

Terrestrial avoidance behaviour tests as screening tool to assess soil contamination

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Avoidance Behaviour Tests with earthworms and isopods can be used as screening tools in the evaluation of soil contamination.

Abstract

To assess soil quality and risk assessment, bioassays can be useful tools to gauge the potential toxicity of contaminants focusing on their bioavailable fraction. A rapid and sublethal avoidance behaviour test was used as a screening tool with the earthworm *Eisenia andrei* and the isopod *Porcellionides pruinosus*, where organisms were exposed during 48 h to several chemicals (lindane, dimethoate and copper sulphate, for isopods and carbendazim, benomyl, dimethoate and copper sulphate for earthworms). Both species were also exposed to soils from an abandoned mine. For all bioassays a statistical approach was used to derive EC₅₀ values. Isopods and earthworms were able to perceive the presence of toxic compounds and escaping from contaminated to clean soil. Furthermore the behaviour parameter was equally or more sensitive than other sublethal parameters (e.g. reproduction or growth), expressing the advantages of Avoidance Behaviour Tests as screening tools in ERA.

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1. Introduction

Soil is a dynamic and complex system functioning as habitat for microorganisms, flora, animals and humans (Hund-Rinke et al., 2002). Nowadays contaminated soils have become a primordial problem since they will probably lead to, for example, groundwater contamination and biomagnification of chemical compounds through food webs, and sometimes will affect human health. Contaminants in soil have some distinct fractions depending on the contamination and soil type. Some of these chemical fractions are bioavailable and

thus can be absorbed by organisms which are dependent of the soil physicochemical conditions (e.g. pH, clay content, cation exchange capacity, amount of organic matter) and on the chemical form of the element. Therefore the determination of the total chemical contents is not sufficient to evaluate the ecological risk that is inherent to a contaminated soil. To assess soil quality, bioassays can be useful tools to gauge the potential toxicity of contaminants focusing on their bioavailable fraction.

In soil ecotoxicology acute and chronic standardized tests have been developed using soil dwelling invertebrates, like earthworms (ISO, 1998a; ISO, 1998b), potworms (ISO, 2004) and collembolans (ISO, 1999). Edaphic invertebrates play a crucial role in maintaining the structure and fertility of soils, recycling nutrients, increasing aeration and drainage, and can constitute an

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important component of the diet of birds, reptiles or small mammals (Allen, 2002). Nevertheless, the use of edaphic invertebrates in acute and chronic ecotoxicological tests have shown some disadvantages: acute tests are not ecologically relevant when compared to chronic ones, because they do not provide insight into effects on the population dynamics, while chronic tests last too long and are very labour intensive, sometimes ranging from 4 to 7 weeks. To obtain quick answers with low costs in contamination problems a first screening tool is required and a rapid and sublethal avoidance behaviour test has been under development and standardization using earthworms (ISO/CD, 2003; Natal da Luz et al., 2004; Stephenson et al., 1997), where organisms have the ability to choose or avoid a soil. The results of avoidance behaviour response tests can increase sensitivity in this evaluation, quickly assessing an ecological endpoint that is not measured by any other test using the soil matrix (Yeardley et al., 1996). Nevertheless, Avoidance Behaviour Tests are not aimed to replace other ecotoxicological tests used nowadays, being just a complementary initial or screening test in soil contamination assessment. Earthworms have been chosen as test-organisms in ecotoxicological tests because they are common in a wide range of soils, representing 60–80% of the total soil animal/invertebrate biomass. Furthermore, by having chemoreceptors in the prostomium and sensory tubercles on their body surface, they can provide a high sensitivity to chemicals in soil (Reinecke et al., 2002).

Like earthworms, isopods are widely distributed and are a key species that play an important role in soil dynamics, mainly in leaf litter decomposition. They also have chemoreceptors located in an apical organ in the second antennae called aesthetascs, that can perceive chemicals and test stimuli. The antennae move continuously and a fluid excreted through channels mediates chemoreception. There is also some evidence of the existence of tricorn sensillae that are contact chemoreceptors in the tegument of isopods (Hoese, 1989; Warburg, 1993). Isopods have shown sensitivity to several chemicals present in soils because they can intake water from soil through uropods by capillary action, ingest soil or even absorb water through the cuticle (Sutton, 1980). The uptake of the chemical compound can influence physiological processes in isopods because these edaphic organisms are known to have low excretion rates. Zinc and Copper are two examples of elements that are not excreted by isopods and are deposited in granules in their internal organs (Donker, 1992). Another characteristic of isopods is their ability to avoid environment limiting factors like extreme humidity, light and others (Takeda, 1980). For all these reasons they have been used in soil bioassays.

The objectives of this study were to test if avoidance behaviour responses by edaphic organisms can be used

as a first screening tool for soil Environmental Risk Assessment and to compare the performance of two test organisms (earthworms and isopods) in choosing between two different substrates. Avoidance behaviour response tests were carried out using the earthworm *Eisenia andrei* and the isopod *Porcellionides pruinosus* exposed to organic and non-organic toxicants and also tested with natural soils from an abandoned mine to assess their performance in a real scenario.

