Universidade de Aveiro Departamento de Biologia
Ano 2012

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MECANISMOS DE ECOTOXICIDADE EM *FOLSOMIA CANDIDA*

MECHANISMS OF TOXICITY IN *FOLSOMIA CANDIDA*

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Aplicada, ramo Toxicologia e Ecotoxicologia, realizada sob a orientação científica da Doutora Mónica João de Barros Amorim (Investigadora Auxiliar do Departamento de Biologia & CESAM da Universidade de Aveiro).

Dedico este trabalho aos meus pais.

o júri

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palavras-chave

collembola, pesticidas, metais, mistura binaria, efeitos crónicos, comportamento de “evitamento”, atividade de cholinesterases

resumo

Uma enorme variedade de contaminantes pode ser encontrada no ambiente, podendo estes afetarem o ecossistema terrestre. Sendo por isso, importante, o estudo de diferentes contaminantes e de diferentes parâmetros de avaliação. O presente estudo abrange dois assuntos diferentes com diferentes abordagens, na área de ecotoxicologia terrestre: 1) toxicidade de misturas binaria de químicos com diferentes modos de ação usando a sobrevivência e a reprodução da *Folsomia candida* como parâmetros de avaliação e 2) comportamento de “evitamento” e atividade de colinesterases (ChE) (após previa caracterização). Esta espécie teste é um inseto de solo, pertence a um grupo ecologicamente relevante com um importante papel nos processos de mineralização e decomposição no solo. Estão disponíveis procedimentos padronizados para avaliar efeitos na sobrevivência, reprodução e comportamento de “evitamento”. Os resultados dos testes de toxicidade de misturas foram interpretados com base nos modelos conceptuais da Adição da Concentração e da Ação Independente, incluindo os desvios destes modelos: sinergismo, antagonismo, dependente do nível-dose e do rácio-dose. Diferentes padrões de resposta a toxicidade de misturas em *F. candida* foram observados em diferentes parâmetros de avaliação (sobrevivência e reprodução). Sinergismo foi frequentemente observado após exposição a misturas de pesticidas (lindano e atrazina, e dimetoato e lindano) ou quando um dos compostos da mistura era um pesticida (cadmio e dimetoato, quando o parâmetro de avaliação foi a reprodução). A caracterização revela que a *Folsomia candida* contém principalmente acetilcolinesterases. Em relação ao efeito do dimetoato, no comportamento de “evitamento” dos organismos ocorreu uma preferência pelo solo contaminado. Observações mostram que ocorreu imobilização associada a inibição das ChE – sinais de paralisia com o aumento da concentração está correlacionado com a diminuição da atividade das colinesterases. Se tivéssemos em conta apenas os resultados do teste de “evitamento” estaríamos perante um resultado falso negativo devido aos efeitos diretos na locomoção.

keywords collembola, pesticides, metals, binary mixture, chronic effects, avoidance behaviour, cholinesterase activity

abstract A variety of contaminants can be found in the environment and can affect terrestrial ecosystems. It is important to study not only the effects of different chemicals but also at various levels and endpoints. The present studies covers two different topics and approaches to the soil system: 1) toxicity of binary mixtures of chemicals with different modes of action using survival and reproduction of *Folsomia candida* as endpoints and 2) avoidance behaviour and cholinesterases (ChE) activity (after previous characterization). This test species is a wingless terrestrial insect, an ecologically relevant group with a role in soil mineralization and decomposition processes. Standard guideline test procedures are available to assess effects on survival, reproduction and avoidance behaviour. Results of the mixture toxicity test were interpreted in light of conceptual models of Concentration Addition and Independent Action, including synergistic, antagonistic, dose-level and dose-ratio dependent deviations from these models. Differences on mixture toxicity response patterns in *Folsomia candida* were observed in the different endpoints used (survival and reproduction). Synergism was frequently observed upon exposure to pesticide mixtures (lindane and atrazine, and dimethoate and lindane) or when one of the components of the mixture was a pesticide (Cd and dimethoate, when the endpoint was reproduction). Characterisation revealed that *Folsomia candida* presents mainly acetylcholinesterases. In regard to the effects of dimethoate, on the avoidance behaviour of the organisms there was a preference for the spiked soil. Observations showed that immobilization occurred associated with ChE inhibition - signals of paralysis with increasing concentrations were correlated with a decrease in the ChE activities. Results from the avoidance test alone would be in fact a false negative of avoidance due to direct effects on the locomotion.

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Context and main objectives

Soil is the habitat of many organisms, playing a key role in suporting biodiversity in terrestrial ecosystems (Edwards 1998). It is formed by mineral particles, organic matter, water, air and living organisms. It also absorbs rainwater and act as a filter and a buffer, promoting clean groundwater (van Straalen 2002) and is the ground for many agricultural activities and major economic importance.

Terrestrial ecosystems are often affected by pollution of various sources, e.g. wastes of industrial activities, domestic wastes, pesticides, fertilizers, metals, and can also occur as complex mixtures (van Straalen 2002, Loureiro et al. 2009).

To assess the effects of this span of pollutants different approaches are required depending on numerous factors. In ecotoxicology, a most traditional procedure includes the assessment of effects organisms, e.g. on survival, reproduction or avoidance behaviour.

The present thesis is organized in two main chapters (publications):

1. Amorim, M. J. B., C. Pereira, V. B. Menezes-Oliveira, B. Campos, A. M. V. M. Soares, and S. Loureiro. 2012. Assessing single and joint effects of chemicals on the survival and reproduction of Folsomia candida (Collembola) in soil. Environmental Pollution **160**:145-152.

2. Dimethoate affects cholinesterases in *Folsomia candida* and their locomotion - false negative results of an avoidance behaviour test (Cecília M. S. Pereira, Sara C. Novais, Amadeu M.V.M. Soares & Mónica J.B. Amorim), submitted.

The selected test species, *Folsomia candida,* is a widely used standard test species. Belongs to the class Collembola, an ecologically relevant group, with an important role in soil mineralization and decomposition processes (Fountain and Hopkin 2005). It is a small soil arthropod, a blind unpigmented eudaphic collembolan with a worldwide distribution (Fountain and Hopkin 2005). Reproduces parthenogenetically and is sexually mature after approximately 22 days (Fountain and Hopkin 2005, Campiche et al. 2007). *Folsomia candida* has a well-developed furca (jumping organ) and an active running movement and jumps readily if disturbed, presents high locomotors ability (Fountain and Hopkin 2005).

Standard guidelines test procedures are available for reproduction and for avoidance behaviour of *Folsomia candida* (ISO 1999, 2011).

In chapter 1 the problematic of toxicity of mixtures has been studied using effects at reproduction level as endpoint – my contribution was in part of an already ongoing study.

In chapter 2, a more mechanistic study has been carried out, where the effect of the insecticide dimethoate on the behaviour was assessed and further understood with subcellular analysis.

