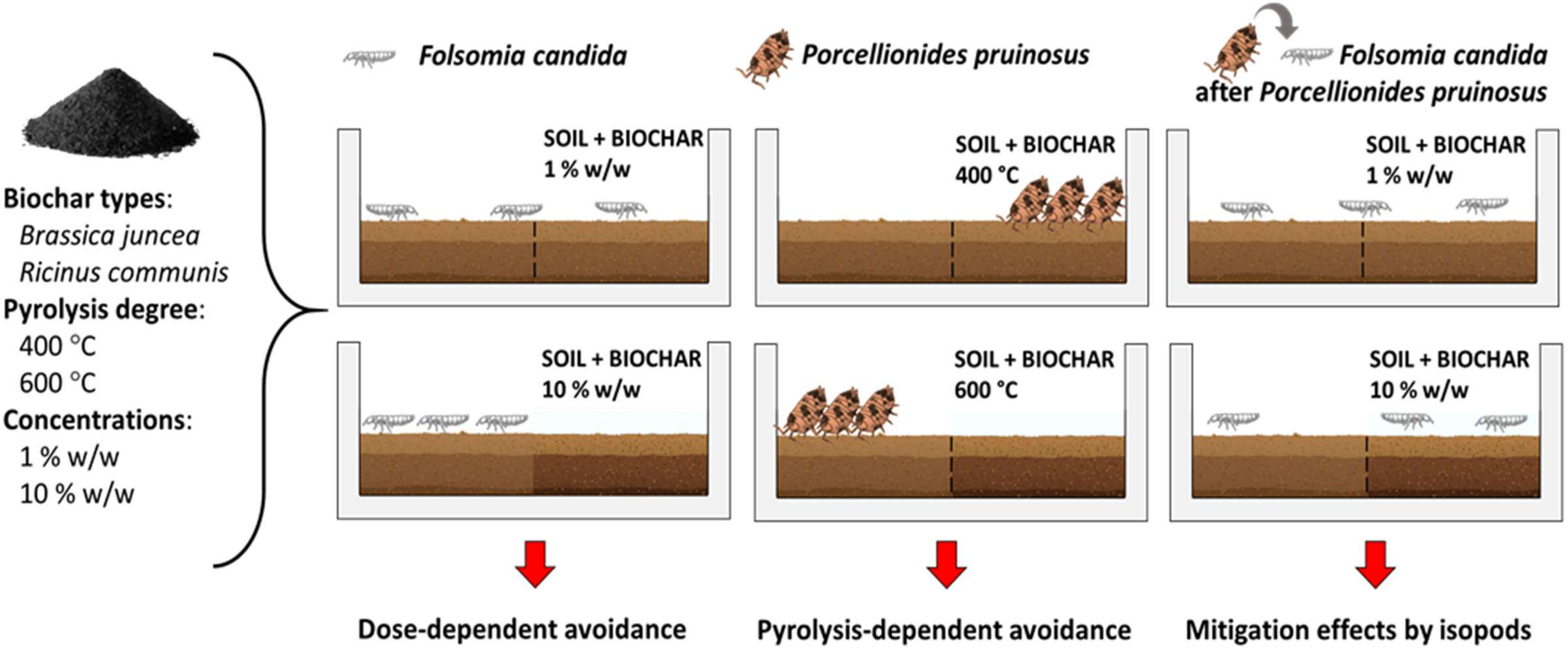
Škarková, P., Kos, M., Drobne, D., Vávrová, M., Jemec, A. 2016. Effects of food salinization on terrestrial crustaceans *Porcellio scaber*. *Appl Soil Ecol.*, 100, 1-7. https://doi.org/10.1016/j.apsoil.2015.11.007

Snyder, B. A., Hendrix, P. F. 2008. Current and potential roles of soil macroinvertebrates (earthworms, millipedes, and isopods) in ecological restoration. *Restor. Ecol*., 16(4), 629-636. https://doi.org/10.1111/j.1526-100X.2008.00484.x

Stachowicz, J. J. 2001. Mutualism, facilitation, and the structure of ecological communities: positive interactions play a critical, but underappreciated, role in ecological communities by reducing physical or biotic stresses in existing habitats and by creating new habitats on which many species depend. *Bioscience*, 51(3), 235-246. https://doi.org/10.1641/0006-3568(2001)051[0235:MFATSO]2.0.CO;2

Tammeorg, P., Parviainen, T., Nuutinen, V., Simojoki, A., Vaara, E., Helenius, J. 2014. Effects of biochar on earthworms in arable soil: avoidance test and field trial in boreal loamy sand. *Agr. Ecosyst. Environ*., 191, 150-157. https://doi.org/10.1016/j.agee.2014.02.023

