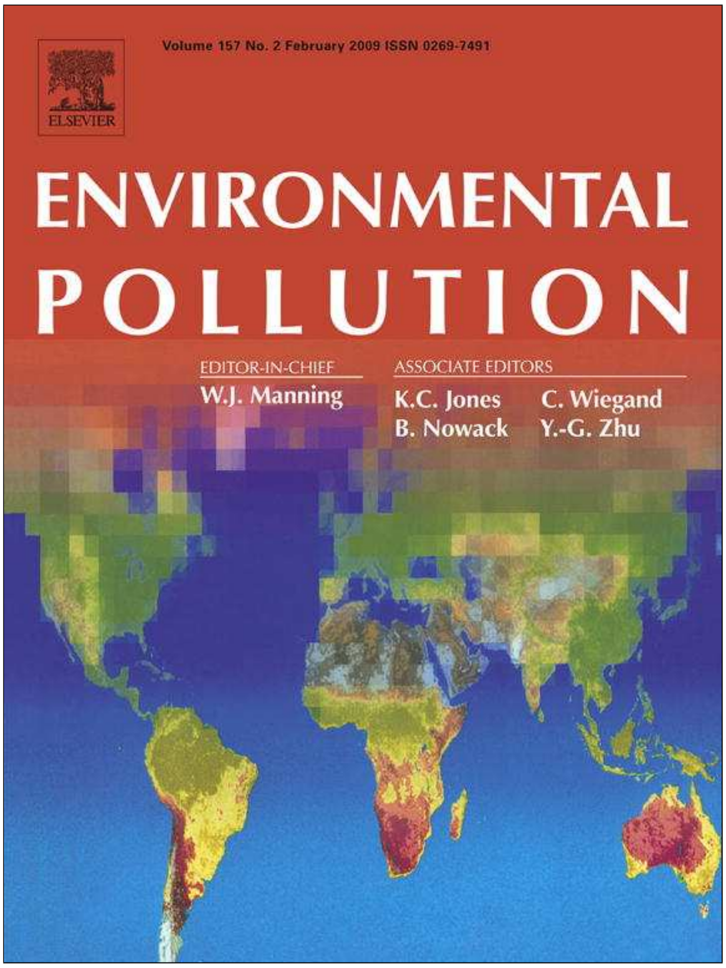
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Assessing joint toxicity of chemicals in *Enchytraeus albidus* (Enchytraeidae) and *Porcellionides pruinosus* (Isopoda) using avoidance behaviour as an endpoint

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Avoidance behaviour to binary mixtures of chemicals in two edaphic species.

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ABSTRACT

Contamination problems are often characterized by complex mixtures of chemicals. There are two conceptual models usually used to evaluate patterns of mixture toxicity: Concentration Addition (CA) and Independent Action (IA). Deviations from these models as synergism, antagonism and dose dependency also occur. In the present study, single and mixture toxicity of atrazine, dimethoate, lindane, zinc and cadmium were tested in *Porcellionides pruinosus* and *Enchytraeus albidus*, using avoidance as test parameter. For both species patterns of antagonism were found when exposed to dimethoate and atrazine, synergism for lindane and dimethoate exposures (with the exception of lower doses in the isopod case study) and concentration addition for cadmium and zinc occurred, while the exposure to cadmium and dimethoate showed dissimilar patterns.

This study highlights the importance of dose dependencies when testing chemical mixtures and that avoidance tests can also be used to asses the effects of mixture toxicity.

1. Introduction

Nowadays, conflicts between agricultural production and environmental quality are growing, focusing on agrochemicals' use, biodiversity, genetic resources, climate changes and water quality. Contamination is a major problem for agriculture since it impairs human health and environmental quality. Agrochemicals and manure are integral components of modern agriculture systems, leading to high levels of contamination by organic compounds and also metallic elements (Dach and Starmans, 2005). Contamination problems are often characterized by complex mixtures of chemicals belonging to the same or to different compound classes (e.g. organochlorides, organophosphates, carbamates, heavy metals). As recently pointed out by some European Commission reports on harmonisation procedures for Environmental Risk Assessment (ERA) there is a need for shorter and cost-effective tests that will enable us to understand how chemicals interact with living beings.

Avoidance tests are based on the fact that organisms have chemoreceptors highly sensitive to chemicals in their environment (Edwards and Bohlen, 1996; Römbke and Schmidt, 1998). Avoidance behaviour is an ecologically relevant measurement endpoint, because it influences the energy budget of animals by dispending energy on detecting and escaping from hazard and also decreasing their feeding activity in contaminated areas. Several studies using this behavioural response endpoint have been performed, also using different organisms that show a behavioural response, such as earthworms, collembolans, enchytraeids and isopods (e.g. Natal da Luz et al., 2004; Amorim et al., 2005a; Loureiro et al., 2005; Lukkari and Haimi, 2005; Aldaya et al., 2006). Data from these tests refer to mono-contaminated soils, i.e. soil with single chemical contamination. So far, there is no information available regarding the effects of contamination by more than one chemical simultaneously.

Because of the potential importance of chemical mixture effects in biological systems, (eco)toxicologists have developed a number of approaches to mixture toxicity assessment. Among these approaches, mathematical models previously developed and used in pharmaceutical research have been adapted for the toxic evaluation of chemical mixtures in the environment: the Concentration Addition (CA) and Independent Action (IA) models. The CA model assumes that chemicals with the same mode of action (MoA) will act additively. This conceptual model is defined as a summation of the relative toxicities of the individual components in mixture (Groten, 2000; Ferreira et al., 2008). On the other hand, the IA model is usually used if the question asked is whether the probability of toxicity to one chemical is independent from the probability of toxicity exposure to another chemical (Backhaus et al., 2003; Altenburger et al., 2004; Jonker et al., 2004, 2005). Deviations from the models can also be tested. Some compounds can interact synergistically becoming more toxic than expected from the toxicity of single compounds or enhancing the probability of effect of one another. Different

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compounds may also interact antagonistically, defined as situations where the mixture toxicity is lower than expected from the toxicities of single compounds or where the compounds decrease the probability of effect to one another. The interaction between different compounds might also depend on the mixture dose level or on the dose ratio (Jonker et al., 2005). The dose level-dependency means that observed deviations from the reference models are different at low dose levels than at high dose levels. The dose ratio-dependency means that the proportion of the compounds in the mixture affects the deviation from the reference model in an asymmetric fashion, with one of the chemicals being the major responsible for the observed deviation (Jonker et al., 2005).

In this paper we investigated if enchytraeids and isopods could avoid several chemical substances, singly and in binary mixtures. The following four chemicals with similar and/or dissimilar MoA were chosen: 1) atrazine is a triazine-ring herbicide and its MoA in animals is not yet well described but it is known to act as an endocrine disruptor in frogs and fish (Hecker et al., 2005); 2) the organophosphate dimethoate is a cholinesterase inhibitor after metabolism, and it acts at the cholinergic synapses (IPCS, 1989); 3) lindane (organochlorine insecticide) is a GABA-gated chloride channel antagonists (Casida, 2005), acting as a central nervous system stimulant and also an inducer of DNA fragmentation (Olgun et al., 2004); 4) zinc and cadmium are known as oxidative stress inductors, causing lipid peroxidation (Pinto et al., 2003; Badisa et al., 2007).

The following two hypotheses were tested: 1) Do chemicals with the same MoA in avoidance behaviour tests cause the response pattern stated by the conceptual model CA? 2) Do chemicals with dissimilar MoA act according to the IA model in avoidance behaviour tests?

2. Materials and methods

2.1. Test organisms

The test organisms used for this study belong to the species *Enchytraeus albidus* Henle, 1837 and *Porcellionides pruinosus*, Brandt 1833. Enchytraeids were cultured in the laboratory, kept at 18 °C with a photoperiod of 16:8 h light:dark and were fed once a week with finely ground and autoclaved rolled oats (Cimarrom, Portugal). More details for culturing are given in Römbke and Moser (2002). Isopods were obtained from horse and cow compost pills and brought to the laboratory where they were fed *ad libidum* with alder leaves (*Alnus glutinosa*) and maintained at 25 °C, with a 16:8 h (light:dark) photoperiod for 3 months before use in experiments. Only adult animals (15–20 mg wet weight) with antenna were selected for the tests; sexes were not distinguished but pregnant females were not used.