To our knowledge, there is no reliable method to estimate EC_x values for avoidance behavioural responses (Heupel, 2002; Hund-Rinke and Wiechering, 2001; ISO/CD, 2003; Natal da Luz et al., 2004; Schaefer, 2004), as required in the standardized protocol, which is currently under development from the The International Organization for Standardization (Heupel, 2002; Hund-Rinke and Wiechering, 2001; ISO/CD, 2003; Natal da Luz et al., 2004; Schaefer, 2004). Hence the comparison between avoidance behaviour tests and the classical acute/chronic bioassays is difficult. In Avoidance Behaviour Response Tests, where 50% presence in one of the soils is considered as a non-preferential behaviour (endpoint), the term of EC_{50} requires to be adapted to the avoidance results. Here we present an adaptation of a standard methodology to the calculation of an EC_{50} for Avoidance Behaviour Test data.

2. Materials and methods

2.1. Test organisms and test chemicals

The isopods *Porcellionides pruinosus* were obtained from a two-year laboratory culture, maintained at 25 °C with a 16:8 (light: dark) photoperiod. Only adult animals (15–20 mg wet weight) with antenna were selected for the tests and sexes were not distinguished.

The earthworms *Eisenia andrei* were obtained from a culture for compost use in the Alentejo region, in the South of Portugal. Only adult animals with a developed clitellium were selected for the test (50–60 mm length).

In these experiments organic chemicals were chosen based on their use in agriculture and on the existence of data on acute and/or chronic tests with earthworms and isopods, as well as with other edaphic invertebrates. For the isopod experiments lindane (Merck, 95% pure) and dimethoate (Sigma-Aldrich, 99.9% pure) were used, while in the earthworm's experiments the test chemicals were carbendazim (100% pure) (AgrEvo), benomyl (100% pure) (DuPont) and dimethoate. The inorganic chemical used for both test organisms was copper (II) sulphate pentahydrated (Merck), a constituent of some fungicides used in vineyards. All chemical compounds were incorporated in Lufa 2.2 soil and several concentrations were tested (Table 1).

Table 1
Mean nominal and real concentrations of the organic chemical compounds used in the Avoidance Behaviour Tests (– data not determined)

Test organism	Control	Test chemical in Lufa 2.2 soil	Nominal concentration (mg/kg)	Real concentration (mg/kg)
<i>Eisenia andrei</i>	Lufa 2.2	Carbendazim	1	3.90
			10	9.23
			100	82.73
<i>Eisenia andrei</i>	Lufa 2.2	Benomyl	1	–
			10	–
			100	–
<i>Porcellionides pruinosus</i>	Lufa 2.2	Dimethoate	2.5	2.20
			5	5.70
			10	10.67
<i>Eisenia andrei</i>	Lufa 2.2	Lindane	2	2.70
			10	7.60
			20	22.00
<i>Porcellionides pruinosus</i>	Lufa 2.2	Lindane	53	59.10
			113	108.20
			200	190.40

Soil contamination was controlled by chemical analysis by Gas Chromatography-tandem Mass Spectrometry (GC-MS/MS) for lindane and dimethoate using a Varian 3800 gas chromatograph with Electronic Flow Control (EFC) and fitted with a Saturn 2000 ion-trap mass spectrometer (Varian Instruments, Sunnyvale, CA, USA). Liquid Chromatography-mass Spectrometry (LC-MS) was used for carbendazim analysis, using an Alliance 2695 equipped with autosampler, degasser, and heater column purchased by Water. Mass spectrometer system was a ZQ 2000 single quadrupole purchased by water-Micromass (Manchester, UK). Two soil samples were analysed per each contaminant.

Soil samples were extracted with selective organic solvents that were concentrated until dryness and later re-dissolved in a small aliquot of cyclohexane (for GC analysis) and in mobile phase (for LC analysis). In the case of LC analysis, the extract was previously passed through a Solid Phase Extraction cartridge Oasis HLB for cleaning up the extract before instrumental determination.

Benomyl was not determined because of its high degradation rate. The recovery of the extraction method was of 95% ($\pm 14\%$ std. dev.) for lindane, 89% ($\pm 14\%$ std. dev.) for dimethoate and 81% ($\pm 11\%$ std. dev.) for carbendazim. Chemical analyses were only carried out at the beginning of the experiment. No extra analysis was carried out at the end of the experiment due to the short test period of 48 hours.

To evaluate the performance of these tests in real scenarios, two soils from the abandoned mine Mina de Jales, located in the northeast of Portugal (N 41.47403; W 7.57876), were chosen, due to their different heavy metal contents. JC soil is a soil located 150 m from the mine spoil and is contaminated with a high amount of heavy metals; JNC soil is located 3 km from the spoil with a low amount of heavy metals (Table 2). Heavy metal analysis was performed by ICP-OES. A prior soil digestion was made, with an oxidation in HCl 0.1 N.

2.2. Experimental set up

In this study, two section chambers were used as test containers and two behaviour avoidance tests were carried out (Fig. 1). Earthworms and isopods were exposed to chemicals in group tests where 10 animals were placed in each test box. Additionally, an isopod individual test was performed with just one individual per box, due to the ability of isopods to produce the aggregation pheromone and consequently forming groups (aggregation behaviour).