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CHAPTER I

ASSESSIN SINGLE AND JOINT EFFECTS OF CHEMICALS ON THE SURVIVAL AND REPRODUCTION OF *FOLSOMIA CANDIDA* (COLLEMBOLA) IN SOIL

Assessing single and joint effects of chemicals on the survival and reproduction of *Folsomia candida* (Collembola) in soil

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Abstract

Chemicals are often found in the environment as complex mixtures. There has been a large effort in the last decade to assess the combined effect of chemicals, using the conceptual models of Concentration Addition and Independent Action, but also including synergistic, antagonistic, dose-level and dose-ratio dependent deviations from these models. In the present study, single and mixture toxicity of atrazine, dimethoate, lindane, zinc and cadmium were studied in *Folsomia candida*, assessing survival and reproduction. Different response patterns were observed for the different endpoints and synergistic patterns were observed when pesticides were present. Compared with the previously tested *Enchytraeus albidus* and *Porcelionides pruinosus*, the mixture toxicity pattern for *F. candida* was species specific. The present study highlights the importance of studying toxicity of chemicals mixtures due to the observed potentiation of effects and confirms that for an adequate ecologically relevant risk assessment different organisms and endpoints should be included.

Capsule: Exposure to chemical mixtures in *Folsomia candida* showed potentiation of effects. Mixture toxicity patterns differ among species and endpoint measured.

Keywords: binary mixture; pesticides, metals; chronic effects;

1. Introduction

For several decades chemicals have been produced and released in the environment by anthropogenic activities, namely industrial, urban, and agricultural applications, leading to complex mixtures that can be found as cocktails in the environment (Loureiro et al. 2009). The US EPA has described a Framework for Cumulative Risk Assessment (CRA) where an analysis, characterization, and possible quantification of the combined risks to human health or the environment from multiple agents or stressors is carried out in addition to single exposure effects (USEPA 2003). Nevertheless, effect and risk assessment have been mainly based on exposures to single chemicals. Therefore in the last 15 years, several European Union projects like BEAM (EVK1-CT-1999-00012), MIXTOX (ENV4-CT97-0507) and NoMiracle (FP6 contract nº 003956) made efforts and developed several tools tackling this issue through the use of described models (Loewe and Muischnek 1926, Bliss 1939), allowing a better interpretation and prediction of mixture toxicity (e.g. Altenburger et al. 2003, Backhaus et al. 2003, Jonker et al. 2004, Jonker et al. 2005, Loureiro et al. 2010).

Studies on mixture toxicity are available, mostly on aquatic species (e.g. Deneer 2000, Van der Geest et al. 2000, Barata et al. 2006, Cedergreen et al. 2008, Loureiro et al. 2010) and fewer on terrestrial organisms (e.g.Loureiro et al. 2009, Santos et al. 2010a, Santos et al. 2010b). Nevertheless, there is still lack of information on exposure, addressing and discussing the combined effects of mixtures of chemicals in soils.

Therefore, the present study focused on the assessment of binary mixture toxicity to a terrestrial species, the collembolan *Folsomia candida* at survival and reproduction levels. This is a widely used standard test species (ISO 1999), belonging to an ecologically relevant group, with an important role in soil mineralization and decomposition processes (Hopkin 1997). Reproduction is a robust endpoint for effect assessment and representative for ecological risk.

The following five chemicals were selected due to a) their importance and priority use, b) being found in the edaphic system and c) comparison with a mixture toxicity previous study carried out on isopods and enchytraeids (Loureiro et al. 2009): 1) atrazine, a triazine-ring herbicide, known to act as an endocrine disruptor in frogs and fish (Hecker et al. 2005); 2) dimethoate, an organophosphate, cholinesterase inhibitor after metabolism, and it

acts at the cholinergic synapses (IPCS 1989); 3) lindane (organochlorine insecticide), a GABA-gated chloride channel antagonist (Casida 2005), acts as central nervous system stimulant and is also an inducer of DNA fragmentation (Olgun et al. 2004); 4) zinc and 5) cadmium, known as oxidative stress inductors, causing lipid peroxidation (Pinto et al. 2003, Badisa et al. 2007).

The main objectives of this study were to evaluate response patterns of combined chemical exposures to *Folsomia candida*, using the available conceptual models for Concentration Addition and Independent Action. In addition, deviations from synergism (i.e. more toxicity than expected) or antagonism (i.e. less toxicity than expected), or dose level or ratio were investigated. Dose level or dependent ratio deviations have been described and modeled by Jonker et al (2005) and have been observed in several studies where chemical mixtures or combined chemical and natural stressors where evaluated in terrestrial and aquatic invertebrates (e.g. Loureiro et al. 2009, Loureiro et al. 2010, Santos et al. 2010b, Pérez et al. in press). Regarding these two deviations synergism and antagonism are observed at different levels or ratios, depending on the dosage used or depending on the chemical that dominates the mixture (i.e. chemical at higher dose).

In this study two endpoints (survival and reproduction) will be reported, giving the possibility to distinguish patterns of response depending on the endpoint, but also to compare with literature data to check the consistency of the mixture interactions seen for other organisms.

2. Materials and Methods

2.1 Test organism

The standard test species *Folsomia candida* (Collembola) was used. Organisms were cultured on a moist substrate of plaster of Paris and activated charcoal (in a 8:1 ratio), at 18 °C, under a photoperiod regime of 16:8 (light:dark) and fed weekly with dried baker's yeast (*Saccharomyces cerevisae*).

2.2 Test chemicals and soil

Five chemicals were used in these experiments: the herbicide atrazine [Sigma-Aldrich, 97.4% purity], the insecticides dimethoate [Sigma-Aldrich (Riedel-de Haën), 99.8%

purity] and lindane [g-HCH, Sigma-Aldrich, 97% purity] and the metals zinc chloride [$ZnCl_2$, Sigma-Aldrich (Riedel-de Haën), 98% purity] and cadmium chloride [$CdCl_2$, Sigma-Aldrich, 99% purity]. Experiments were done in the certified loamy sand soil LUFA 2.2 (Lokke 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%, water holding capacity 45.2±5 g/100g (weight per volume), texture: 6% clay; 17% silt; 77% sand.

2.3 Experimental procedure

Chemicals were spiked into the pre moistened soil as aqueous solutions, in the single chemical exposure tests, each test concentration into the whole batch of soil.,in the binary mixture toxicity tests, chemicals were added to the soil one after the other and then homogeneously mixed. In the case of atrazine and lindane, these were solved in acetone and homogeneously mixed with the soil. 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, all soils were spiked with the same amount of acetone and 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). For single exposure tests with Zn, Cd and lindane, data was available from literature (Smit and Van Gestel 1996), (Van Gestel and Hensbergen 1997a), (Lock et al. 2002)) as can be depicted in table 1.

Concentrations used in the mixture toxicity experiment were as follows (given as mg chemical per kg soil (dry weight)): dimethoate (from 0.22 to 1.77), atrazine (from 5.91 to 47.3), lindane (from 0.02 to 0.189), zinc (from 43.5 to 348) and cadmium (from 7.22 to 57.8).