2.2. Chemical compounds and soil

Five chemicals were used in these experiments: the herbicide atrazine (Sigma-Aldrich, 97.4%), the insecticides dimethoate [Sigma-Aldrich (Riedel-de Haën), 99.8%] and lindane (γ-HCH, Sigma-Aldrich, 97%) and the metals zinc chloride [$ZnCl_2$, Sigma-Aldrich (Riedel-de Haën), 98%] and cadmium chloride anhydrous ($CdCl_2$, Sigma-Aldrich, 99%).

Experiments were done in the certified loamy sand soil LUFA 2.2 (Løkke and Van Gestel, 1998). This soil type is commercially available at the German institution LUFA Speyer. The properties of this soil can be summarised as follows: pH = 5.5, organic matter = 3.9%, texture = 6% clay; 17% silt; 77% sand.

2.3. Experimental procedure

In the single chemical exposure tests, chemicals were spiked into the pre-moistened soil as aqueous solutions, each test concentration into the whole batch of soil (Table 1). In the binary mixture toxicity tests, chemicals were added to the soil as aqueous solutions, one after the other, and then homogeneously mixed. In the case of atrazine and lindane, these were solved in acetone (in the case of enchytraeids) or in ethanol (in the case of isopods) and homogeneously mixed with the soil (Table 1). Solvent was left to evaporate overnight and then deionised water was added to moisten the soil to 40–60% of the water holding capacity (WHC). In this case, the control soil was also spiked with the same amount of acetone or ethanol; therefore, tests were run with a solvent control. In the case of metals, the spiked soil was allowed to equilibrate for three days previous to the start of the test, as recommended by McLaughlin et al. (2002). All concentrations are given as active ingredient (a.i.) per kg soil (dry weight) (Table 1).

Table 1
Summary of avoidance behaviour experiments performed to determine the effects of selected chemicals, single and in binary mixtures, to *Porcellionides pruinosus* and *Enchytaeus albidus* in LUFA 2.2 soil

Control soil	Individual/ mixture tests	Species and concentration ranges
LUFA 2.2	Dimethoate	*E. albidus* (3–235 mg/kg)[a] *P. pruinosus* (10–60 mg/kg)
LUFA 2.2 and solvent	Atrazine	*E. albidus* (0.32–100 mg/kg)[a] *P. pruinosus* (100–500 mg/kg)
LUFA 2.2 and solvent	Lindane	*E. albidus* (32–180 mg/kg)[a] *P. pruinosus* (0.5–40 mg/kg)
LUFA 2.2	Zinc	*E. albidus* (5.6–180 mg/kg)[a] *P. pruinosus* (100–1000 mg/kg)
LUFA 2.2	Cadmium	*E. albidus* (5.6–560 mg/kg)[a] *P. pruinosus* (100–500 mg/kg)
LUFA 2.2 and solvent	Dimethoate and atrazine	*E. albidus*: dimethoate (7.30–58.3 mg/kg) atrazine (4.71–38 mg/kg) *P. pruinosus*: dimethoate (4.13–33 mg/kg) atrazine (25–200 mg/kg)
LUFA 2.2 and solvent	Dimethoate and lindane	*E. albidus*: dimethoate (7.30–58.3 mg/kg) lindane (21.25–170 mg/kg) *P. pruinosus*: dimethoate (4.13–33 mg/kg) lindane (4.42–35.33 mg/kg)
LUFA 2.2	Zinc and cadmium	*E. albidus*: zinc (14.25–114 mg/kg) cadmium (49–392 mg/kg) *P. pruinosus*: zinc (40.84–326.73 mg/kg) cadmium (65.42–523 mg/kg)
LUFA 2.2	Cadmium and dimethoate	*E. albidus*: cadmium (49–392 mg/kg) dimethoate (7.30–58.3 mg/kg) *P. pruinosus*: cadmium (62.5–500 mg/kg) dimethoate (4.19–33.54 mg/kg)

[a] From Amorim et al. (2008).

Avoidance behaviour tests were performed using two-section vessels (ISO, 2007 (draft)). The material used in these experiments included circular plastic boxes (7.8 cm diameter×4.3 cm height) for *E. albidus* experiments or rectangular plastic boxes (14.3 cm × 9.3 cm; 4.7 cm height) for *P. pruinosus* and a removable plastic wall to divide the boxes into two equal sections. The control soil (25 g wet weight) was placed on one side of the test vessel and the same amount of test soil was placed on the opposite side. After this, the wall was gently removed and a group of adult enchytraeid worms or isopods were left on the contact line of the soils. The box was covered with a lid (containing small holes) and kept, for 48 h, at 20(±2) °C for *E. albidus* or 25(±2) °C for *P. pruinosus* with a photoperiod of 16:8 h (light:dark). In the enchytraeids single and mixture exposure ten organisms per replicate were used; in the isopods avoidance tests a group of 5 and 8 organisms were used in the single and mixture exposures, respectively. Five and three replicates per treatment were used in the enchytraeids and isopods single exposure tests, respectively. Only one replicate per treatment was used in the binary mixture tests to allow the use of more treatments in each test, and obtain a reliable coverage of the response surface. According to Jonker et al. (2005), the increase in the number of treatments in this type of tests and approaches will increase the reliability and power of the analysis. For that one has to decrease the number of replicates so that experiments can become feasible. This test design will not bring any problem because the methodology used is based on a regression model where variances are calculated between data and model values.

At the end of the test, the movable wall was again placed in the centre and each side of the box was independently searched for animals. Table 1 resumes the experiments carried out in this study.

2.4. Test design

In the single exposure bioassays, five concentrations per chemical compound were used. In the binary mixture experiments, individual and mixture exposures were carried out simultaneously so that responses could be controlled and also to correct differences in organisms' responses due to sensitivity variations. Chemical

Table 2
Analysis of mixture toxicity data and interpretation of additional parameters (*a* and *b*) that define the functional form of deviation pattern from the reference models concentration addition (CA) and independent action (IA) (adapted from Jonker et al., 2005)

Deviation pattern	Parameter *a* (CA and IA)	Parameter *b* (CA)	Parameter *b* (IA)
Synergism/Antagonism (S/A)	$a > 0$: Antagonism $a < 0$: Synergism		
Dose-ratio dependent (DR)	$a > 0$: Antagonism except for those mixture ratios where negative *b* value indicates synergism $a < 0$: Synergism except for those mixture ratios where positive *b* value indicates antagonism	$b_i > 0$: Antagonism where the toxicity of the mixture is caused mainly by toxicant *i* $b_i < 0$: Synergism where the toxicity of the mixture is caused mainly by toxicant *i*	
Dose-level dependent (DL)	$a > 0$: Antagonism low dose level and synergism high dose level $a < 0$: Synergism low dose level and antagonism high dose level	$b_{DL} > 1$: Change at lower EC50 level $b_{DL} = 1$: Change at EC50 level $0 < b_{DL} < 1$: Change at higher EC50 level $b_{DL} < 1$: No change but the magnitude of S/A is DL dependent	$b_{DL} > 2$: Change at lower EC50 level $b_{DL} = 2$: change at EC50 level $1 < b_{DL} < 2$: Change at higher EC50 level $b_{DL} < 1$: No change but the magnitude of S/A is effect level dependent

mixture concentrations were based on the AC_{50} (concentration inducing an avoidance of 50%) for the single exposure experiments previously carried out. These AC_{50} values were converted into Toxic Units (TUs), and the toxic potency of the mixture was used for the calculation of chemical concentrations in the mixture. In these experiments the $\sum TU$ used for every mixture combination was ≤ 1 with the exception of two equitoxic mixtures of $\sum TU = 1.5$ and $\sum TU = 2$ (corresponding to $0.75 + 0.75$ and $1 + 1$ TUs, respectively). The experimental design was based on a ray design.