Rectangular plastic containers were used (210×123 mm) in the group test. They were divided in two compartments by a removable plastic split. On the outside of the container a line representing the split place was drawn (Fig. 1). The standardized Lufa 2.2 soil (± 200 g dw), used as control, was placed in one of the compartments, and the test-soil (± 200 g dw) was placed in the opposite compartment. The test-soil was obtained by mixing the contaminants to Lufa 2.2 soil in a water solution form (copper sulphate and dimethoate) or by mixing the chemical powder directly to the soil (lindane, carbendazim and benomyl) (Table 1). An extra test was

Table 2
Physico-chemical characteristics and main contaminant levels of soils used: Lufa 2.2, JNC and JC from Mina de Jales (Portugal) (– data not determined)

Parameters	Units	Lufa 2.2 soil	JNC soil	JC soil
pH	–	5.03	4.14	4.47
Soil organic matter	%	1.28	5.07	2.88
Cation exchange Capacity	cmol/kg	11	4115	5492
Sand	%	77.10	20.96	23.08
Clay	%	8.0	16.8	13.6
Silt	%	14.90	27.51	25.02
As	mg/kg	–	71	251
Al	mg/kg	–	14,000	10,000
Cd	mg/kg	<0.2	1.9	8.2
Cr	mg/kg	9.6	6.0	15.0
Cu	mg/kg	1.5	8.0	24.0
Fe	mg/kg	–	7370	17,800
Hg	mg/kg	0.07	<0.05	<0.05
Mn	mg/kg	–	99	255
Ni	mg/kg	2.7	5.0	9.0
Pb	mg/kg	16.8	33.0	209.0
Zn	mg/kg	19	33	97

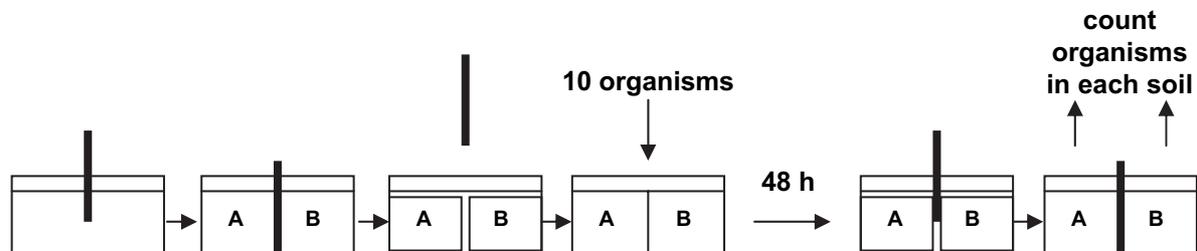


Fig. 1. Scheme of the Avoidance Behaviour Response test set up.

also performed where isopods were confronted with clean Lufa 2.2 soil in both sides of the containers. The soil humidity was adjusted to 60% of the Water Holding Capacity (WHC) in the earthworm test and to 40% WHC in the isopod tests.

The same methodology was adapted and applied to screen the quality of the field soils from Mina de Jales, by opposing Lufa 2.2 soil to these two soils. Four additional treatments for each soil were also used. The treatments were obtained by diluting the mine soils with the control soil Lufa 2.2, obtaining treatments of 12.5% (12.5% of test-soil + 87.5% Lufa 2.2), 25% (25% of test-soil + 75% Lufa 2.2), 50% (50% of test-soil + 50% Lufa 2.2), 75% (75% of test-soil + 25% Lufa 2.2) and 100% (only the test-soil). The pH values were measured in a KCl (1M) solution (ISO, 1994) in all dilutions, ranging from 4.61 to 4.14 in JNC soil (12.5% to 100%) and from 5.02 to 4.47 in JC soil (12.5% to 100%).

In all group tests, for each treatment or concentration, 5 replicates were used with 10 animals per replicate. In the isopod individual tests 10 replicates were used and only one animal was placed in a cylindrical box (8 cm diameter and 4.5 cm high). All other procedures were similar to the group tests. Earthworms were kept at $20\text{ }^{\circ}\text{C} \pm 2\text{ }^{\circ}\text{C}$, with a photoperiod of 16:8 (light: dark); isopods were exposed to chemicals at $25\text{ }^{\circ}\text{C} \pm 2\text{ }^{\circ}\text{C}$, with the same photoperiod.

After the 48 h test period, the split was reintroduced in the marked position and the individuals were counted in each compartment containing the control and the test soil. If any animal was not found it was assumed that its death was caused by the test soil. Animals that were cut by the split were considered as being in the soil to which the animal's head was directed.

2.3. Statistical analysis

Statistical approaches in Avoidance Behaviour Tests are usually restricted to Mann–Whitney *U*-test, when data are not normally distributed (Schaefer, 2004), or comparing the number of test-organisms in the control soil or in the test-soil in different concentrations, using Analysis of Variance or a one tailed Student's *t*-test (ISO/CD, 2003). Another approach also used is based only on the mean values and standard deviations of the

number of organisms in the two sections of the test-box (Heupel, 2002; Hund, 1998; Hund-Rinke et al., 2003; Hund-Rinke and Wiechering, 2001). More recently, the Fischer Exact Test has been also used to analyse results from avoidance tests with collembolans and earthworms, comparing the obtained animal distribution with an expected distribution of animals which showed no avoidance (Natal da Luz et al., 2004).