Test procedures followed the ISO guideline 11267 for toxicity testing with *Folsomia candida* (ISO 1999). Organisms were obtained from controlled cultures established for more than 5 years at the Department of Biology, University of Aveiro. Ten organisms of 10 to 12 days old were used per test container, containing the test soil (25gr dry weight per test vessel) plus food supplied (2 mg dried baker's yeast). Four replicates per treatment were used. Food was replenished at day 14 and water (based on weight loss) was added

weekly. After 4 weeks, the test ended and each test vessel was filled with distilled water, gently stirred with a spatula, causing floatation of the organisms. Through digital imaging and using appropriate software (SPSS 1997), adults and juveniles were automatically counted.

2.4 Test design

In the single exposure bioassays, five to six concentrations per chemical compound were used in four replicates. In the binary mixture experiments, individual and mixture exposures were carried out simultaneously so that responses could be controlled and also to correct for differences in organisms' responses due to sensitivity variations. Only one replicate per treatment level was used in order to allow covering as much as possible the exposure range of both chemicals. Therefore effort was devoted to the number of binary combinations at the expense of the number of replicates. This was possible because the response surface analysis carried out is based on a regression model and therefore it will not compromise experimental validation (Jonker et al. 2004, Jonker et al. 2005). Chemical mixture concentrations were based on the EC50 (concentration inducing a reproduction decrease of 50%) from the single exposure experiments previously performed. These EC50 values were used as a basis for calculating 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 ΣTU used for every mixture combination was ≤1 with the exception of two equitoxic mixtures of ΣTU= 1.5 and ΣTU= 2 (corresponding to 0.75+0.75 and 1+1 TUs, respectively). The experimental design was based on a fixed ray design.

2.5 Statistical analysis

ECx (Effect Concentration) and NOEC (No Observed Effect Concentration) values were determined for the single exposures to dimethoate and atrazine using the best fitting models (probit or two parameter logistic curve) (ToxRat® 2003) and p(Chi2) (goodness of fit measure) and p(F) (test for regression) are given (Table S2). From the mixture experiments, EC50/LC50 values were also calculated considering the trials carried out individually for each chemical, using a three-parameter logistic curve (equation 1) (SystatSoftwareInc 2002).

$$Y_i = \frac{\max}{1+\left(C_i / EC_{50i}\right)^{\beta_i}}$$

(equation 1)

Where the response of a given parameter (Y_i) at a concentration (C_i) of a chemical (i) can be calculated using the maximum response value (max) for that parameter, the EC_{50i} (for mortality replace with LC_{50i}), and the slope (β_i) for the chemical.

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. The two reference of models Concentration Addition (CA) and Independent Action (IA) were used to derive patterns for joint effects of mixtures(Loewe and Muischnek 1926, Bliss 1939). The CA model assumes that the mixed chemicals have the same mode of action (MoA), and consequently can be regarded as dilutions of one another. The Concentration Addition model (CA) is mathematically described by:

$$\sum_{i=1}^{n} \frac{Ci}{ECxi} \quad \text{(equation 2)}$$

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.

Therefore in this study we used the CA model for data obtained in the cadmium and zinc mixture, for effects on survival and reproduction endpoints.

The Independent Action model (IA) is mathematically described by:

$$Y = umax \prod_{i=1}^{n} qi(Ci)$$

(equation 3)

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, u*max* the control response for the selected endpoint and $\prod$ the multiplication function.

Both conceptual models were used for all mixture toxicity data and final patterns obtained from modelling were compared.

Deviations from these two reference models such as synergism/ antagonism, and dose-ratio and dose-level dependent deviations were obtained by additional parameters used in the

mathematical models that describe CA and IA, and tested within a nested framework [Table S1 and more details 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 [for details see Jonker et al (2005)]. 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 supplementary material (Table 1) and more details can be found in Jonker et al. (2005).

3. Results

3.1 Single toxicity

Results of the single toxicity tests with dimethoate and atrazine can be seen in Table 1. Further details are provided as supplementary information (Figure 1 and Table 2)

Table 1: Summary of 28-day effect concentrations (EC_{50}) in *Folsomia candida*, following the standard ISO guideline 11267 (ISO, 1999), for the toxicity of $ZnCl_2$, $CdCl_2$. lindane (from literature), dimethoate and atrazine (present study), which were used as a basis for the experimental setup of binary mixture toxicity bioassays. (CI =95% Confidence Interval).

Chemical substance	**EC_{50} (mg/kg)**	**Soil Type**	**Reference**
$ZnCl_2$	348 (307<CI<393)	LUFA 2.2	(Smit and VanGestel 1996)
$CdCl_2$	40 (25<CI<65)	OECD	(Van Gestel and Hensbergen 1997b)
Lindane	0.189	OECD	(Lock et al. 2002)
Dimethoate	1.6 (n.d.)	LUFA 2.2	Present study
Atrazine	43.2 (34-54)	LUFA 2.2	Present study

Both tests fulfilled the validity criteria of the test guideline regarding control survival and reproduction. Survival was not affected by atrazine and only significantly reduced at the highest dimethoate concentration; therefore no LC50 could be calculated. For atrazine exposure, reproduction showed a gradual dose-response within the tested concentration range, while for dimethoate it was only significantly reduced at the highest tested concentration. EC50 values were 43.2 and 1.6 mg/kg respectively.

In the mixture experimental setup, single exposures were also carried out and an EC50 value for reproduction calculated for each chemical. For cadmium, in the single exposures the obtained EC50 values were of 6.6mg/kg (Cd+Zn) and 46.4 mg/kg (Cd+dimethoate); zinc single toxicity showed EC50 values of 262 mg/kg in the experiment of Cd+Zn; for dimethoate, EC50 values obtained were of 1.3 mg/kg (Cd+dimethoate), 1.1 mg/kg (dimethoate+atrazine) and 0.5 mg/kg (dimethoate+lindane); in the combination of

dimethoate and atrazine, atrazine single exposure showed an EC50 value of 105 mg/kg and lindane showed an EC50 of 0.8 mg/kg when exposed singly in the experiment for dimethoate+lindane.

3.2 Binary mixture toxicity

After fitting our data to both CA and IA conceptual models, the output pattern was almost always similar, showing additivity (with CA or IA models) or deviations from the conceptual models (Table 2). In addition, the best fit obtained with CA or IA or deviations were also comparably similar to real/raw data, as it can be seen on Figures 1-4.