2.5. Statistical analysis

The avoidance effect was expressed as the percentage of affected animals (i.e. those which avoided the treated part of the test vessel). AC_{50}s were calculated using a Probit regression (statistical software SPSS 12.0), and assuming that in the control 50% of the animals were in each side of the test vessel (no effect). For the Probit analysis, when non-avoidance exceeded 100% (cases where the test soil was preferred), values were transformed into 100%.

In accordance with the previously mentioned Guideline for the Earthworm Avoidance Test (ISO, 2007 (draft)), the habitat function of soils is considered to be limited if on average >80% of worms are found in the control soil (indication of an impact on behaviour), which is approximately the AC50 (Loureiro et al., 2005).

To address the toxic effects in the mixtures experiments, the observed effect was compared to the expected effect of mixtures calculated from the single compound exposure toxicities. This procedure was based on the above described conceptual models. The concentration addition model (CA) is mathematically described by:

$$\sum_{i=1}^{n} C_i / EC_{xi} = 1 \qquad (1)$$

where C_i is the dose used for stressor *i* in the mixture and EC_{xi} is the effect dose of stressor *i* that produces the same effect (*x*%) as the whole mixture.

The independent action model (IA) is mathematically described by:

$$Y = \mu_{max} \prod_{i=1}^{n} q_i(C_i) \qquad (2)$$

where *Y* denotes the biological response, C_i is the concentration of chemical *i* in the mixture, $q_i(C_i)$ the probability of non-response, μ_{max} the control response for the selected endpoint.

Although these two conceptual models take into consideration the information available on the chemicals' MoA, there is always some ambiguity when discussing chemical MoA because 1) some chemicals have several MoA; 2) sometimes the information available for the chemical MoA refers to other species than the ones used. So, in this study both conceptual models were fit to data from the four mixtures tested and the best fit is discussed. Deviations from these two reference models such as synergism/antagonism, dose ratio and dose level were obtained by additional parameters used in the mathematical models that describe CA and IA, and tested within a nested framework [Table 2 and more detail in Jonker et al. (2005)]. The models were fitted to the data using the method of maximum likelihood, by minimizing the objective function (*L*) and statistically compared through likelihood testing. The best fit was chosen using a Chi-square test based on the minimization of the objective function based on the binominal log likelihood. The biological interpretation of these additional deviation parameters are summarized in Table 2 and can be found in more details in Jonker et al. (2005).

3. Results and discussion

3.1. Single toxicity assessment

Table 3 shows the AC50 values calculated from single exposure experiments and from single assessments in mixture exposures. These AC50 values were based on nominal exposure concentrations.

All the exposure tests were carried out in LUFA 2.2 soil due to consistency and comparability of results. The use of soils with other properties, for the same experiments would lead to a different exposure scenario situation for the test species and, therefore, to a different evaluation of the risk of the test substance. This was observed in the studies of Amorim et al. (2005a,b) where the exposure of several chemicals in different soil types showed different toxicities to enchytraeids.

In the isopod avoidance tests, the AC50 value for lindane was lower than the one obtained by Loureiro et al. (2005) (48.6 mg/kg), although within the same range.

Table 3
AC50 values (mg/kg dry soil, with corresponding 95% confidence intervals) for the effect of selected chemicals on the avoidance behaviour of *Porcellionides pruinosus* and *Enchytaeus albidus* exposed for 48 h in LUFA 2.2 soil

AC_{50} (95% CL)	Experiment	*Porcellionides pruinosus*	*Enchytaeus albidus*
Dimethoate	Single exposure experiments	34.0 (32.7–35.5)	58.3 (–)
	Mixture experiment with atrazine	41.6 (38–46.5)	94.9 (75.7–117.5)
	Mixture experiment with cadmium	47.5 (35.3–133)	110 (98–123)
	Mixture experiment with lindane	18.2 (14.4–25.0)	229 (–)
Atrazine	Single exposure experiments	153.1 (–)	38.0 (16–238)
	Mixture experiment with dimethoate	333 (322–346)	18.2 (15.0–22.0)
Lindane	Single exposure experiments	18.8 (15.2–25.5)	173 (133–241)
	Mixture experiment with dimethoate	37.7 (36.4–39.1)	220 (–)
Cadmium	Single exposure experiments	523 (490–572)	362 (244–871)
	Mixture experiment with zinc	105 (–)	571 (–)
	Mixture experiment with dimethoate	140 (84.7–189)	363 (–)
Zinc	Single exposure experiments	321 (299–345)	92.0 (68.0–139)
	Mixture experiment with cadmium	303 (267–336)	215 (174–293)

Results for *E. albidus* from Amorim et al. (2008).

Results on the avoidance tests in enchytraeids for lindane showed AC50 values of *ca.* 173 and 220 mg/kg. Information is available on the toxicity in OECD standard soil for survival (Lock et al., 2002) and reproduction (Römbke, 2003) with values of LC50 of 107 mg/kg and EC50 of 10 mg/kg, respectively. In this case one of the AC50 value was close to the LC50 value reported by Lock et al. (2002).

For the dimethoate exposure the values obtained is this study for *P. pruinosus* (Table 3) were higher but in the same range as the one