Verheijen, F., Jeffery, S., Bastos, A. C., Van der Velde, M., Diafas, I. 2010. Biochar application to soils. A critical scientific review of effects on soil properties, processes, and functions. *Eur*., 24099(162), 2183-2207. https://dx.doi.org/10.2788/472



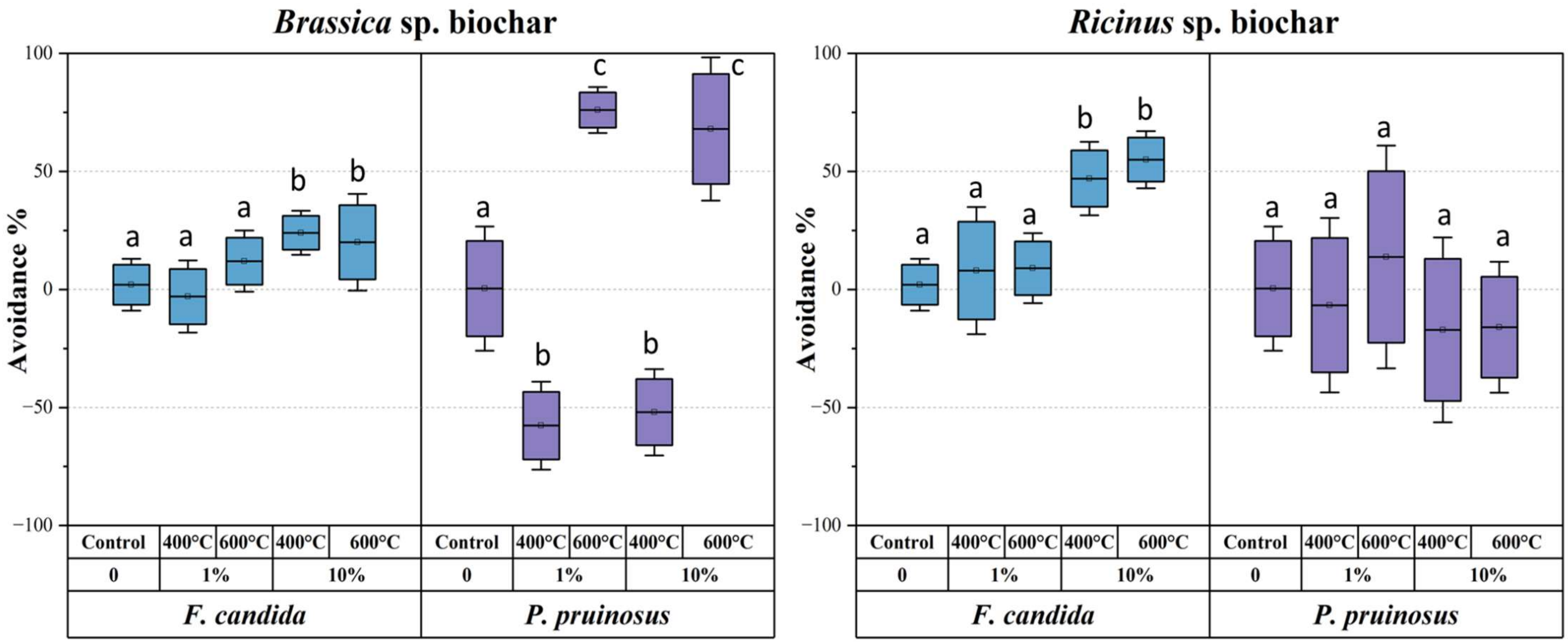
- **Biochar types**:
  - *Brassica juncea*
  - *Ricinus communis*
- **Pyrolysis degree**:
  - 400 °C
  - 600 °C
- **Concentrations**:
  - 1 % w/w
  - 10 % w/w