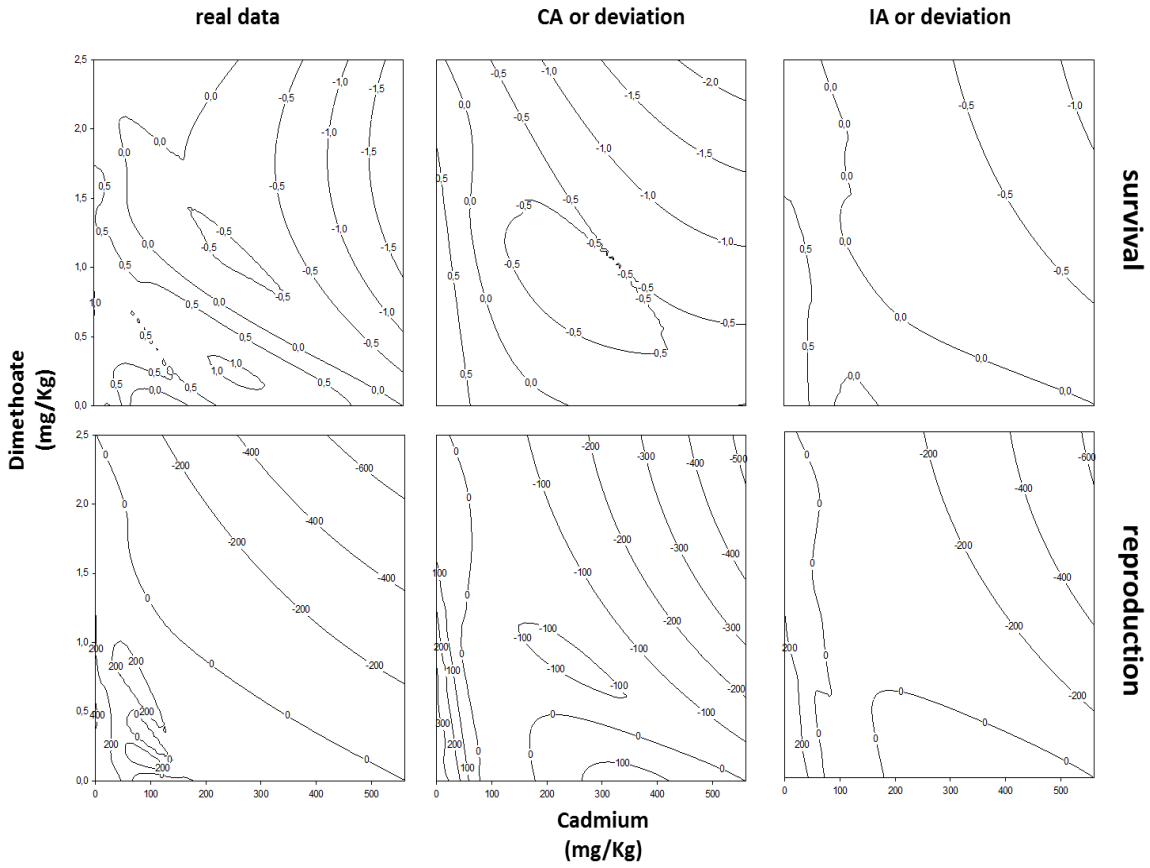

Figure 1: Isobolograms of the mixture of cadmium and dimethoate tested on the survival (top graphs) and reproduction (bottom graphs) of *Folsomia candida*. The graphs on the left represent real data; graphs located in the middle refer to responses where the best model fit was CA; graphs on the right side are related to modelled data with IA (survival) or deviations from the model to synergism (reproduction). For details on best model fit, please see Table 2.

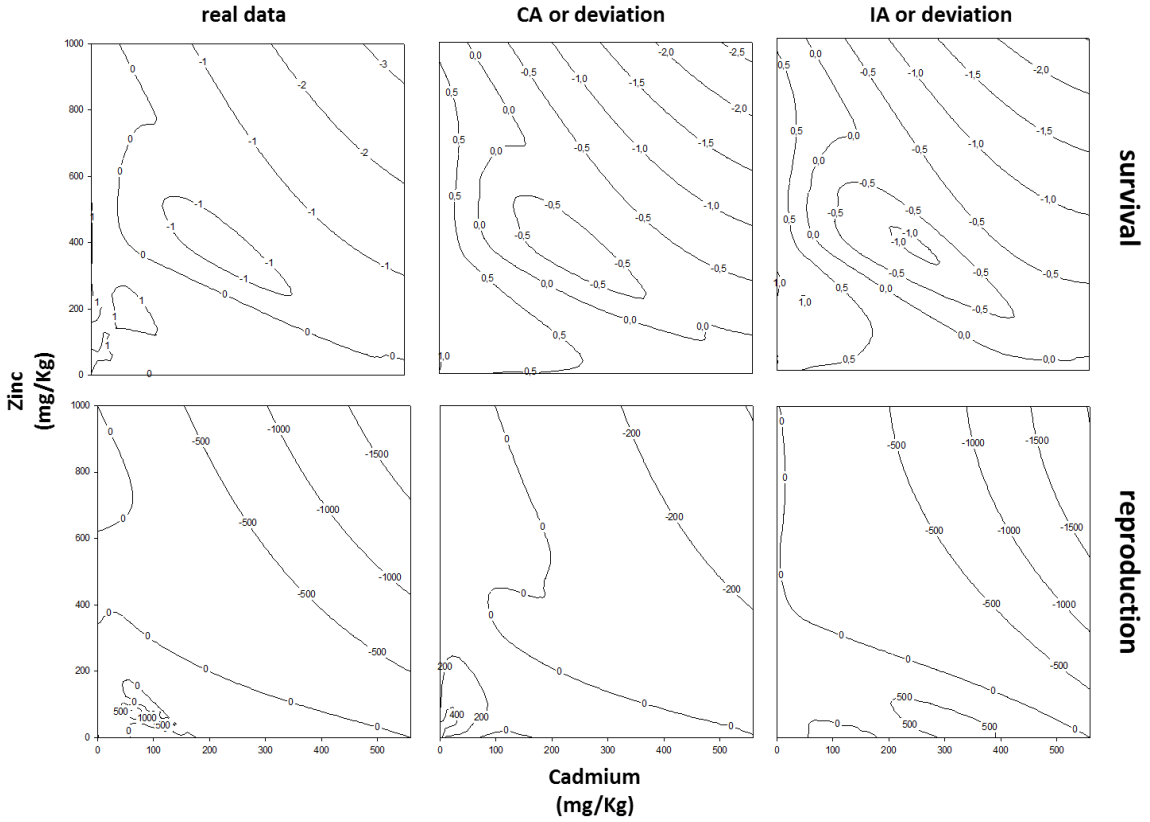

Figure 2: Isobolograms of the mixture of cadmium and zinc tested on the survival (top graphs) and reproduction (bottom graphs) of *Folsomia candida*. The graphs on the left represent real data; graphs located in the middle refer to responses showing dose level deviations after CA modelling ; graphs on the right side are related to modelled data with IA (reproduction) or deviations from the model to dose level (survival). For details on best model fit, please see Table 2.