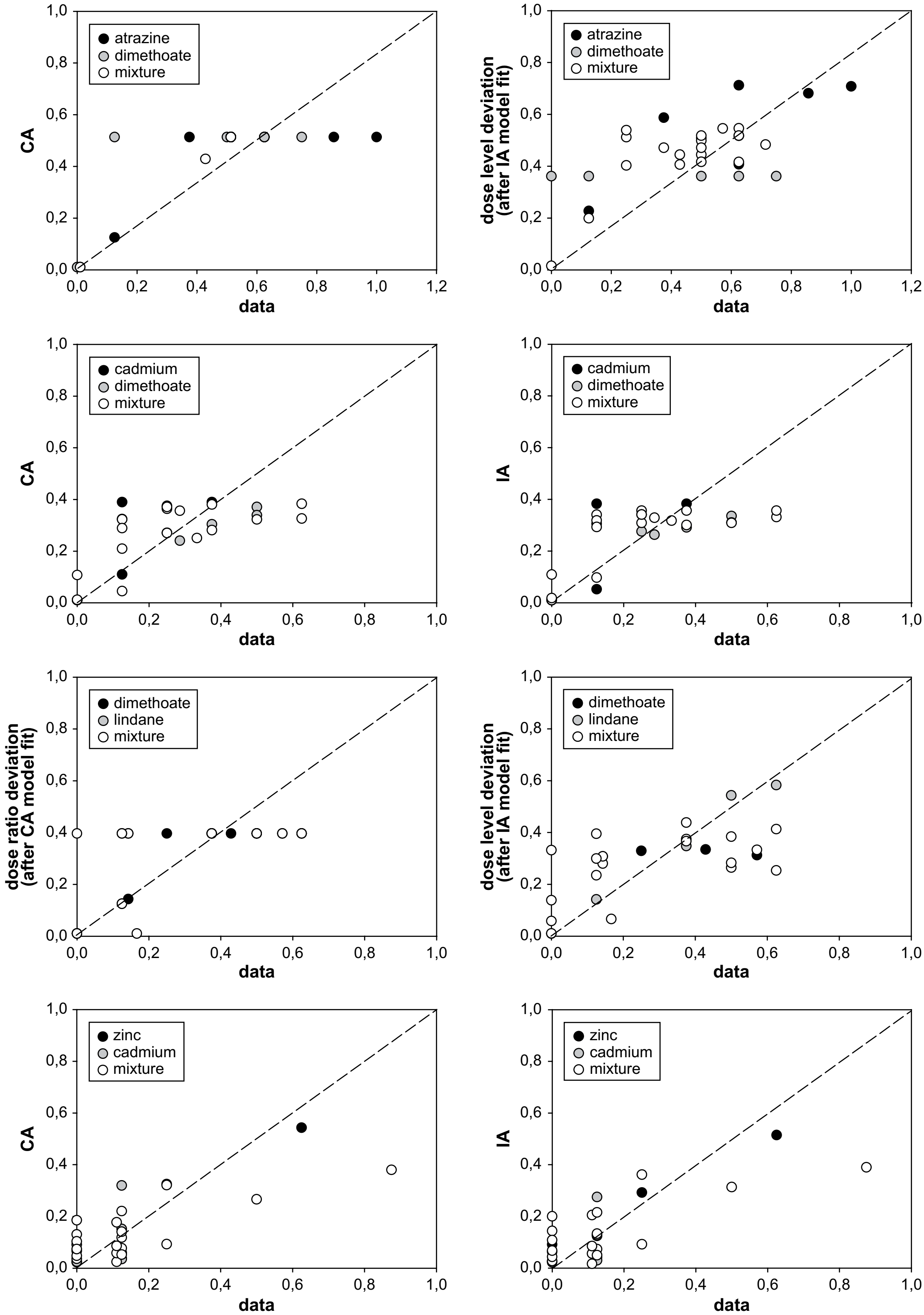

Fig. 1. Relationship between observed data from *Porcellionides pruinosus* exposures (expressed as avoidance ratio: number of animals in the test soil/exposed animals) and the modelled values for single exposures and mixtures of dimethoate + atrazine, cadmium + dimethoate, dimethoate + lindane and zinc + cadmium. Left column: data vs modelled values using the CA reference model or deviations; right column: data vs modelled values using the IA reference model or deviations.

reported by Hornung et al. (1998) for the isopod *Porcellio scaber* (EC50 value for growth of 17.5 mg/kg). Although parameters (avoidance and growth) and species are different, their sensitivity towards dimethoate was quite similar. It is known that dimethoate induces significant changes in reproductive traits, increasing sorption and consequently decreasing the number of juveniles released. In addition, it has been shown that dimethoate induces hyperactivity in woodlice. During a dimethoate exposure (140 g/ha), Bayley (1995) studied the locomotor behaviour of *P. scaber* and observed that exposed isopods showed an excited state that was related to stress. Enchytraeid avoidance behaviour to dimethoate was reported by Amorim et al. (2008), showing an AC50 of 58.3 mg/kg. When repeating the single exposure in the mixture experiments, the AC50 value was within the same range in the dimethoate + atrazine

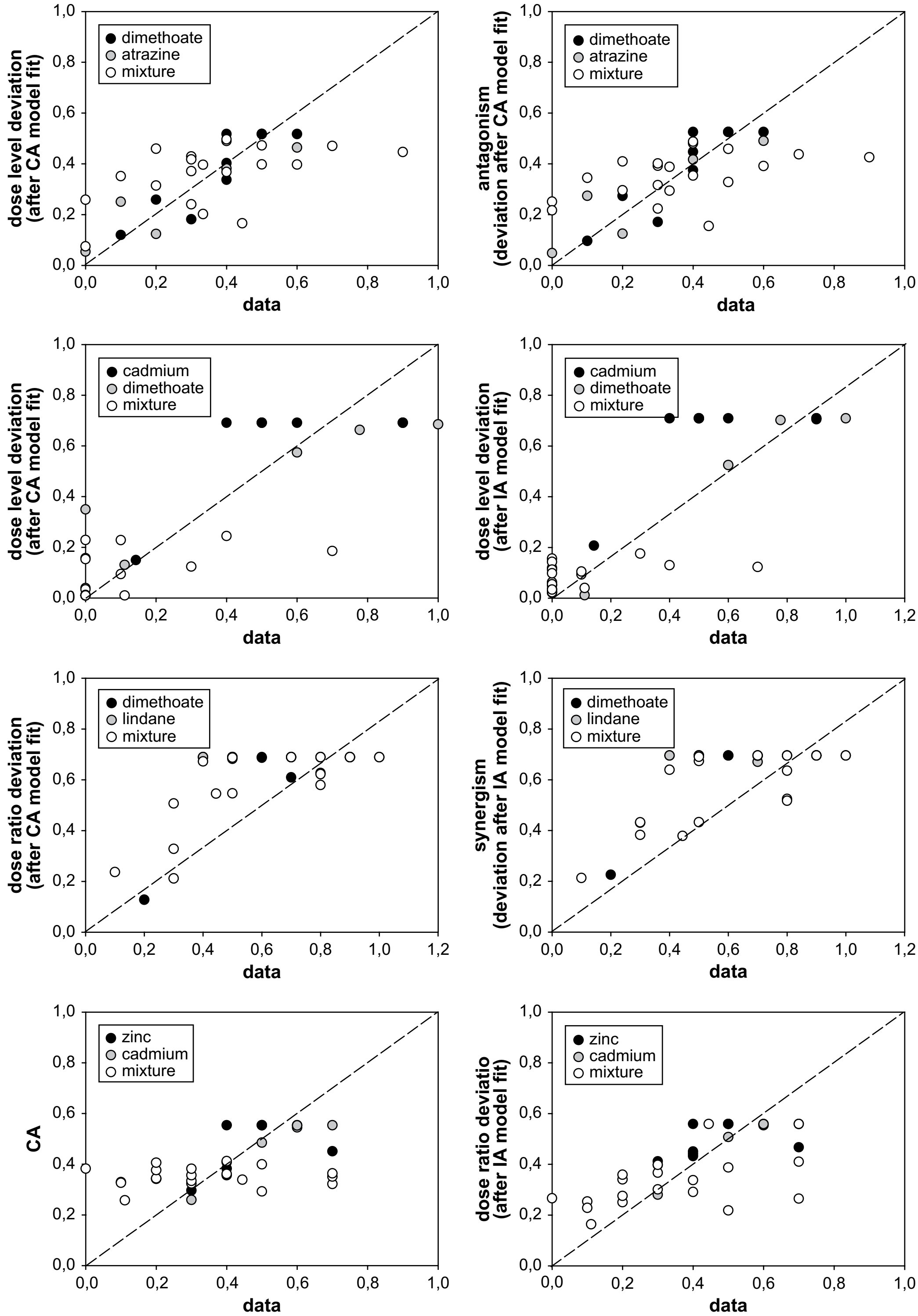

Fig. 2. Relationship between observed data from *Enchytraeus albidus* exposures (expressed as avoidance ratio: number of animals in the test soil/exposed animals) and the modelled values for single exposures and mixtures of dimethoate + atrazine, cadmium + dimethoate, dimethoate + lindane and zinc + cadmium. Left column: data vs modelled values using the CA reference model or deviations; right column: data vs modelled values using the IA reference model or deviations.

combination. On the other hand, in the mixture experiments with dimethoate and cadmium and dimethoate and lindane, the AC50 values for dimethoate were higher. No further toxicity information could be found for other parameters.

To the best of our knowledge there is no available information about the toxicity of atrazine to isopods. For enchytraeids, avoidance data was traduced in AC50 values of 38 and 18.2 mg/kg, showing that enchytraeids are more sensitive to this chemical than the isopod species used here. In a study where the earthworm *Eisenia fetida* was exposed to the formulation of atrazine Hungazin-PK, growth and reproduction was significantly decreased at 100 mg/kg, and the cocoon production was null at 200 mg/kg after 2, 4 and 6 weeks of exposure (Fischer, 1989).

For the zinc exposure, Posthuma et al. (1997) reported an EC50 value for enchytraeid juvenile production of 188 mg/kg which is within the two values found here. Furthermore, Lock and Janssen (2002b) showed that avoidance was more sensitive than mortality and reproduction, LC50 = 610 mg/kg and EC50 = 345 mg/kg, respectively.