In this kind of test, the calculation of an EC_x value is required, but till now no methodology has been presented to obtain these values. So, in Avoidance Behaviour Tests and for the evaluation of soil toxicity, the use of the “habitat function” of soil “limited” definition (ISO/CD, 2003) has been proposed by several authors. In this case, the habitat function of soils is considered to be limited if less than 20% of the test organisms (on average) are found in the test soil, which indicates an impact on behaviour. This is also related to the advisable measurement of the EC_{50} value presented in the currently under development standardized protocol (ISO/CD, 2003), because when the mean avoidance is 50%, it means that 75% of the animals are found (on average) in the control soil. This statement assumes that when 50% of the animals are in the test-soil it is considered that the animals show no preference for both soils. Considering a total of 12 animals, it would mean that 6 animals are in the contaminated and 6 in the control soil. If at the end of the bioassay there are 3 animals in the contaminated soil and 9 in the control soil, that will mean that a total of 50% (3/6) avoided the contaminated soil, hence a 50% of avoidance. Also, if at the end of the experiment only 2 animals are found in the contaminated soil and 10 in the control soil, it will mean that 66% (2/6) avoided the contaminated soil. This last example represents the “habitat function” of soil “limited” definition, where 80% of the total animals are found in the control soil and 20% in the contaminated soil.

Two statistical approaches were adopted to treat the results obtained in this study.

First, and using the “habitat function” definition, if the percentage of live animals positioned in the contaminated/test soil was lower than 20%, the contaminated/test soil was considered to have an impact on the behaviour of the test organisms and therefore

the soil was considered to be toxic or with less quality (Hund-Rinke et al., 2003). A One-Way ANOVA was also performed to assess the differences between the percentages of live animals in different treatments. Data for this analysis were transformed using Eq. (1), because avoidance tests are based on the ability of an organism to move away from contaminated sites, and the proportion of individuals responding (avoiding the test-soil) can be calculated as A ,

$$A = \frac{N - 2 * T}{N} \quad (1)$$

where N is the number of individuals per trial and T is the number of individuals observed in the test soil. Negative responses were treated as no avoidance.

Second, the EC_{50} value was calculated with the probit method, using the statistical package Minitab® (Minitab, 2000). Data was also previously transformed using Eq. (1).

3. Results

For all group Avoidance Behaviour Response Tests EC_{50} values and 95% confidence limits were calculated (Table 3). The EC_{50} values for the JC soil exposure for *P. pruinus* and for JNC and JC soil for *E. andrei* were higher than 100 (i.e. the soil without dilution) due to the non avoidance behaviour response towards these exposures. Additionally the EC_{50} value for earthworms' exposure to carbendazim and benomyl was not calculated because it presented values lower than the lowest concentration used.

3.1. Isopod avoidance behaviour response tests

All tests performed with the isopod *Porcellionides pruinosus* presented less than 20% of mortality, except for the 113 and 200 mg of lindane per Kg of soil exposure, where the former reached 37.5% of mortality and the latter 28%.

When confronting the two portions of Lufa 2.2 soil, isopods showed a random distribution between the two sides of the test-box ($\chi^2 = 5.518$, $df = 4$, $P > 0.05$).

Isopods exposed to copper sulphate showed avoidance behaviour by choosing the control soil Lufa 2.2, at the concentration 1500 mg/kg of soil, in both the individual and group tests (Fig. 2). When exposed to the organophosphorus insecticide dimethoate, isopods in the group test avoided the test soil at 20 and 40 mg/kg of soil, showing a significant difference in the number of isopods present in 2.5 mg/kg and 20 mg/kg (One-Way ANOVA $F_{4, 19} = 12.096$, $P < 0.05$; Tukey test, $P < 0.05$); in the individual tests, avoidance behaviour patterns were not statistically perceived (Fig. 2), although an EC_{50} of 39.43 mg/kg could be calculated.

For lindane exposure, isopods avoided the contaminated soil in the group tests at 113 and 200 mg lindane/kg of soil, where less than 20% of live animals were in the contaminated portion, while in individual tests the avoidance was observed at 10 mg/kg and above 53 mg/kg of soil (Fig. 2). A high variability in the data was observed, mainly in the two lowest concentrations. Even though, there was a significant difference between 53 and 200 mg/kg of lindane exposures (One-Way ANOVA $F_{5, 22} = 4.127$, $P < 0.05$; Tukey test, $P < 0.05$).

To fulfil one of the objectives of this study, avoidance behaviour tests were used in natural contaminated soils. The evaluation of the two soils was made separately. After dilutions with Lufa 2.2, isopods avoided JNC soil

Table 3

EC_{50} values (and 95% CL) obtained in all Avoidance Behaviour Tests and Bioassays with *Eisenia andrei* and *Porcellionides pruinosus* (– data not determined)

Test organism	Test soil	Test type	EC_{50} (95% CL)	Concentrations with <20% ind.
<i>Eisenia andrei</i>	Carbendazim	Collective	<1	10 and 100
	Benomyl	Collective	<1	10 and 100
	Dimethoate	Collective	50.07 (37.98–66.53)	40
	Copper	Collective	181.10 (168.54–195.39)	320
	JNC	Collective	> 100	–
	JC	Collective	> 100	75
<i>Porcellionides pruinosus</i>	Lindane	Individual	48.32 (41.03–56.06)	10, 53, 113 and 200
		Collective	48.62 (26.66–68.40)	113 and 200
	Dimethoate	Individual	39.43 (35.63–44.63)	–
		Collective	28.67 (25.57–32.60)	20 and 40
	Copper	Individual	1059.85 (983.51–1147.29)	1500
		Collective	802.26 (640.50–966.81)	1500
	JNC	Individual	64.30 (56.06–74.06)	75 and 100
		Collective	57.08 (50.82–63.66)	75 and 100
	JC	Individual	> 100	25, 50 and 75
		Collective	> 100	–