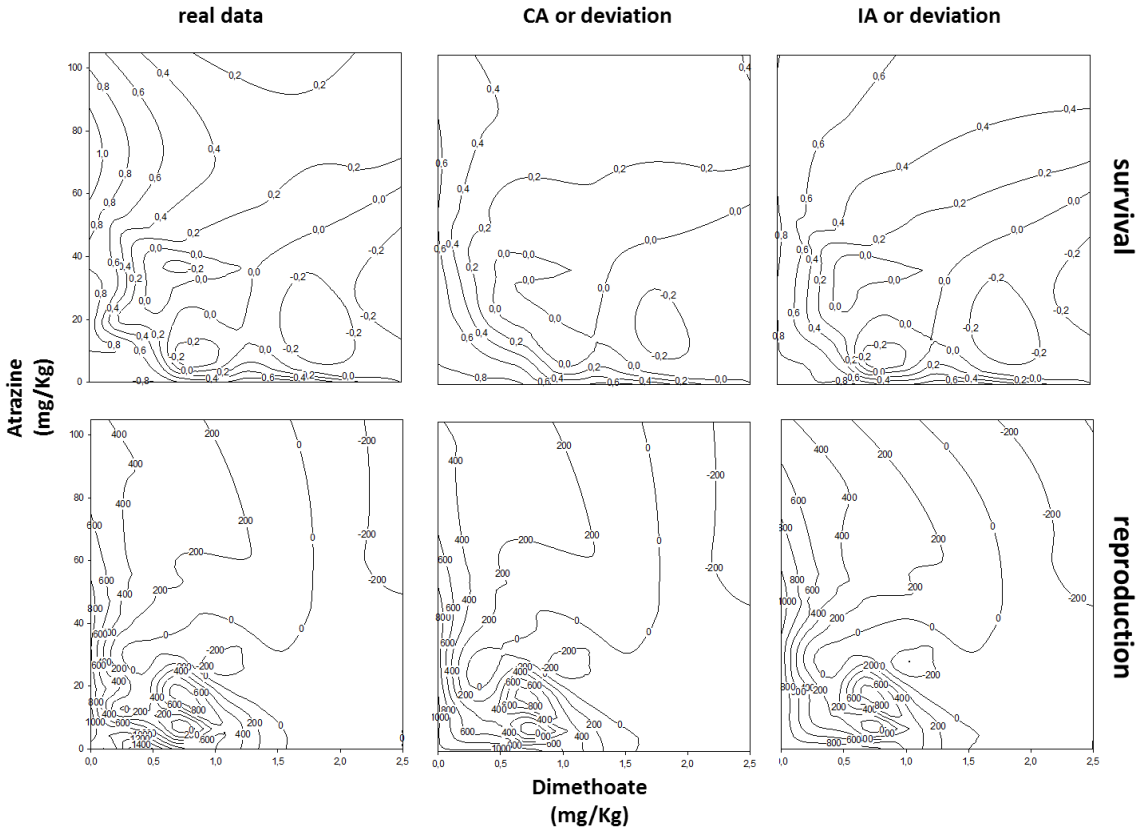

Figure 3: Isobolograms of the mixture of dimethoate and atrazine tested on the survival (top graphs) and reproduction (bottom graphs) of *Folsomia candida*. The graphs on the left represent real data; graphs located in the middle refer to responses showing deviations for dose level (survival) or dose ratio (reproduction) after CA modelling; graphs on the right side refer to responses showing deviations for dose ratio (survival) or dose level (reproduction) after IA modelling. For details on best model fit, please see Table 2.

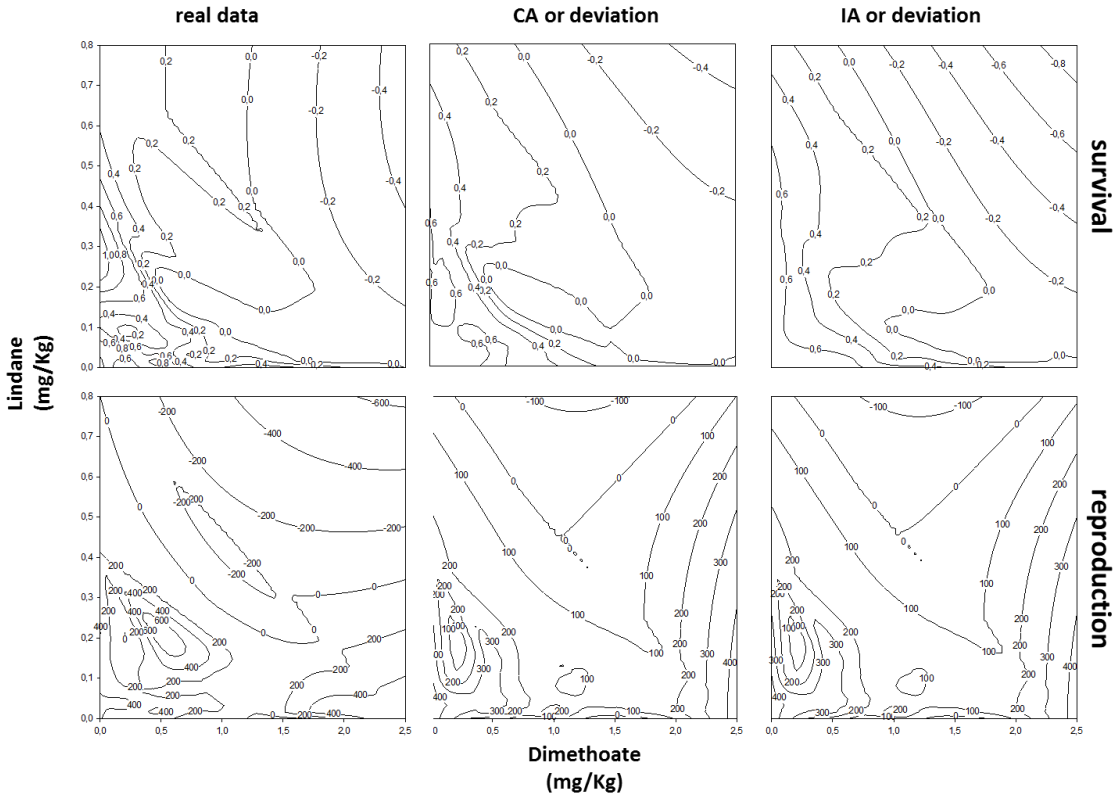

Figure 4: Isobolograms of the mixture of dimethoate and lindane tested on the survival (top graphs) and reproduction (bottom graphs) of *Folsomia candida*. The graphs on the left represent real data; graphs located in the middle refer to responses showing a synergistic deviation after CA modelling (survival) or additivity with CA (reproduction) ; graphs on the right side are related to modelled data with IA (reproduction) or deviations from the model to dose level (survival). For details on best model fit, please see Table 2.

Table 2: Summary of survival and reproduction responses of *Folsomia candida* exposed for 28 days to several chemical mixtures in LUFA 2.2 soil.