Our results for cadmium toxicity showed AC50 values between 362 and 571 mg/kg for enchytraeids. These results are in the same range of acute toxicity (with an LC50 value around 500 mg/kg) (Lock and Janssen, 2002a; Römbke, 2003) and less sensitive than values obtained in chronic toxicity tests using reproduction as endpoint (with an EC50 value around 140 mg/kg) (Lock and Janssen, 2002a,b).

Although there is some information on metal toxicity to isopods, mainly using contaminated food, the available information for soil toxicity is scarce.

Comparing the results obtained in all single exposure experiments, for isopod, AC50 values obtained for each compound were similar between experiments, with the exception for cadmium exposure. For enchytraeids case study, the AC50 values were also similar within the same chemical exposure with the exception of dimethoate and zinc.

This might be due to the variability that can be found in this kind of tests as they present binary responses (e.g. avoid or not avoid).

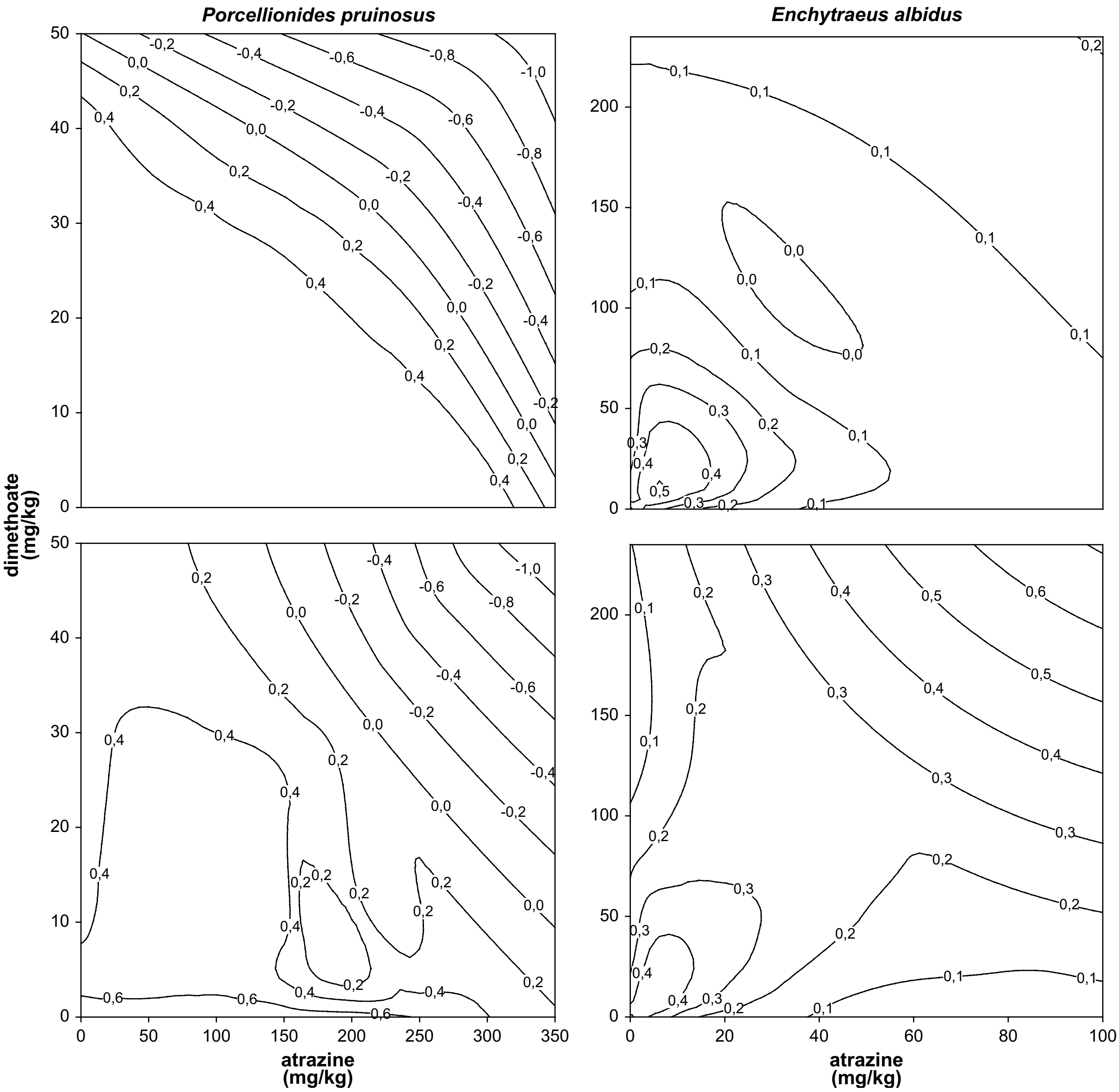

Fig. 3. Dose–response data for avoidance behaviour of *Porcellionides pruinosus* and *Enchytraeus albidus* exposed in LUFA 2.2 soil for 48 h to the combination of dimethoate and atrazine. The top graphs show a Concentration Addition fit for *P. pruinosus* and a dose level deviation, translated to antagonism (after CA model fit) for *E. albidus*. The bottom graphs show a dose level deviation, after IA model fit, translated to antagonism for *P. pruinosus*, and a deviation to antagonism, after the IA model fit, for *E. albidus*. Values in isoboles are the modelled values for the avoidance ratio (number of animals in the test soil/exposed animals).

3.2. Mixture toxicity assessment

The best descriptive model and the relationship between the observed and the modelled data (after CA and IA model fit) are represented in Figs. 1 and 2 for *P. pruinosus* and *E. albidus*, respectively.

From the binary mixture exposure of atrazine and dimethoate both isopod and enchytraeid data showed a significant deviation from the IA conceptual model fit for antagonism. In the case of *E. albidus*, a significant decrease in the mixture toxicity was observed when compared to the expected (Fig. 3; Table 4; χ^2 test, $P < 0.05$). For *P. pruinosus*, a significant dose level dependent deviation from the IA model was observed, showing antagonism at low dose levels and synergism at high dose levels (Fig. 3; Table 4; χ^2 test, $P < 0.05$). As the switch between antagonism and synergism occurred at doses higher than the AC50 level, it is possible that this synergism might be converted in the dead of the exposed organisms, due to the potency of the mixture. So, the biological response might only be translated into antagonism.

Analysing the same data set using the conceptual model of CA, the pattern was similar for *E. albidus* with a dose level-dependency (with antagonism at low dose levels and synergism where concentration levels were higher) that will probably be transposed as antagonism, as explained before, and for *P. pruinosus* no deviation from CA was found (Fig. 3; Table 4).

In a study carried out by Lindstrom and Lydy (1997), the aquatic midge *Chironomus tentans* was exposed to mixtures of atrazine and the organophosphates methyl-parathion, malathion, chlorpyrifos and trichlorfon. The combined actions of mixtures of atrazine and these four organophosphates showed response patterns always greater than additive (synergism) which is opposite to our results for both species.

Regarding the exposure to the binary mixture of cadmium and dimethoate we expected that these two chemicals act dissimilarly, thus we first use the IA conceptual model. Although the terrestrial isopod data did not deviate significantly from the conceptual model IA (Fig. 4; Table 4; c2 test, $P < 0.05$), for the enchytraeids a significant deviation from the IA model with a synergistic effect at low dose levels and antagonism when exposed to higher doses was observed. The switch from synergism to antagonism was observed at doses higher than the AC_{50} values. This might be transposed to a synergistic pattern because the antagonistic pattern at higher doses might be a result of the model extrapolation because our ray design did not cover all high dose levels. We expected an increase in mortality rate and that was the reason for not testing such high doses.

In the equitoxic mixture assessment of the organophosphate diazinon and the metal copper to the larvae of Epheron virgo, Vander Geest et al. (2000) observed that the acute toxicity was suggesting a response for less than concentration additive, i.e. antagonism. But, these authors also provided examples of other studies with similar compounds where synergistic mixture effects of metals and organophosphorous insecticides were observed as it was in this case study.

