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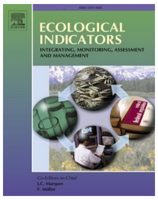

Original Articles

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*.

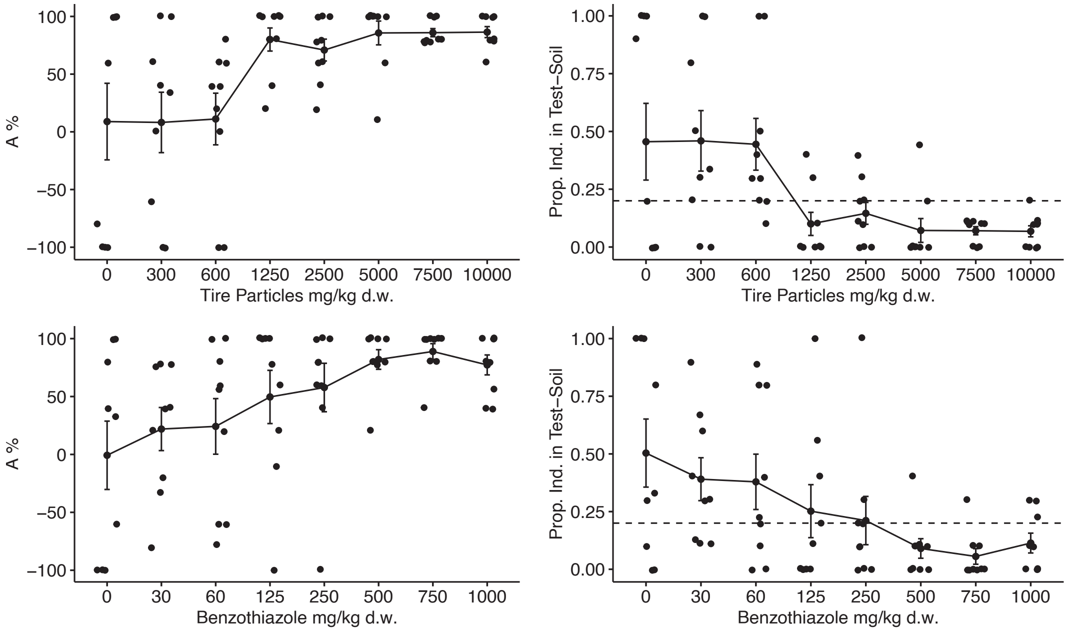

Fig. 1. Avoidance behavior (A%) of *Porcellionides pruinosus* (left) and proportion of individuals in test-soils (right) when exposed to tire particles and benzothiazole (mg/kg d.w.) in LUFA 2.2 soil for 48 h. Avoidance behavior responses are expressed as mean values ± standard error, while the distribution 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.

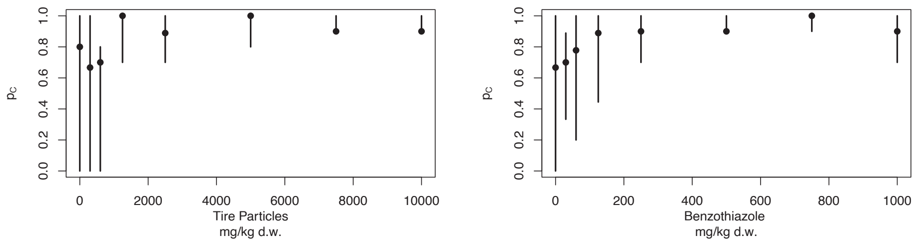

Fig. 2. Robust one-sample test results for the proportion of organisms on uncontaminated soil against a fixed value of 0.5 for each concentration, along with the approximated 95% confidence intervals based on 10,000 bootstrap replicates.

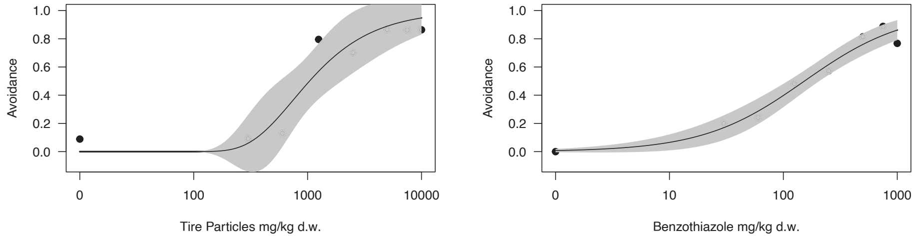

Fig. 3. Dose–response curves for the avoidance behavior of *Porcellionides pruinosus* to the exposure to tire particles (left, Weibull) and benzothiazole (right, log–logistic). Points represent mean observed net response data and line is the fitted regression model (shaded region = 95% confidence bounds).

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 ⩽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 μm) 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]

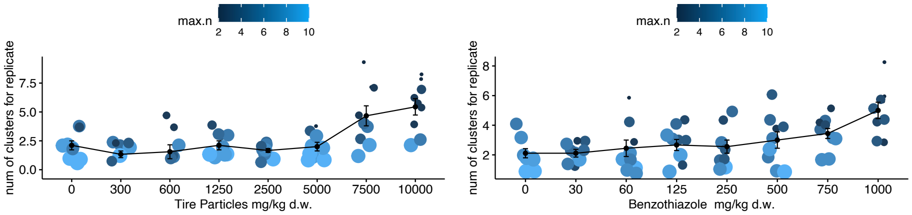

Fig. 4. Identified clusters for each concentration level of tire particles (left) and benzothiazole (right) in control soil. Each point represents the number of clusters identified for each replicate ($n = 9$), and the size-color point is proportional to the cluster size (lighter color identifies larger 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.

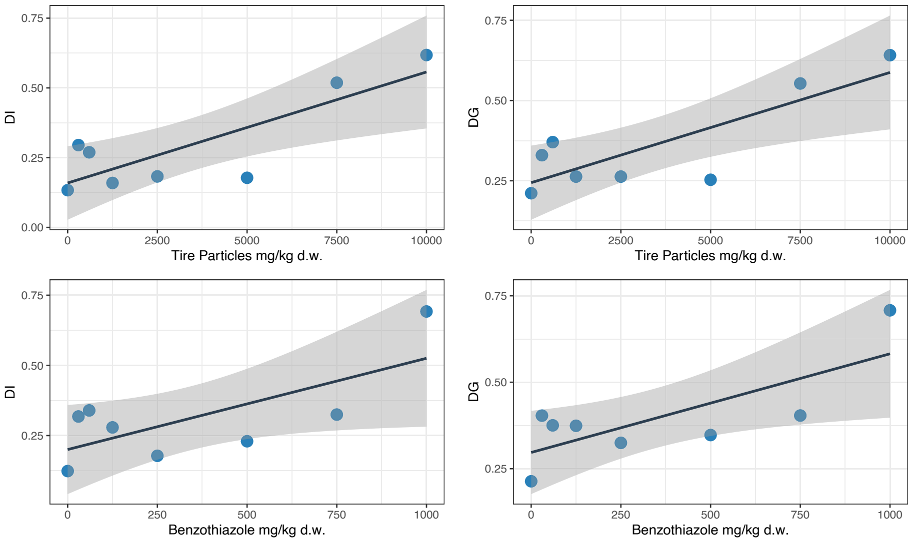

Fig. 5. Linear regression fit (solid line) with the 95% confidence region (shaded region) for DI response (left) and for DG response (right) of *Porcellionides pruinosus* as a function of tire particles and benzothiazole exposure. Points represent mean observed DI and DG data respectively. Both indexes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

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