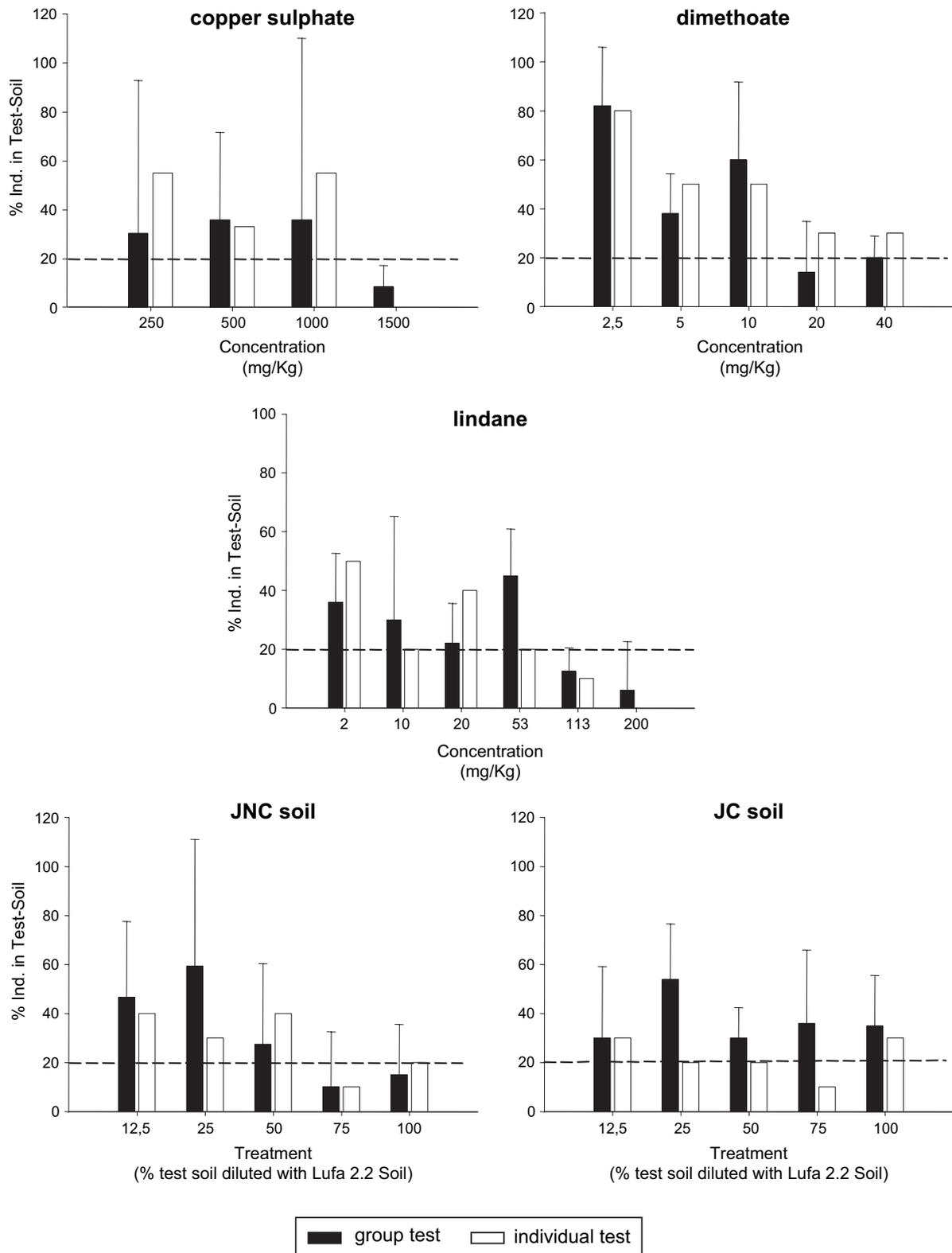


Fig. 2. Percentage of the test-organism *Porcellionides pruinosus* in the test-soil (mean \pm 95% Confidence Limits) exposed to copper sulphate, lindane, dimethoate and the two soil from Jales mine (JNC and JC soil). The dash line states the 20% "habitat function limit". The absence of the open column in the highest copper concentration in individual exposure of copper sulphate means 100% of avoidance in this concentration (i.e., zero organisms in the contaminated soil).

at 75% of the field soil and 100% (soil without dilution), when exposed individually and in group (Fig. 2). Even though, there was no significant difference in the avoidance of isopods between all treatments (One-Way ANOVA, $F_{4,15}=2.188$, $P>0.05$).

Isopods exposed to JC soil treatments in group bioassays did not show any preference by one of the soils. However, when exposed in individual bioassays isopods showed an avoidance performance in the 25%, 50% and 75% treatments (Fig. 2). The average number of individuals in the test soil was similar in all treatments (One-Way ANOVA, $F_{4,19}=1.401$, $P>0.05$), and no EC_{50} could be calculated.