For the dimethoate and lindane mixture exposure experiments, a deviation from the IA conceptual model was also observed but with different patterns when comparing the species behaviour. The terrestrial isopod data showed a dose level dependent response with antagonism at low dose levels and synergism at high dose levels (switching at levels lower than the AC_{50}), but *E. albidus* showed a synergistic response pattern (Fig. 1; Table 4; χ^2 test, $P < 0.05$). When the avoidance data were fit into the CA conceptual model, both species showed a dose ratio-dependency although no accurate *a* and *b* values could be calculated for *E. albidus* (Table 4). For the *P. pruinosus* exposures higher toxicities than expected were obtained when lindane was the dominant chemical in the mixture

Table 4
Summary of avoidance responses of *Enchytaeus albidus* and *Porcellionides pruinosus* exposed for 48 h to several chemical mixtures in LUFA 2.2 soil

Chemical mixture	Species	CA													IA												
		CA		S/A deviation					Final deviation found						IA		S/A deviation					Final deviation found					
		L	r^2	Type	*P*	*L*	r^2	*a*	Type	*P*	*L*	r^2	*a*	*b*	*L*	r^2	Type	*P*	*L*	r^2	*a*	Type	*P*	*L*	r^2	*a*	*b*
Dimethoate and atrazine	*E. albidus*	57.82	0.204	A	3.96×10^{-4}	45.3	0.377	8.37	DL	4.6×10^{-4}	41.14	0.434	22.2	0.16	48.4	0.333	A	0.05	44.6	0.386	6.02	A	0.05	44.6	0.386	6.02	–
	P. pruinosus	39.9	0.395	–	–	–	–	–	–	–	–	–	–	–	49.8	0.245	–	–	–	–	–	DL	0.012	40.9	0.380	333	1.98
Dimethoate and lindane	*E. albidus*	72.2	0.187	–	–	–	–	–	DR	4.3×10^{-4}	56.7	0.361	>1000	<-1000	72.1	0.187	S	1.04×10^{-4}	57.12	0.357	−23.2	S	1.04×10^{-2}	57.1	0.357	−23.2	–
	P. pruinosus	37.4	0.369	–	–	–	–	–	DR	0.046	31.2	0.473	−3.03	4.84	40.9	0.323	–	–	–	–	–	DL	0.017	32.8	0.446	85.0	2.10
Zinc and cadmium	*E. albidus*	46.2	0.213	–	–	–	–	–	–	–	–	–	–	–	45.6	0.223	–	–	–	–	–	DR	0.009	36.2	0.383	−463	>1000
	P. pruinosus	34.5	0.511	–	–	–	–	–	–	–	–	–	–	–	34.7	0.508	–	–	–	–	–	–	–	–	–	–	–
Cadmium and dimethoate	*E. albidus*	96.4	0.526	S	2.49×10^{-7}	70.8	0.653	−5.98	DL	1.84×10^{-4}	66.4	0.677	−8.08	0.59	119	0.416	S	5.7×10^{-5}	103.76	0.495	−4.73	DL	1.27×10^{-1}	69.8	0.660	−15.0	1.02
	P. pruinosus	21.6	0.576	–	–	–	–	–	–	–	–	–	–	–	23.3	0.542	–	–	–	–	–	–	–	–	–	–	–

L is the objective function used for avoidance (as discrete data); r^2 is the coefficient of determination; *a* and *b* are parameters of the deviation functions (see Table 2); *P* value indicates the significance of deviations found from the conceptual models; CA is concentration addition; IA is independent action; S/A is synergism/antagonism, DR is dose-ratio deviation and DL is dose-level deviation from the reference.

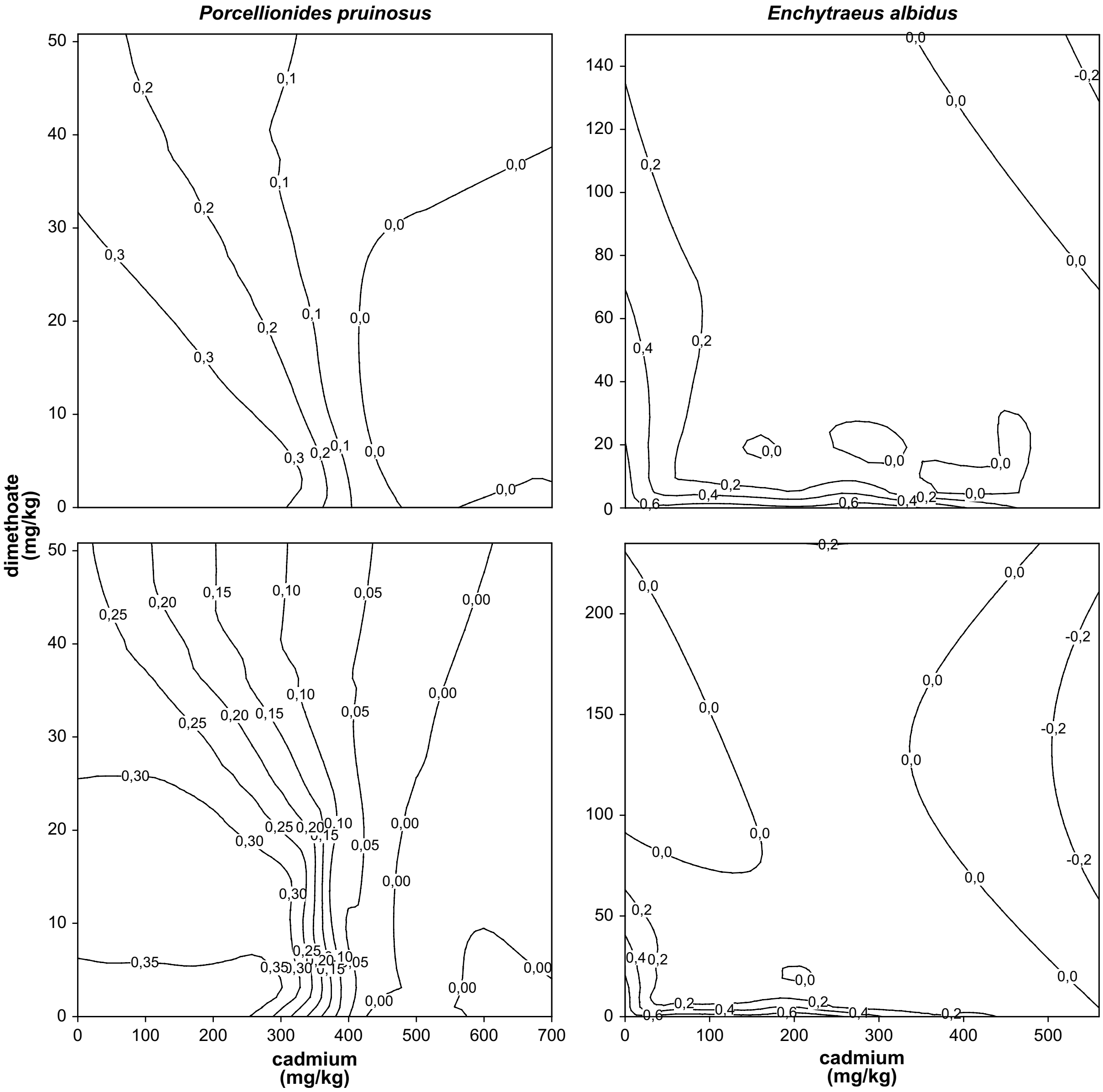

Fig. 4. Dose–response data for avoidance behaviour of *Porcellionides pruinosus* and *Enchytraeus albidus* exposed in LUFA 2.2 soil for 48 h to the combination of cadmium and dimethoate. The top graphs show a CA model fit for *P. pruinosus*, and a dose level deviation, translated to synergism (after CA model fit) for *E. albidus*. The bottom graphs show an IA model fit for *P. pruinosus*, and a dose level deviation, after the IA model fit, for *E. albidus* where there is synergism at low doses and antagonism at higher doses. Values in isoboles are the modelled values for the avoidance ratio (number of animals in the test soil/exposed animals).