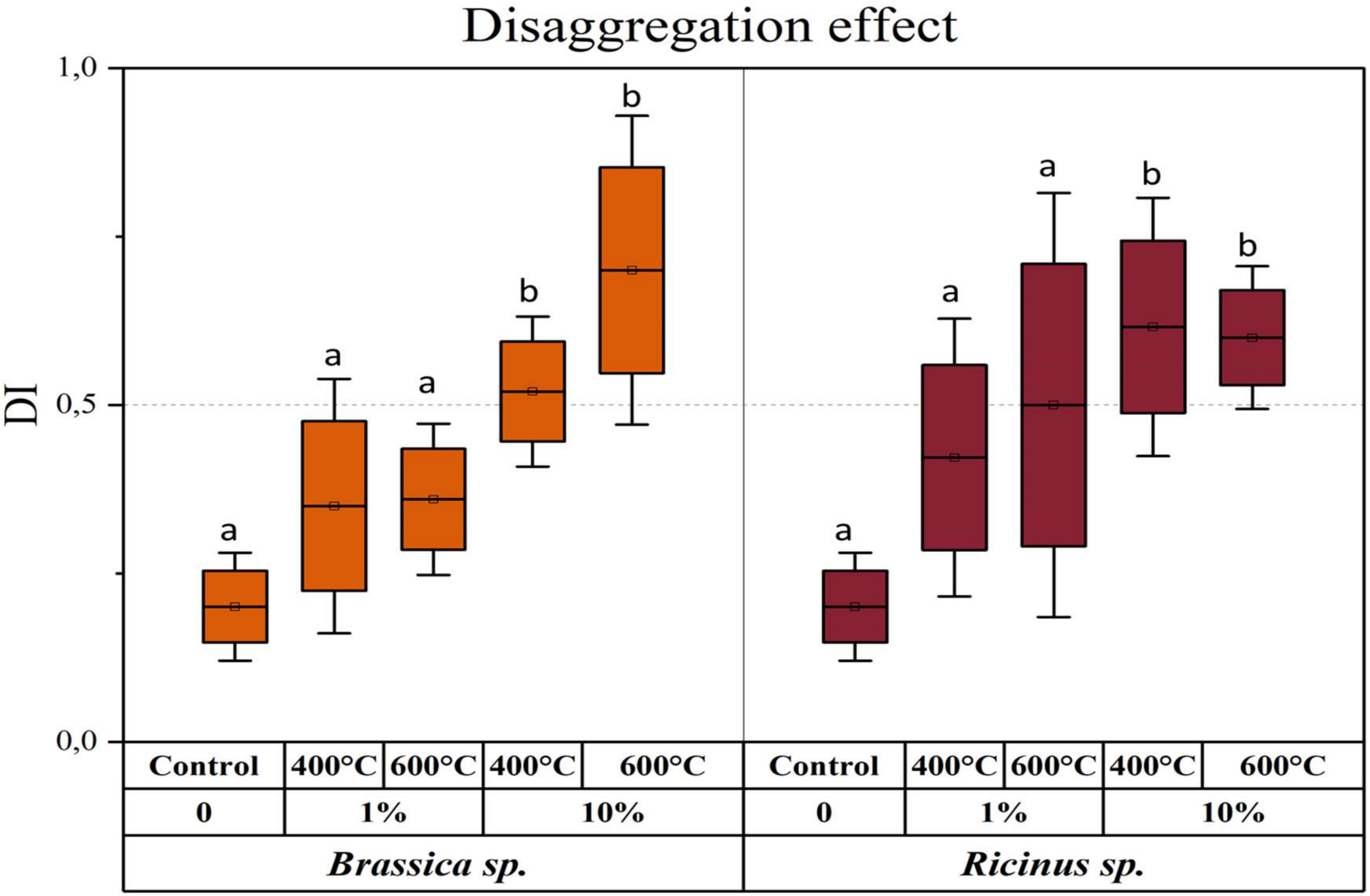
Dose-dependent avoidance

Pyrolysis-dependent avoidance

Mitigation effects by isopods

fig.1


Brassica sp. biochar
Ricinus sp. biochar
Avoidance %
100
50
0
−50
−100
a
a
a
b
b
a
b
c
b
c
a
a
a
b
b
a
a
a
a
a
Control
400°C
600°C
400°C
600°C
0
1%
10%
F. candida
P. pruinosus


Disaggregation effect
DI
1,0
0,5
0,0
a
a
a
b
b
a
a
a
b
b
Control
400°C
600°C
400°C
600°C
Control
400°C
600°C
400°C
600°C
0
1%
10%
0
1%
10%
Brassica sp.
Ricinus sp.