3.2. Earthworm avoidance behaviour response tests

Although in the Avoidance Behaviour Tests with *Eisenia andrei* some organisms were not found after the test period, and were considered dead, mortality never reached 20% in any of the concentrations used.

When exposed to copper sulphate the earthworm *Eisenia andrei* showed an avoidance behaviour at 320 mg/kg of soil (Fig. 3). The avoidance in copper exposure showed significant differences between 320 mg/kg and all the other copper sulphate concentrations (One-Way ANOVA, $F_{3,16}=15.412$, $P<0.05$; Tukey test, $P<0.05$), and also between 40 and 160 mg/kg (Tukey test, $P<0.05$).

When exposed to the pesticide dimethoate, earthworms showed avoidance behaviour at 40 mg/kg of soil, where 20% of the animals (average) were found in the contaminated test soil (Fig. 3). Nevertheless, there were no significant differences between avoidance values in all concentrations (One-Way ANOVA, $F_{4,18}=1.127$, $P>0.05$).

Earthworms exposed to carbendazim and benomyl showed the same pattern, avoiding the soil at concentrations equal or higher than 10 mg/kg of soil (Fig. 3), which is in agreement with Hund-Rink and Wiechering (Hund-Rinke and Wiechering, 2001). Although exposure to benomyl showed a significant difference between the lowest concentration and 10 and 100 mg/kg (Kruskal–Wallis One-Way ANOVA, $H=10.274$, $df=2$, $P<0.05$), carbendazim exposure did not present a significant difference between the concentrations used (One-Way ANOVA, $F_{2,12}=1.867$, $P>0.05$).

When soils from the abandoned mine were tested, organisms showed no avoidance in all JNC soil treatments, including the 100% treatment (soil with no dilution). For JC soil (considered a contaminated soil), earthworms only showed some preference for Lufa 2.2 soil when they were also exposed to the treatment “75% JC soil+25% Lufa 2.2 soil”: more than 80% were present in the Lufa 2.2 soil, hence less than 20% of the earthworms were found in the test soil “75% JC soil+25% Lufa 2.2 soil” (Fig. 3).

4. Discussion

Isopod mortality reached values higher than 20% in some chemical concentrations (113 and 200 mg/kg lindane) which may indicate that these can be considered lethal to isopods, even if they have tried to avoid them. Despite Avoidance Behaviour Tests being considered chronic tests (evaluating a sublethal parameter), mortality can also be considered an important evaluation endpoint in these tests. This is supported by the fact that for some chemical doses isopods were found dead in the control portion. The fact that some of these chemical compounds affect the nervous system and therefore will disorientate the isopods can explain their inability to escape. Lindane is considered a persistent chemical with bioaccumulation potential and dimethoate is a dithiophosphorous insecticide that has a broader spectrum effect on arthropods by inhibiting their cholinesterase activity. Dimethoate is considered a volatile chemical compound and it might enter into organisms' body via air (e.g. respiration), in addition to pore water and soil particles ingestion. By acting as an inhibitor of acetylcholinesterase, dimethoate can also influence isopods and earthworms behaviour (Martikainen, 1996; Ribeiro et al., 1999). Fábrián and Petersen (1994) observed that *Folsomia fimetaria* exposed to dimethoate exhibited motionless and/or uncoordinated motion. The isopod *Oniscus asellus* when exposed to lindane showed some changes in a stress protein level, which would comprise the adjustment of the nervous system (Köhler et al., 1999). Hence, when present in very high concentrations chemical compounds with these characteristics can affect orientation and consequently animals survivorship.

4.1. Isopod avoidance behaviour tests

From the results from the group and the individual isopods' tests, it is possible to hypothesize that the production of an aggregation pheromone can be also important for the recognition and evasion from chemical compounds. However, this remains to be tested. In some situations isopods can escape from contaminated places only by accompanying others that have perceived the presence of chemicals. The active principle responsible for this aggregation phenomenon in isopods was observed on their faeces and it is thought that they communicate by a response to chemical stimuli through their antennae (Takeda, 1980). The aggregation pheromone is secreted from the epithelial tissues on the mid or hind gut into the lumen where it will impregnate the faeces. For this reason animals with anosmia (animals with no antenna) were not used. Individual tests were run to give a more conclusive answer because aggregation was observed in some trials, producing high variability in the results. A possible example of this