(Table 4). The relationship between observed and modelled data from isopods is stronger for the dose level dependent deviation (after IA model fit) than for the dose ratio one (after CA model fit) (Fig. 1). This might be the reason why the top left graph in Fig. 5 is difficult to interpret. From the *a* and *b* parameter values obtained for the DR deviation fit on enchytraeid data (after CA model fit), it might be concluded that the deviation for synergism (after IA model fit) is more adequate. In a study on mouse thymocytes exposed *in vitro* to lindane and malathion, Olgun et al. (2004) also observed a pattern of more than additive increase in both apoptotic and necrotic cell populations.

When the two metals zinc and cadmium were present in the mixture, we expected them to act similarly hence we fitted the CA model. This was confirmed and the pattern observed for both species was similar with no deviation from the CA reference model (Table 4; χ^2 test, $P < 0.05$). When trying to fit our data to the IA conceptual model, there was also no deviation for the isopod exposure. But for the enchytraeids, although a dose ratio-dependency was observed with cadmium acting as principal toxicant in the mixture, nothing can be concluded due to the inaccurate values obtained for parameters a and b ($a = -463$ and $b > 1000$) (Fig. 6). Isopod and enchytraeid data are probably better explained by the CA pattern (Fig. 6). Looking at the graphs in Fig. 6 CA seems to represent better isopod data; the CA model fit for the enchytraeid data set seems graphically not accurate at doses higher than the EC50 which might be explained by a possible extrapolation of the model to these higher doses.

In another work where the isopod *Oniscus asellus* was exposed to a binary mixture of cadmium and zinc (among other metallic mixtures), stress proteins (hsp) were induced more than expected (synergism) at small dose levels of both metals. Although Kohler and Eckwert (1997) also used high cadmium and zinc concentrations, they were not able to model effects because responses at higher doses were quite different from the one at low exposure concentrations. Their model was not able to detect or fit data that shift in pattern responses in different dose levels.

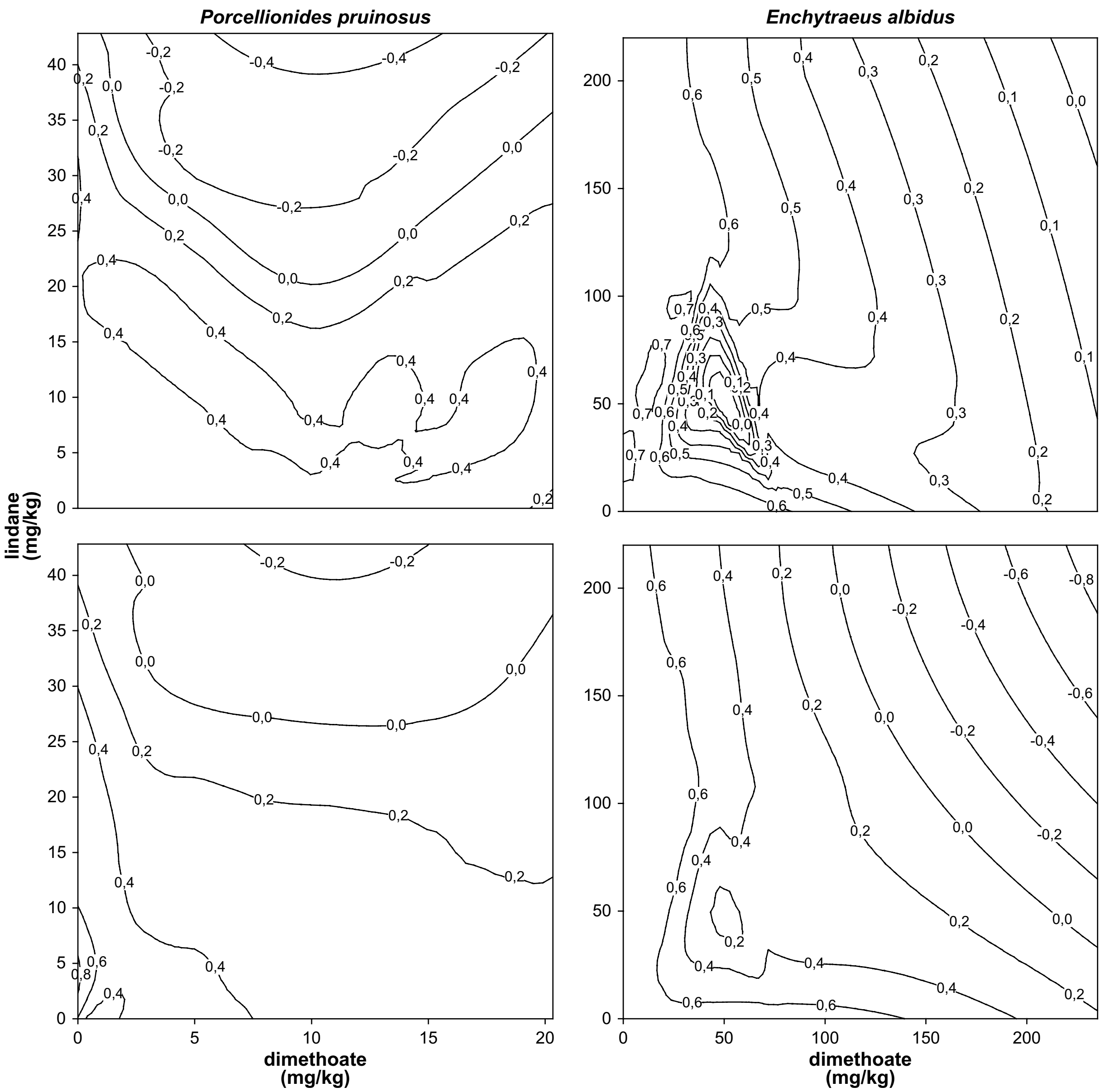

Fig. 5. Dose–response data for avoidance behaviour of *Porcellionides pruinosus* and *Enchytraeus albidus* exposed in LUFA 2.2 soil for 48 h to the combination of dimethoate and lindane. The top graphs show a dose ratio deviation (after CA model fit) for both species. The bottom graphs show a dose level deviation (after IA model fit), translated to antagonism for *P. pruinosus*, and a synergism deviation, after the IA model fit, for *E. albidus*. Values in isoboles are the modelled values for the avoidance ratio (number of animals in the test soil/exposed animals).

When modelling the effects of cadmium and zinc on the reproduction of *Folsomia candida* to the CA conceptual model, Jonker et al. (2005) observed a dose ratio dependent deviation where cadmium dominancy was responsible for an antagonistic pattern, while zinc induced a synergistic effect. The same toxicity pattern was observed, in the same study, with data from a survival test with *Eisenia andrei*.

3.3. Avoidance tests in mixture toxicity assessment

From the results (single and mixture exposures) obtained in this study we can conspicuously state that the ability of the tested organisms to detect chemicals plays an important role in the environment because it may allow them to avoid toxicants and other stress factors. In certain scenarios, organisms may even escape from being affected by chemicals due to their ability to detect polluted soils. When only avoidance occurs, without any detoxification using biochemical procedures, it is difficult to discuss results in terms of detoxification inhibition or induction. Moreover, some chemicals like those acting on the nervous system can affect invertebrates, causing a loss of their ability to escape. In the latter case, high mortality rates may occur.

In the avoidance behaviour tests carried out with the terrestrial isopods and enchytraeids, concentrations used in the mixture exposure did not induce significant mortality.