Mixture		Survival						Reproduction					
		SS	r^2	p (x^2)	a	b	pattern	SS	r^2	p (x^2)	a	b	pattern
Cd + dimethoate	IA	28.3	0.8				IA	$77x10^3$	0.8				
	deviation							$62x10^3$	0.9	$7x10^{-3}$	-4.2		synergism
	CA	30.8	0.8				CA	$68\ x10^3$	0.9				CA
Cd + Zn	IA	66.5	0.6					$3.8x10^5$	0.7				IA
	deviation	25.0	0.8	$1x10^{-9}$	1061	1.6	DL						
	CA	71.9	0.5					$5.6x10^5$	0.5				
	deviation	32.2	0.8	$2x10^{-9}$	53.7	1.5	DL	$3.2x10^5$	0.7	$5x10^{-4}$	21.7	0.04	DL
dimethoate + atrazine	IA	92.9	0.6					$21x10^5$	0.6				
	deviation	45.9	0.8	$6x10^{-11}$	-9	-11	DR	$74x10^4$	0.8	$2x10^{-7}$	-19	-46	DL
	CA	87.5	0.6					$14x10^5$	0.8				
	deviation	45.4	0.8	$7x10^{-10}$	-0.14	-91	DL	$7x10^5$	0.9	$5x10^{-5}$	-1.6	-4.2	DR
dimethoate + lindane	IA	45.7	0.7					$17x10^4$	0.8				IA
	deviation	30.5	0.8	$5x10^{-4}$	-12.9	-6.7	DL						
	CA	49.5	0.6					$17x10^4$	0.8				CA
	deviation	42.0	0.7	$6x10^{-3}$	-2.5		synergism						

r^2 is the coefficient of determination; a and b are parameters of the deviation functions (see Table S1, from supplementary material); p value indicates the significance of deviations found from the conceptual models; CA is concentration addition; IA is independent action; DR is dose-ratio deviation and DL is dose-level deviation from the reference.

The only two cases where this was not observed were on the effects on juvenile production for: Cd and dimethoate, where synergism was observed after IA modelling, and additivity with the CA conceptual model; and for Cd and Zn, where a dose level deviation from CA was observed and on the other hand additivity was attained with IA modelling (Table 2).
For cadmium and dimethoate exposure additivity was the best approach to describe the results obtained for adult survival (Table 2). The same additivity with CA modelling was observed for the juveniles produced, but an increase in toxicity, higher than expected, was observed after the IA model fit, showing therefore a synergistic pattern (Fig. 1 and Table 2).

Modelling responses from the cadmium and zinc combinations with the CA model revealed a significant dose-level dependent deviation ($p=5x10^{-4}$) for the number of juveniles produced (Fig. 2, middle bottom graph). In this case antagonism was observed at low concentrations but with a $b_{DL}<0$, changes for synergism did not occur on the range of the tested concentrations. On the other hand, with IA modelling, additivity was observed and no deviations were found to fit more significantly. Survival showed a pattern for dose level deviation, where antagonism was also observed at low concentrations, changing to synergism (i.e. increasing toxicity more than expected) at lower levels than the EC50 (after CA fit) or at higher levels than the EC50 (after IA fit) (Fig. 2, top graphs, Table 2).
Upon the combined exposures to dimethoate and atrazine, results from adult survival showed to be dose-ratio or dose level dependent (Fig. 3, top graphs). But regarding *a* (<0) and *b* (<0) values from the models, the overall pattern was synergism (Tables 2 and S1). For reproduction effects, deviations dependent on the chemical level or ratio were also observed, with synergism being the main pattern (Table 2 and S1).
For dimethoate and lindane exposure the additivity models (CA and IA) were the best descriptors of the effects on the number of juveniles produced (Fig. 4, bottom graphs, Table 2). On the other hand, a deviation from the IA model to dose-level for the effects on adult survival as can be concluded by the values for parameters *a* and *b* (Table 2), and in this case a<0 indicates that synergism was observed at low levels (concentrations) of both chemicals. This pattern might change to antagonism ($b_{DL}<1$) but only at higher levels than the ones used. This pattern for synergism is in accordance with the one found after fitting these results to the CA conceptual model (Fig. 4, top graphs).

4. Discussion

4.1 Single toxicity

Results from single toxicity tests showed that dimethoate and lindane were the most toxic compounds. This fact is hardly surprising given that both are insecticides, therefore highly effective in insects such as collembolans. Atrazine and Cd were in the same toxicity range with similar EC50s (tab. 1) and Zn was approx.10 times less toxic.

Regarding dimethoate toxicity results from the present study are also in accordance to the result of Santos et al. (2010b) when exposing *Folsomia candida* in a mixture toxicity experiment to the commercial formulation AGROR® (40% dimethoate). For AGROR® single exposures (during mixture experimental setup), the several EC50 values calculated were 0.05 (0.03<CI<0.07), 0.15 (0.08<CI<0.21) and 0.37 (0.19<CI<0.21) mg/kg, which are in the same range of the ones calculated here, although approximately 6 times lower. This might be related to the chemical purity used in the different studies where 99.8% pure dimethoate was used in our study while the formulation AGROR® of 400 g a.i. L^{-1} contains dimethoate (40%), cyclohexanone (28.4%), nonylphenol ethoxylate (2.2%), petroleum naphta (26.1%) and calcium alkyl benzene sulphonate inpropil 2-ol (0.4%).

For atrazine exposure, the reproduction test with *Folsomia candida* showed toxicity at the same level as the avoidance tests (individual and mixture) with *Porcellionides pruinosus* (EC50 of 153 and 333 mg/kg) and *Enchytraeus albidus* (EC50s of 38 and 18.2 mg/kg) (Loureiro et al. 2009).

Comparison of EC50 values between literature (Table 1) and the tests carried out in this study, confirms the reproducibility of the EC50 values from the mixture toxicity studies (e.g. Santos et al. 2010b). In the mixture toxicity approach, observed data are compared to the modelled one, and modelling is based on single exposure results. To assure reproducibility of results in mixture exposures, the similarity of results of single exposure effects within tests should be verified. Given this, the results of the single exposure can also be used as control responses. In the present study, this reproducibility was achieved and the mixture toxicity experimental setup should be considered reliable.

4.2 Binary mixture toxicity

In the presence study the approach of modelling all results with both conceptual models showed some coherence on the patterns found. In addition to the statistics and parameters obtained after modelling, graphically isoboles obtained after CA or IA modelling are very similar and comparable to real/raw data. Therefore discussion will be based on the overall pattern obtained.

The combined effects of Cd and Zn have been mainly described as following additive or antagonistic patterns (Loureiro et al. 2009, Zidar et al. 2009). In the study of Zidar et al. (2009), equitoxic mixtures of Cd and Zn were provided via food to the terrestrial isopod *Porcellio scaber* and additive effects were observed based on total and water extractable concentrations but antagonistic effects were found when considering internal metal concentrations in isopods. For *Enchytraeus albidus* and *Porcellionides pruinosus* in avoidance behaviour experiments to Cd and Zn, mainly additivity was found, based on the Concentration Addition model (Loureiro et al. 2009). In our study the exposure of *Folsomia candida* to Cd and Zn provided patterns of response that are mainly dose level dependent for both survival and number of juveniles produced. As a general trend, antagonism is observed at lower doses and synergism at higher doses. Recently a study from Xu et al. (2011), on the interactions of metalllic elements in sea urchin embryo and larvae in binary, ternary and quaternary mixture exposures, showed that exposures to the combination of Cd and Zn mainly lead to synergistic effects. But, when organisms were exposed to ternary or quaternary combinations where Cd and Zn were included, additivity and antagonism were the main tendencies observed. The antagonistic pattern observed in the present study might be due to the known inter-regulation of cadmium and zinc when present simultaneously. In a study of Demuynck et al (2007) where the earthworm *Eisenia fetida* was exposed to Cd and Zn, it was showed that Zn regulated Cd accumulation at low concentrations of Cd: the increase of cytosolic metallothionein levels regulated Zn concentrations in the worms and could limit Cd toxicity.