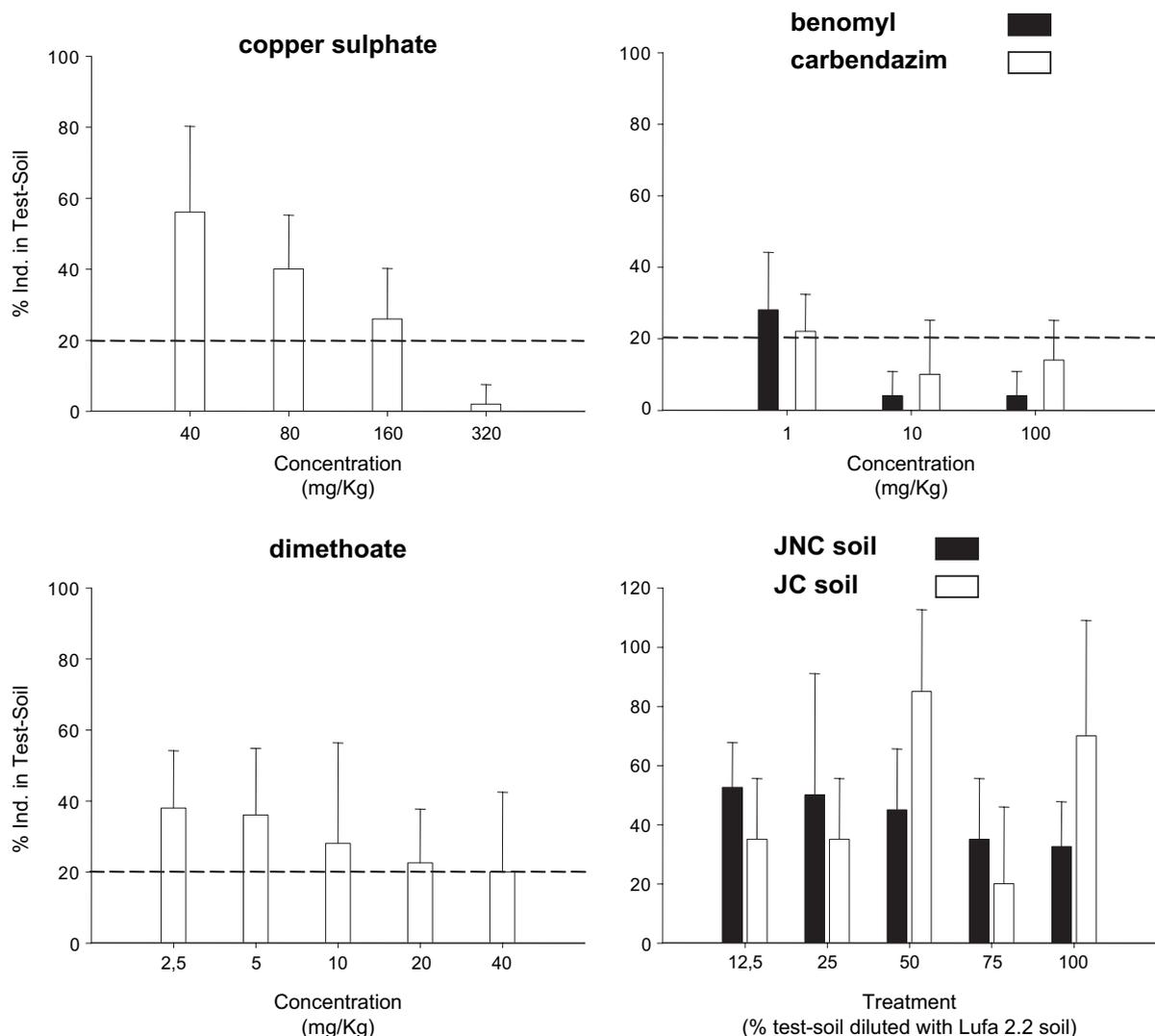


Fig. 3. Percentage of the test-organism *Eisenia fetida* in the test-soil (mean \pm 95% Confidence Limits) exposed to copper sulphate, dimethoate, carbendazim, benomyl and the two soil from Jales mine (JNC and JC soil). The dash line states the 20% "habitat function limit".

phenomenon was observed by the high confidence limits obtained in the exposure to copper sulphate, demonstrating the variability between replicates. Furthermore when exposed to dimethoate isopods avoided the soil with the highest contaminant levels only in group exposure, while individually they did not show any clear preference.

Some studies with edaphic species exposed to lindane have been performed, mainly considering bioaccumulation of this persistent compound (Amorim et al., 2002; Loureiro et al., 2002; Santos et al., 2003; Sousa et al., 2000) and its effects on reproduction (Lock et al., 2002; Römbke, 2003). In this study the exposure of isopods to lindane showed the same trend as former studies observing avoidance at 20 mg/kg and with EC_{50} values of 48.32 and 48.62 mg/kg in the individual and group tests, respectively. This was also supported by the bioaccumulation study with *P. pruinus* (Santos et al.,

2003) where the authors found an LC_{50} value of 76.3 mg/kg after 2 days and 23.5 mg/kg after one week. The sensitivity of terrestrial isopods to lindane was also confirmed by the influence of this chemical compound on the activity of the stressor protein *hsp 70*, showing an initial peak of the *hsp 70* activity during the first three days of exposure to lindane (Köhler et al., 1999).

When exposed to dimethoate in this study, *P. pruinus* showed the same trend as the isopod *Porcellio scaber* exposed to dimethoate in Lufa 2.2, where an EC_{50} value on growth of 17.5 mg/kg was calculated and an LC_{50} higher than 75 mg/kg was also found (Fischer et al., 1997; Løkke and Van Gestel, 1998).