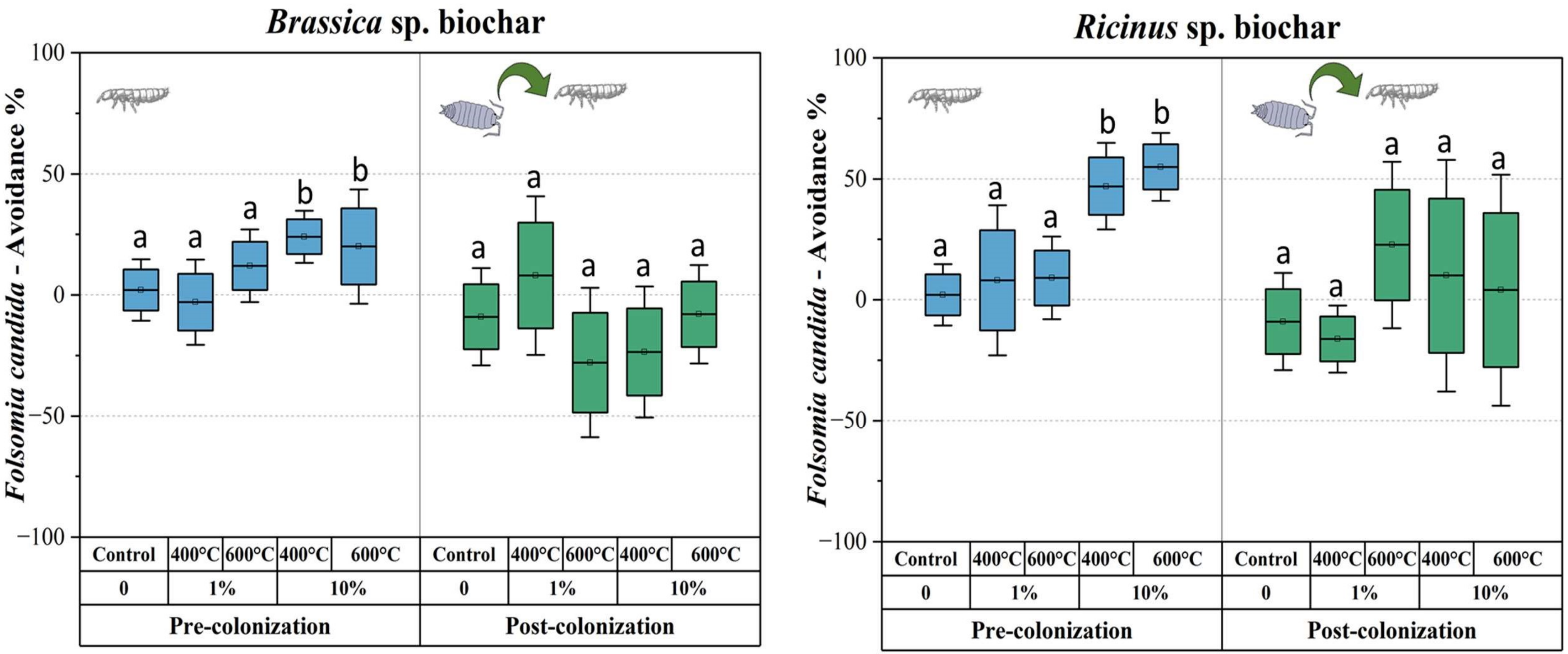
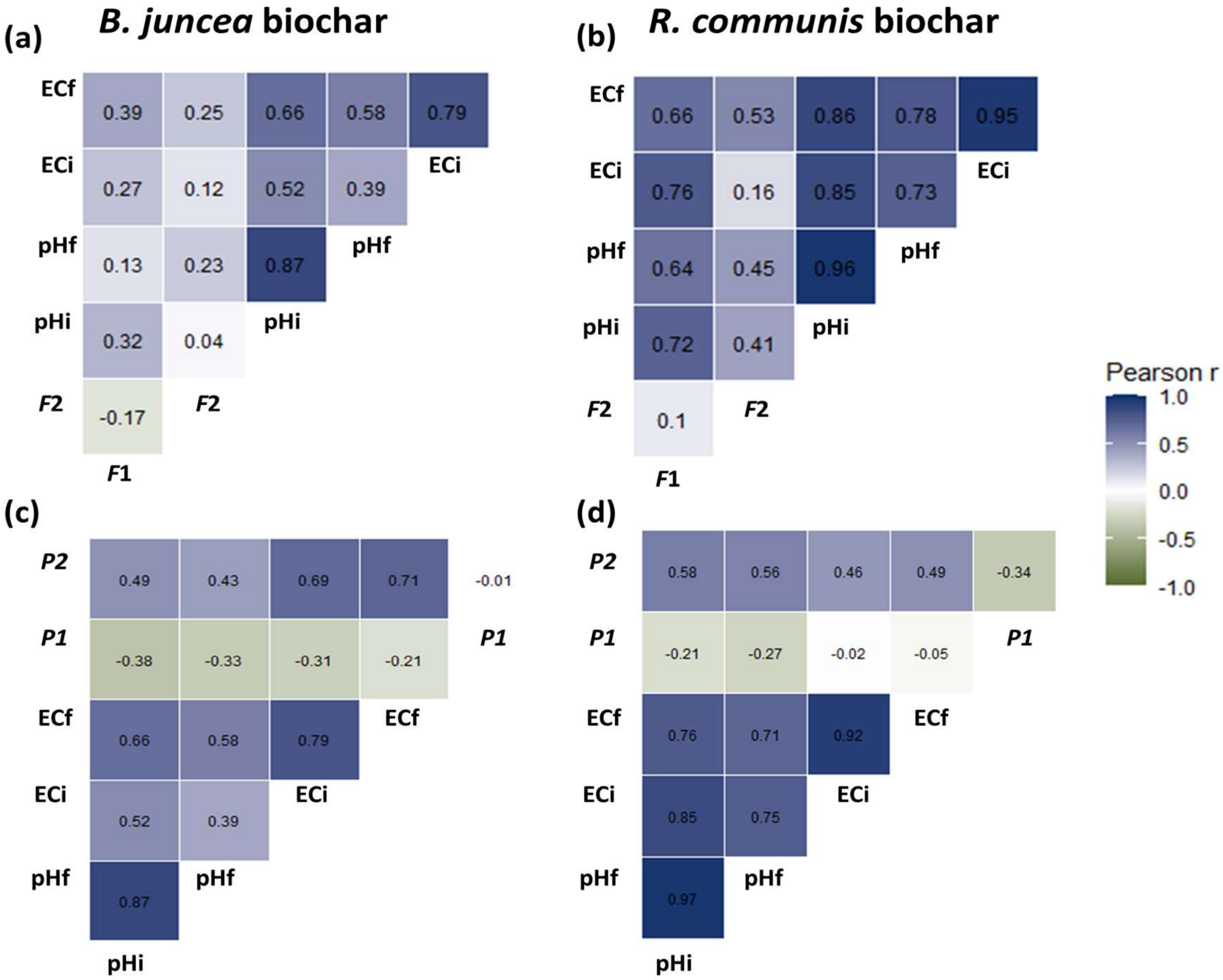