This study has shown the importance of using different species in the evaluation of chemical mixture toxicity, as it has been already advocated for single chemical exposures (Amorim et al., 2005a, 2008; Cedergreen et al., 2006; Badisa et al., 2007). Different life-traits and consequently different behaviours (e.g. biochemical, locomotion) of the two species used here might justify the different sensitivities to chemical exposures. This can be applied to single chemical exposure, as mentioned before for the case of the metal physiological adaptations, but can also be transposed to mixtures of chemicals. Both organisms have chemoreceptors which enable the

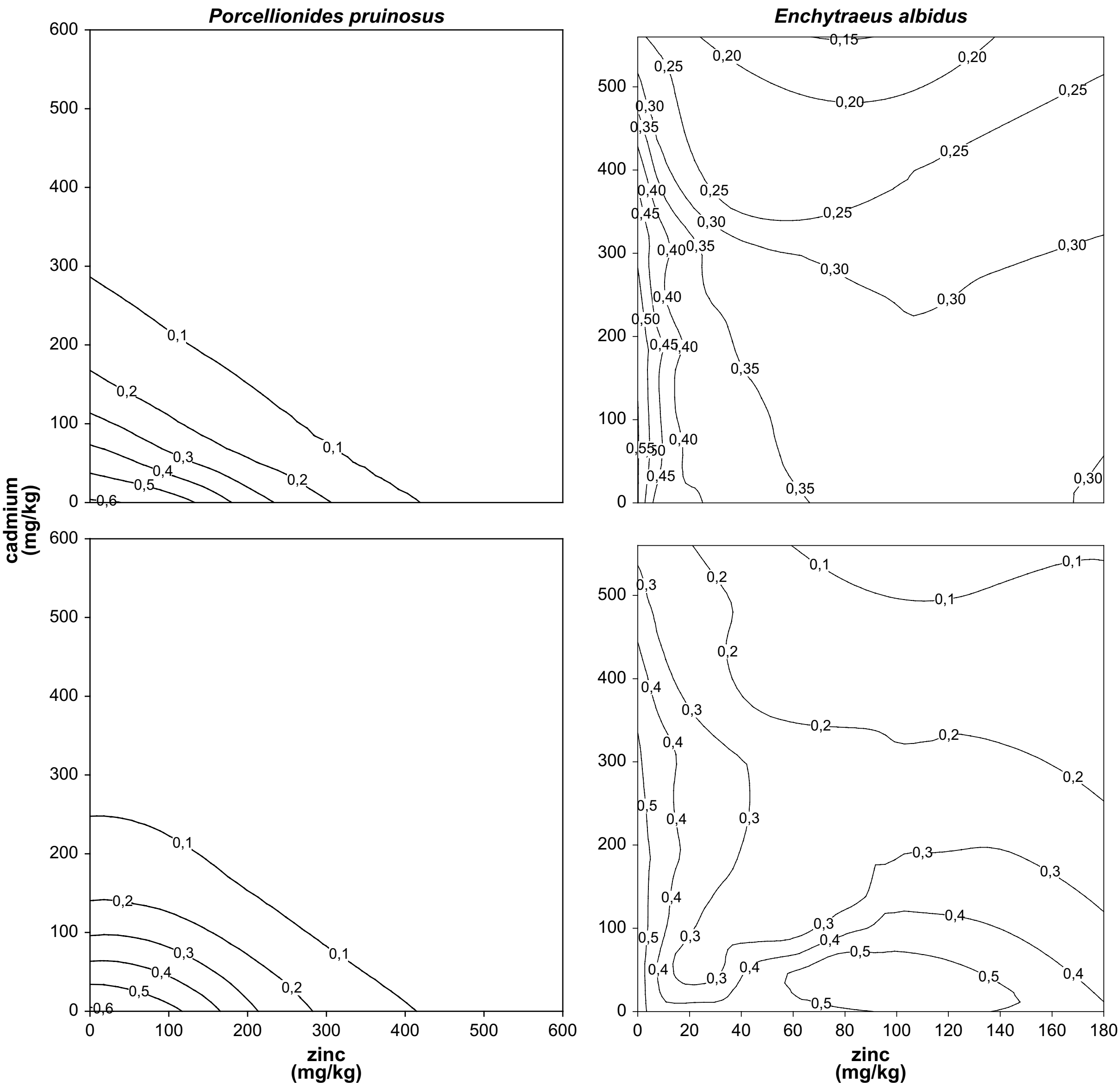

Fig. 6. Dose–response data for avoidance behaviour of *Porcellionides pruinosus* and *Enchytraeus albidus* exposed in LUFA 2.2 soil for 48 h to the combination of zinc and cadmium. The top graphs show CA model fit for both species and the bottom graphs show IA model fit for *P. pruinosus*, and a dose ratio deviation, after the IA model fit, for *E. albidus*. Values in isoboles are the modelled values for the avoidance ratio (number of animals in the test soil/exposed animals).

detection of chemical compounds, but their location is different: enchytraeids have cuticular sense organs while isopods have their alarm organs in their antenna (Hoese, 1989; Warburg, 1993; Römbke and Schmidt, 1998; Amorim et al., 2005a; Loureiro et al., 2005). In addition, isopods and enchytraeids can avoid environmental limiting factors that can also act as stressors like extreme/lack of humidity, light or temperature. Using their pheromone detection isopods are also able to form groups, a "social" behaviour that helps them to avoid danger (Takeda, 1980).

Behavioural parameters have been considered good biomarkers when assessing soil contamination (Bayley, 1995; Bayley et al., 1997; Sorensen et al., 1997). However, the interpretation and the statistics of behavioural data is sometimes difficult due to the binary responses obtained, usually responses of "yes or no" choices, "turn right or left" or, as in avoidance tests "avoid or not". This also results in a high variability and worse fit (e.g. low r^2 values) in mathematical models when compared to other ecological parameters. Nevertheless, this kind of studies are very important because locomotor behaviour is related to crucial processes like feeding, predation, reproduction and migration which link individual patterns to population stress.

3.4. CA and IA models and deviations in avoidance tests

Regarding the mathematical methodology used to fit the data to conceptual models, our results show that simple deviations for synergism or antagonism are not enough to explain response patterns in mixture exposures. Dose ratio or level dependencies are shown here to play an important role in mixture toxicity responses, as previously demonstrated by other studies, such as the exposure of the isopod *O. asellus* to the binary mixture of cadmium and zinc (Kohler and Eckwert, 1997). This confirms the importance of using our approach which will also provide information on dose ratio and dose level dependent patterns of response to chemical mixture exposures.

In addition, the use of ray designs is essential in this kind of approaches, when it is aimed to detect DR or DL dependent deviations from the conceptual models. The use of equitoxic concentrations in mixture is not sufficient to allow the detection of such deviations.

As explained before, avoidance data fit to the models can be considered "poor" as we are dealing with binary and behavioural data. Although AC50 values for single chemical exposure have shown few differences when the same chemical was tested, one cannot guaranty the reproducibility of mixture experiments. This was advocated by Cedergreen et al. (2007), who state that experimental reproducibility can be sometimes difficult in this kind of experimental setups. This will not only depend on the setup but also on the species that is used: if we consider clone individuals as test species experiments will have more chances to be reproducible than if the test organisms have genetically variability among specimens.

4. Conclusions

Although with only two test species, this study restates the differences in species sensitivity in ecotoxicological approaches, and alerts for the lack of information that single chemical exposures give to the actual needs of ERA procedures.

The chemical binary mixtures tested showed an accurate fit to the conceptual models (IA or CA), but also that deviations for synergism, antagonism or a toxicity level dependent on the dose or on the chemical ratio are also found.

Both species showed similar patterns for some of the tested mixtures. Patterns of antagonism were found for both species when exposed to dimethoate and atrazine. Synergism for lindane and dimethoate exposures (with the exception of lower doses in the isopod case study) and concentration addition for cadmium and zinc were also found for both test species.

The exposure to cadmium and dimethoate showed dissimilar patterns for both species. Whereas independent action was found for *P. pruinosus*, a pattern for synergism was observed for the enchytraeids case study.

As other tests used in ecotoxicology, avoidance behaviour tests can also be used in this kind of approaches because test organisms like isopods and enchytraeids are able to detect single chemicals and also chemical mixtures.

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