Copper is known to be essential for the production of hemocyanine in isopods. Its high accumulation and storage in the mid gut lobes can also be explained as a detoxification process (Zimmer and Topp, 1998). Due to this, ecotoxicological studies with isopods exposed to

copper have reported very high EC_x values (Løkke and Van Gestel, 1998; Zidar et al., 2003). The exposure of *P. scaber* to Lufa 2.2 soil contaminated with copper chloride resulted in an EC_{50} value of 1858 mg/kg for juvenile growth and a LC_{50} value of 3755 mg/kg (Løkke and Van Gestel, 1998). This study showed that *P. pruinosus* was more sensitive than other isopod species. Moreover, avoidance behaviour of this species was shown to be more sensitive than other ecotoxicological parameters. The EC_{50} value of 802 mg/kg (for group tests) and the avoidance of soil with a concentration of copper higher than 1500 mg/kg were lower than the results that have been reported for *P. scaber* (Løkke and Van Gestel, 1998).

4.2. Earthworm avoidance behaviour tests

Earthworms exposed to benomyl exhibited the same behaviour pattern as in a study by Hund-Rink and Wiechring (Hund-Rinke and Wiechering, 2001) that also used an Avoidance Behaviour test. Earthworms avoided the soil test when contaminant levels were 10 mg/kg or higher. For carbendazim, earthworms also showed the same pattern. A range of concentrations of benomyl (8.3, 56, 112 mg/kg) used in a spermatogenesis study caused abnormalities in the ultrastructure of sexual organs of *E. fetida* (Sorour and Larink, 2001). In other studies, using a soil microcosm, the exposure of earthworms to carbendazim showed an EC_{50} value for the biomass loss of 1.9 mg/kg and a LC_{50} of 6.2 mg/kg (Burrows and Edwards, 2002). The latter values were in agreement with the results obtained for this study with carbendazim exposure, where less than 20% of the animals were found in the 10 and 100 mg/kg concentrations, although it was not possible to calculate the EC_{50} value.

The Avoidance Behaviour Tests with dimethoate showed similar results when compared with other endpoints. Martikainen (1996) observed IC_{50} values for biomass change in the range of 14.4 to 42.9 mg/kg of soil for the earthworm *Aporrectodea caliginosa tuberculata* which were in the same range of the EC_{50} value observed in this study. In another study the number of cocoons and juveniles produced by the earthworm *E. fetida* exposed to dimethoate decreased, showing NOEC values of 10 mg/kg and 32 mg/kg, respectively, what is in concordance with what was found in our study (Løkke and Van Gestel, 1998).

The EC_{50} value found for the earthworm avoidance tests with copper is in agreement with the study of earthworm population (*Lumbricus rubellus* and *Dendrodilus rubidus*) where concentrations above 100 mg/kg of soil were found to be toxic. In that study it was also stated that earthworms can still be found in places where the copper concentration is above this value (Langdon et al., 2001). Burrows and Edwards also showed that

earthworm biomass was reduced at 200 mg Cu/kg of soil and that an exposure of 100 mg/kg revealed no effects in this parameter (Burrows and Edwards, 2002). As expected, in this experiment with copper sulfate earthworms were more sensitive than isopods.

4.3. Avoidance behaviour bioassays

The performance of Avoidance Behaviour Bioassays in the evaluation of the quality/toxicity of soils from one real scenario, an abandoned mine, was also tested. When isopods and earthworms were exposed to the JNC soil their behaviour was quite different. Although individuals of *E. andrei* did not show any avoidance behaviour, the isopods *P. pruinosus* presented a clear avoidance in the 75% and 100% treatments, in individual and group bioassays, being therefore considered more sensitive. This fact was not expected because JNC soil is a silt loam soil with low amount of heavy metals, almost all below the Ecological Screening Values (Savannah River Site, 1999) or the Canadian Environmental Quality Guidelines (in http://www.ccme.ca/assets/pdf/e1_06.pdf) with high organic matter content, and could be considered clean by the Portuguese legislation (Portaria n° 176/96 (II Série), 3/10/1996).

On the other hand, JC soil was expected to be toxic because of its chemical characteristics. Nevertheless it was found that when test-organisms were in contact with this soil they did not avoid it and the majority seemed in fact to prefer JC soil when confronted with Lufa 2.2 soil. This might be explained by a low bioavailable fraction of heavy metals present in this soil or by an interaction between chemical compounds that would be traduced in a lower toxicity. Additional chemical information not provided by the chemical analysis (i.e. the existence of a non-expected compound in soil) may also explain the results. Anyway, this reinforces the fact that chemical analyses, per se, are not sufficient to evaluate the potential toxicity of soils.

Using the terminology from BioQuest International (Carter et al., 1998), the avoidance bioassay classified JNC soil as a soil with slight toxicity.

5. Conclusions

The results observed in this study show that Avoidance Behaviour Tests can be regarded as a valuable tool in the screening evaluation of soil contamination. The use of these tests as first approaches for contaminated sites evaluation will bring rapid information for future decisions on the evaluation procedure.

Additionally, different species should be used in this kind of tests because species react and respond differently to chemical stimulus, as shown in the mine soil bioassays.

For these reasons statistics for this kind of test have to be improved and established, so that more sound information can be derived from the ISO standardized protocol that is currently being developed (ISO/CD, 2003) using the statistical methodology for calculating EC₅₀s (derived for standard methodologies) presented here.

This will mean that Avoidance Behaviour Tests can be used, with clear advantages, both as first screening tools in Terrestrial Risk Assessment and also in Soil Quality Criteria studies, warranting quantitative assessment of the contaminant(s) bioavailability and toxicity.

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