Brassica sp. biochar
Folsomia candida - Avoidance %
100
50
0
−50
−100
a
a
a
b
b
a
a
a
a
a
Control
400°C
600°C
400°C
600°C
0
1%
10%
Pre-colonization
Post-colonization
Ricinus sp. biochar
Folsomia candida - Avoidance %
100
50
0
−50
−100
a
a
a
b
b
a
a
a
a
a
Control
400°C
600°C
400°C
600°C
0
1%
10%
Pre-colonization
Post-colonization

fig.4

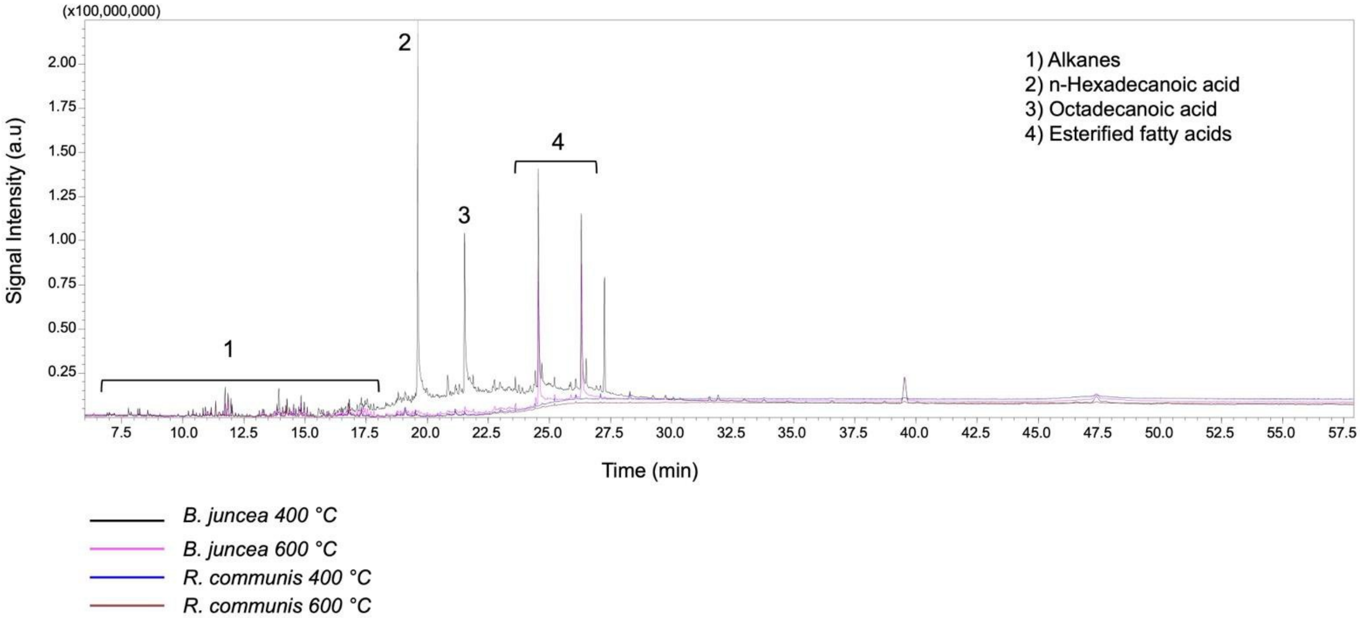

(a)
B. juncea biochar
ECf
0.39 0.25 0.66 0.58 0.79
ECi
0.27 0.12 0.52 0.39
pHf
0.13 0.23 0.87
pHi
0.32 0.04
F2
-0.17
F1
(b)
R. communis biochar
ECf
0.66 0.53 0.86 0.78 0.95
ECi
0.76 0.16 0.85 0.73
pHf
0.64 0.45 0.96
pHi
0.72 0.41
F2
0.1
F1
Pearson r
1.0
0.5
0.0
-0.5
-1.0
(c)
P2
0.49 0.43 0.69 0.71 -0.01
P1
-0.38 -0.33 -0.31 -0.21
ECf
0.66 0.58 0.79
ECi
0.52 0.39
pHf
0.87
pHi
(d)
P2
0.58 0.56 0.46 0.49 -0.34
P1
-0.21 -0.27 -0.02 -0.05
ECf
0.76 0.71 0.92
ECi
0.85 0.75
pHf
0.97
pHi

Ecological Facilitation of Terrestrial Isopods indirectly Modulate Collembolan Behaviour exposed to Biochars

Supplementary material

Table of Content

Figure S1 Overlay of the GC–MS chromatograms obtained for the four biochar extracts: black, *B. juncea* 400 °C; pink, *B. juncea* 600 °C; blue, *R. communis* 400 °C; dark-orange, *R. communis* 600 °C. The chromatograms show a region between approximately 7 and 17 min mainly characterized by alkanes (both linear and branched) for all samples. Free fatty acids were detected only in *B. juncea* biochar pyrolyzed at 400 °C, while *B. juncea* biochar at 600 °C showed only esterified fatty acids. In contrast, the GC–MS profiles of *R. communis* biochar extracts were predominantly characterized by alkanes, with no evident other contributions. In *B. juncea* 400 °C traces of steroid derivatives were additionally detected.



Chapter 8

Conclusions

8.1 General Overview of PhD Thesis

The progressive loss of soil functions and ecosystem services represents one of the major environmental challenges of the 21st century, with direct implications not only for biodiversity conservation but also for food provisioning, human health, climate resilience, and the sustainability of socio-economic systems. In this context, there is a need to move beyond monitoring approaches based solely on soil physicochemical descriptors, which are often insufficient to capture functional and biological alterations of soil ecosystems.

This PhD thesis addresses this critical context by proposing a methodological and conceptual shift, based on the idea that soil health cannot be fully assessed without careful analysis of the behavioural responses of the organisms that constitute its living component. From this premise, the PhD thesis explored the potential of behavioural ecotoxicology as a rapid and ecologically relevant operational tool to bridge the gap between laboratory assessments and the ecological complexity of soil ecosystems. In particular, this work argues that population-level behavioural bioassays constitute key indicators of soil habitat functionality and ecological integrity. Such indicators can be effectively derived from the use of highly gregarious species, such as terrestrial isopods, which serve as particularly suitable sentinels for studying the spatial distribution and social cohesion of populations in response to different soil stressors.