The combined effects of dimethoate and atrazine showed mainly synergistic patterns in our experiments. In the study of Pérez et al. (in press), several herbicide binary mixtures were tested in the green algae *Pseudokirchneriella subcapitata* and the main tendencies observed in mixtures with atrazine were also based on synergistic effects caused by this triazine herbicide. In addition, synergism was also the main pattern observed for the midge

Chironomus tentans upon exposure to atrazine and methyl-parathion, malathion, chlorpyrifos and trichlorfon (Lindstrom and Lydy 1997) which is in accordance with our study.

In previous studies with *Enchytraeus albidus* and *Porcellionides pruinosus*, where the same set of chemical mixtures used in this study were tested, the patterns were not coincident with the ones described here, showing antagonistic patterns for the pot worm and dose level deviations (antagonism at low concentrations and synergism at high concentrations) for the terrestrial isopod. Such differences could be due to species and endpoint specificity.

The effects caused by a 1:1 mixture of dimethoate and fenvalerate to acetylcholinesterase activity and reproductive traits of the spyder *Hylyphantes graminicola* were more than additive considering single toxicity exposures of both insecticides (Peng et al. 2010). This lead to a synergistic trend similar to the one obtained for reproduction of *F. candida* exposed to dimethoate and atrazine and the survival endpoint for dimethoate and lindane. Somehow these chemicals act in the nervous system of invertebrates, either by inhibiting AChE (dimethoate), or by interfering with sodium ion permeability in stimulated nerve membranes (fenvalerate), or by serving as antagonist of the GABA-gated chloride channel inducing also damages in the nervous system (lindane). In addition, atrazine's' mode of action is not well defined for animals but some studies have reported nervous system impairment. Therefore their modes of action are quite independent in terms of specific target, but as a final product they end up inducing similar responses. These responses do not follow additive pattern but a potentiation by for instance inducing transformation of chemicals in their more toxic analogues (Anderson and Lydy 2002). Therefore synergistic patterns seem to appear when atrazine is present in binary mixtures with several types of chemical compounds.

In the study of Santos et al. (2010b), *Folsomia candida* was also exposed to binary mixtures of dimethoate with glyphosate and spirodiclofen and effects on reproduction were evaluated. For the first case, dimethoate and glyphosate showed an antagonism trend after CA and IA modelling; for dimethoate and spirodiclofen, additivity was the tendency for both CA and IA models. In our study, an additive effect was observed for reproduction upon dimethoate and lindane exposure and for survival after cadmium and dimethoate

exposure. Antagonism was never observed in our study when dimethoate was present in the mixture.

In the study of Loureiro et al. (2009) the same binary mixtures with dimethoate were ran and patterns for effects were not consistent and could not derive a response trend. In fact, the mixture tests with cadmium and zinc and with dimethoate and atrazine showed similar trends to what can be found in the literature. As mentioned by Cedergreen and Streibig (2005), the choice of endpoints is crucial for mixture toxicity studies and different choices can lead to contradictory findings. This is also related to chemical MoA, i.e. one chemical can act in a way for one species but throughout a completely different process upon exposure to another one. This is easy to derive or assume when dealing with species from different Kingdom (e.g. plants vs animals), nevertheless when comparing species closely related one should not necessarily assume that chemicals act similarly (e.g. earthworms vs enchytraeids).

In addition, reproducibility of binary mixture studies can be sometimes difficult and repeating an experimental set up might end in different result patterns (Cedergreen et al. 2007). This has been shown by the same authors when comparing responses given by the aquatic plant *Lemna minor* and the terrestrial plant *Tripleurospermum inodorum* upon exposure to four herbicide mixtures (Cedergreen et al. 2007). The variation within and between experiments was different when testing both plants and it was two times higher for the terrestrial system when compared to the aquatic one. Therefore, more studies on mixture toxicity with several test-organisms and different endpoints should be carried out to evaluate why accuracy is or is not verified.

5. Conclusions

Differences on mixture toxicity response patterns in *Folsomia candida* were observed in the different endpoints used (survival and reproduction). The conceptual model used as starting point showed to be of less importance because the final pattern output was overall similar. Synergism was frequently observed upon exposure to pesticide mixtures (lindane and atrazine, and dimethoate and lindane) or when one of the components of the mixture was a pesticide (Cd and dimethoate, when the endpoint was reproduction).

Combining the literature review and our findings, atrazine might be considered a potential synergist in chemical mixtures. Additionally, different invertebrate species, *Folsomia candida*, *Enchytraeus albidus* and *Porcelionides pruinosus*, showed different response patterns to these same chemical combinations, confirming that for an adequate ecological risk assessment several groups of organisms and endpoints should be included.

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7. Supporting Information Available

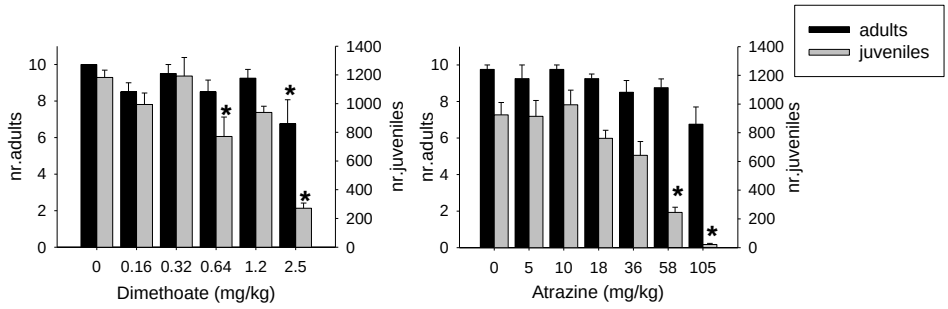

Figure S1: Results from the toxicity reproduction test with *Folsomia candida* exposed for four weeks to LUFA 2.2 soil spiked with dimethoate and atrazine. Survival (number of adults) and reproduction (number of juveniles) is shown as average + standard error.*Dunnett's; $p \leq 0.05$.

Table S1: 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 indicate synergism **a<0:** synergism except for those mixture ratios where positive b value indicate 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	b_{DL}**>1:** change at lower EC50 level	b_{DL}**>2:** change at lower EC50 level
		b_{DL}**=1:** change at EC50 level	b_{DL}**=2:** change at EC50 level
	a<0: synergism low dose level and antagonism high dose level	**0**<b_{DL}<**1:** change at higher EC50 level	**1**<b_{DL}<**2:** change at higher EC50 level
		b_{DL}<**0:** No change but the magnitude of S/A is DL dependent	b_{DL}<**1:** No change but the magnitude of S/A is effect level dependent

Table S2: Effect concentrations (ECx, with corresponding 95% confidence intervals) and No Observed Effect Concentrations (NOEC) for the effects of dimethoate and atrazin on the survival and reproduction of *Folsomia candida* after 4 weeks exposure in LUFA 2.2 soil. p(Chi2) = goodness of fit measure; p(F) = test for regression; n.d.: not determined due to mathematical reasons). EC50 for survival was exceeding the highest test concentration and is therefore not reported.