The guiding thread throughout this work is the development and validation of a novel ecotoxicological endpoint based on the combined analysis of avoidance and disaggregation behaviours in the gregarious edaphic organism *Porcellionides pruinosus* (order Oniscidea). The choice of this species was motivated not only by ecotoxicological sensitivity but also by its ecological relevance and eco-ethological traits, which make aggregation behaviour a central functional trait for survival and population stability. In this sense, social alteration is interpreted as a direct signal of population fragmentation and impairment of soil habitat function, complementing population dynamics studies such as avoidance tests. Through a series of experimental studies presented in Chapter 3 and progressively developed in subsequent chapters, this PhD thesis demonstrates that disaggregation in *P. pruinosus* may occur independently or in conjunction with avoidance of contaminated soil, revealing dimensions of ecological risk that are not detected by traditional behavioural assays. The results highlight that avoidance represents a potentially underestimated migratory response if not integrated with an assessment of population gregariousness, which reflects the loss of social cohesion with direct implications for individual fitness, survival, and population stability. The combination of these two endpoints therefore allows for distinguishing between active spatial redistribution of individuals and impairment of population structure, providing a more nuanced and biologically meaningful interpretation of contaminant effects.

Another significant contribution of this PhD thesis is the systematic analysis of *P. pruinosus* behavioural responses to a wide range of abiotic and biotic stressors, as described in Chapter 4. The identification of distinct response patterns (U-shaped, sigmoidal, bimodal) according to environmental variables demonstrates that aggregation behaviour is finely tuned to soil conditions and responds predictably to stress gradients. In particular, soil temperature and moisture gradients significantly influence population gregariousness, which reaches its maximum under intermediate conditions of both factors. These conditions are complementary to the distribution of maximal population fitness, suggesting that peak gregariousness coincides with peak reproductive output, highlighting the ecological relevance of disaggregation indices. These results reinforce the idea that disaggregation can serve as an integrative biosensor of environmental conditions, reflecting not only the presence of contaminants but also physical and biological soil alterations potentially linked to climate change or soil management practices.

A further contribution emerges in Chapter 5, where behavioural responses to a wide spectrum of contaminants identified disaggregation as a predictive endpoint associated with specific physicochemical properties of substances. In particular, the identification of a threshold Henry's law constant beyond which gregariousness is compromised provides a mechanistic criterion with potential regulatory applications, suggesting that the water–air partitioning of chemical contaminants plays a crucial role in exposure to terrestrial isopods and the onset of disaggregation. The absence of predictive descriptors for avoidance responses, in contrast, underscores the complexity of mechanisms governing migration and highlights the need to integrate multiple behavioural endpoints within a single bioassay.

Chapters 3 to 5 primarily serve an investigative purpose. Complementarily, Chapters 6 and 7 demonstrate the potential applied value of disaggregation tests in terrestrial isopods. Chapter 6 extended the application of behavioural bioassays to real urban soils, showing that model invertebrate responses are consistent with reductions in faunal biodiversity along urban degradation gradients. Soils inducing marked population-level behavioural responses were the same where structural and functional diversity was low. The multispecies approach further showed greater sensitivity of *P. pruinosus* compared to *F. candida* and *E. fetida* in avoidance bioassays, likely due to the species' gregarious nature amplifying the population-level response. Moreover, the use of disaggregation indices helped interpret population-level effects in cases of attractive behaviours; high aggregation in attractive contexts suggests preference (e.g., due to high organic matter content, as observed in

Chapter 6), whereas attractive behaviour coupled with altered gregariousness indicates loss of social cohesion and impaired infochemical functionality due to individual disorientation. Overall, the results indicate that behavioural bioassays provide an effective early-warning system for biodiversity loss and habitat fragmentation in terms of pedofaunal diversity, while the discrepancy between behavioural responses and microbial community structure suggests lower predictive power of behavioural tests for bacterial communities.

While behavioural bioassays in Chapter 6 had primarily diagnostic value, in Chapter 7 they were used for their prognostic potential to assess the quality of soils amended with biochar varying in concentration and pyrolysis degree. The analysis of responses to biochar amendments shows that effects on soil invertebrates depend on a complex combination of factors, including feedstock type, pyrolysis temperature, application rate, and species identity. Populations of *F. candida* were particularly sensitive to biochar dose, correlated with changes in soil conductivity and pH post-amendment. In contrast, *P. pruinosus* avoidance responses were more influenced by pyrolysis degree and biochar composition, while alterations in aggregative behaviour primarily depended on biochar concentration. These results indicate that biochar can modulate both the dynamics and population structure of isopods in a species- and context-specific manner. Additionally, observation of interspecific facilitation processes mediated by terrestrial isopods demonstrated that the presence of epiedaphic organisms can mitigate avoidance responses of euedaphic collembolans, highlighting the importance of incorporating biotic interactions into ecotoxicological bioassays and confirming that natural systems possess intrinsic compensatory mechanisms often overlooked in standard assessments.

8.2 Advantages and Applications

Taken together, the chapters of this PhD thesis outline a coherent scientific trajectory, moving from the urgent need to protect soil health and ecosystem services toward the development of innovative, ecologically relevant, and operationally applicable monitoring tools. The adopted approach integrates behavioural ecotoxicology, environmental factor analysis, and biodiversity assessment, overcoming the limitations of traditional indicators based solely on physicochemical parameters. The results of this PhD thesis open multiple avenues for future research and methodological development in soil health assessment and sustainable ecosystem management.

First, behavioural bioassays, already validated as sensitive and integrative indicators, could be further developed into standardized protocols for ecotoxicological monitoring and comprehensive chemical and physical risk assessment. Integrating disaggregation effects with already standardized tests, such as avoidance responses, would allow for a more complete and predictive evaluation of soil habitat condition. Given that standardized avoidance tests are currently limited to *E. fetida* and *F. candida*, an initial standardized protocol based on terrestrial isopods such as *P. pruinosus* could incorporate an integrative avoidance–disaggregation endpoint as a soil quality indicator.

Second, the relationship between contaminants' physicochemical properties and disaggregation behaviours suggests the potential for more accurate predictive models combining molecular descriptors, exposure thresholds, and environmental parameters, extending bioassay applicability to regulatory and in silico risk management contexts. Investigating the underlying molecular, biochemical, and metabolic responses driving disaggregation—currently poorly understood—will be essential. This limitation is partly due to the complexity and limited characterization of the aggregation pheromone in *P. pruinosus*, necessitating collaborative efforts among biologists, chemists, ecologists, and ecotoxicologists.

Finally, while using behavioural bioassays as predictors of depopulated soils with reduced diversity is promising, empirical validation with large, statistically robust datasets is still required. Such validation, across scales from microcosms to field-level assessments, could enhance the ability to detect and predict ecological tipping points, facilitating proactive interventions in forested, urban, and agricultural areas.

8.3 Limitations and Challenges

Despite its innovative contributions and solid experimental framework, several limitations and critical challenges must be acknowledged, which at the same time outline important directions for future research.

First, the mechanistic basis of aggregation and disaggregation responses is not yet fully understood. The biochemical identity of aggregation pheromones in *P. pruinosus*, the mechanisms underlying their synthesis, release, perception, and degradation, as well as the neurophysiological and metabolic processes regulating social cohesion, require further investigation. At present, disaggregation is interpreted as a functional signal of ecological stress; however, the causal chain linking contaminant exposure, individual physiological alteration, and collective behavioural response remains only partially clarified. Without a detailed mechanistic understanding, the capacity to perform robust predictive extrapolations or to fully integrate these endpoints into quantitative risk models remains constrained.

Second, behavioural responses are inherently species-specific and strongly linked to the eco-ethological traits of the model organism. The gregarious nature of *P. pruinosus* represents a strength in terms of population-level sensitivity, but it also implies that extrapolation to non-gregarious taxa, other functional groups, or different trophic levels requires careful comparative validation. The proposed framework therefore cannot be considered universally representative of soil fauna, but rather should be integrated within multi-species and multi-trophic assessment strategies capable of providing a more comprehensive view of soil ecosystem functioning.

Third, environmental context dependency constitutes a significant methodological challenge. Aggregation patterns are strongly modulated by abiotic factors such as soil temperature and moisture, which directly influence water balance, metabolic activity, and reproductive fitness. If not rigorously controlled or statistically incorporated into experimental designs, these variables may interfere with the interpretation of contaminant-induced responses. Although this sensitivity to environmental gradients enhances the ecological relevance of the endpoint, it also increases experimental complexity and requires carefully designed studies across multiple environmental conditions.

An additional limitation concerns the capacity of behavioural endpoints to represent the full spectrum of soil ecosystem functioning. Behavioural alterations observed in macroinvertebrates did not consistently predict variations in microbial community structure and composition, suggesting that microbial dynamics may respond to different drivers or operate at distinct temporal scales. This highlights the need to integrate behavioural indicators with microbiological, biochemical, and functional parameters in order to achieve a truly holistic assessment of soil quality.

Finally, although the results obtained are robust at laboratory and mesocosm scales, their generalizability across broader spatial scales remains to be confirmed. Extended empirical validation encompassing diverse soil types, climatic regions, and land-use systems is essential to consolidate the reliability, sensitivity, and specificity of the endpoint under real-world conditions. Only through such multi-scale verification will it be possible to fully support the implementation of this framework within regulatory contexts and official environmental monitoring programs.

8.4 Future Directions

Future research should focus on the harmonization and standardization of combined avoidance and disaggregation bioassay, including inter-laboratory validation and ring tests to ensure reproducibility and facilitate regulatory adoption. The integration of this dual endpoint into existing ecotoxicological guidelines would significantly enhance the ecological resolution of soil monitoring frameworks. Furthermore, the development of an experimental prototype equipped with exposure arenas integrated with cameras would allow real-time recording of effects on terrestrial isopods. This would provide a single, integrated, and replicable tool, minimizing errors associated with manual observation, such as disaggregation caused by impacts or sudden movements of the arena.

Mechanistic investigations represent a priority. Interdisciplinary collaboration among ecologists, chemists, and molecular biologists will be essential to identify aggregation pheromones, characterize stress-induced metabolic pathways, and clarify the physiological basis of behavioural disruption. Such knowledge will strengthen causal interpretation and predictive modelling.

In a regulatory context, the development of predictive models integrating behavioural responses with physio-chemical contaminant descriptors, exposure parameters, and environmental variables could support in silico screening tools and regulatory risk assessment applications. The threshold identified for Henry's law constant provides an initial foundation for such integrative modelling approaches.

Finally, future ecotoxicological research should systematically incorporate biotic interactions and community-level processes, recognizing that soil ecosystems possess intrinsic compensatory and facilitative mechanisms. Embedding behavioural ecotoxicology within broader biodiversity and ecosystem-function frameworks will consolidate its role as a scientifically robust and operational pillar of soil health assessment and sustainable ecosystem management.

I would like to express my sincere gratitude to Professor Sara Villa for all the time she has devoted to me, and for the passion, dedication, and faith she has shown throughout my doctoral studies. Thanks to her, I have learned to “*recognize a rose where no one else would have seen it*.”

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