Parameter	**EC10** (mg/kg)	**EC20** (mg/kg)	**EC50** (mg/kg)	**NOEC** (mg/kg)	**p(Chi^2)**	**p(F)**	**Model Used**
Dimethoate							
survival	0.3 (n.d.)	1.6 (n.d.)	-	1.2	0.03	0.3	Probit
Reproduction	0.4 (n.d.)	0.7 (n.d.)	1.6 (n.d.)	0.64	0.99	0.2	2 parameters logistic
Atrazine							
survival	20.5 (7-35)	64.1 (37-202)	-	≥105	0.3	0.04	Probit
Reproduction	21.2 (7-29)	27.6 (13-35)	43.2 (34-54)	36	1	0.01	2 parameters logistic

This material is available free of charge via the internet at http://www.sciencedirect.com.

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CHAPTER II

DIMETHOATE AFFECTS CHOLINESTERASES IN *FOLSOMIA CANDIDA* AND THEIR LOCOMOTION – FALSE NEGATIVE RESULTS OF AN AVOIDANCE BEHAVIOUR TEST

Dimethoate affects cholinesterases in *Folsomia candida* and their locomotion - false negative results of an avoidance behaviour test

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Abstract:

The main mode of action of organophosphate insecticides is to inhibit acetylcholinesterase (AChE), which causes neuromuscular paralysis and uncoordinated movements. The collembolan *Folsomia candida* (Insecta) is a standardized test species with established guidelines to assess, among others, effects on avoidance behaviour. Being insects they represent potential targets of insecticides such as dimethoate, an anti-cholinesterase (anti-ChE) agent. In the present study we exposed *F. candida* to dimethoate and had 2 main aims: 1) to assess the ability of *F. candida* to avoid and 2) to assess its the effect on the cholinergic synapses and its possible link. For the latter several sub-steps were needed: a) to characterise the ChE forms, b) assess ChE activity *in vitro* and c) *in vivo.* Results showed that no avoidance occurred within the tested range (0-0.32-1-3.2-10-32 mg/kg). The main form is acetylcholinesterase. There was a significant decrease of ChE activities with an increase of dimethoate dose (IC_{50}=1.4mg/kg). A positive and significant correlation between AChE reduction and immobilization was found. This was confirmed with post-exposure video records that show clearly a behavioural effect of exposure: organisms exhibit lack of locomotion ability (immobilized). This observation also confirmed that the "attraction-like" behaviour of the organisms is an indirect effect of being unable to "walk-jump" back due to paralysis and not due to lack of avoiding "intention" itself. This constitutes a confounding factor in an avoidance behaviour test and consequent interpretation, which is not accounted for at present.

Keywords: cholinesterases characterisation; in vivo test; in vitro test;

1. Introduction

Avoidance behaviour towards certain environmental stressors has been known to occur in organisms. This represents an important ecological advantage, e.g. organisms in soil may escape exposure to toxicants given the patchiness characteristics of the media. Avoidance tests have been standardized for terrestrial invertebrates like earthworms and collembolans (ISO 2008, 2011).

Cholinesterase inhibition has been associated with alterations on the behaviour (e.g. locomotion) in several studies with vertebrates and invertebrates (e.g. (Vieira et al. 2009, Xuereb et al. 2009, Azevedo-Pereira et al. 2011), including some studies with organophosphate insecticides (Jensen et al. 1997, Engenheiro et al. 2005, Garcia-de la Parra et al. 2006).

Collembolans are apterygota insects which exist worldwide in various terrestrial environments. Often very abundant, have an important role in soil respiration and decomposition processes (Fountain and Hopkin 2005). In particular, *Folsomia candida* (Isotomidae) has been widely used in ecotoxicology, where standardized tests procedures are available for the assessment of effects on e.g. survival, reproduction and avoidance behaviour (ISO 1999, 2011).

Organophosphate pesticides are widely used in agriculture due to the high sensitivity of insects to these compounds. The main mode of action of these insecticides is the inhibition of acetylcholinesterase (AChE), a key enzyme in the nervous system that promotes the hydrolysis of acetylcholine in cholinergic synapses. Inhibition of AChE leads to an accumulation of the mentioned neurotransmitter, causing overstimulation of cholinergic receptors (Guilhermino et al. 1996, Payne et al. 1996) and ultimately neuromuscular paralysis and uncoordinated movements that can end in the organism's death (Purves et al. 2001, Howcroft et al. 2011). Dimethoate, one of the most used organophosphate insecticides in agricultural fields, has been shown to inhibit cholinesterases (ChEs) in e.g. fish (Frasco and Guilhermino 2002), freshwater shrimps (Kumar et al. 2010), chironomids (Domingues et al. 2007) and earthworms (Dell'Omo et al. 1999).

Being insects, *F. candida* represent potential targets of dimethoate application. This compound affects reproduction and survival at relatively low concentrations (EC_{50}=1.6 mg/kg) (Amorim et al. 2012). There was so far no study, to our knowledge, on its effect on the ChE activity, i.e. the specific target in insects.

Cholinesterases are a family of enzymes, traditionally divided in two classes in vertebrates based on their properties and functions: acetylcholinesterases (AChE) and pseudocholinesterase (PChE), also called butyrylcholinesterase. Contrary to AChE, PChE seems to have no function in cholinergic transmission and its role remains unclear. However, some authors suggest that since it binds to anti-cholinesterase agents, reducing the free amount of these substances in the body, PChE might have a protective role towards AChE (e.g. (Howcroft et al. 2011).

The function and forms of ChE present in many invertebrates are still unknown (Howcroft et al. 2011) [this included *F. candida*]. To assess the effects on this enzyme it is essential to first characterize the ChE forms which may show different sensitivities to anti-ChE agents. The criteria to distinguish AChE from PChE used in vertebrates can be used in invertebrates in most cases, i.e. using selective substrates and specific inhibitors (Massoulié et al. 1993, Rault et al. 2007). Acetylcholinesterase has a high affinity for acetylthiocholine (ATChE), being very sensitive to eserine and selectively inhibited by 1,5-bis-(4-allyldimethylammoniumphenyl)-pentan-3-one dibromide (BW284C51), while relatively insensitive to tetraisopropyl pyrophosphoramide (iso-OMPA) (Eto 1974). Pseudocholinesterases have higher affinity for propionylthiocholine (PTChE) or Butyrylthiocholine (BTChE), it is sensitive to eserine and selectively inhibited by iso-OMPA, while relatively insensitive to BW284C51 (Eto 1974).

In the present study we had two main aims: 1) to assess the ability of *F. candida* to avoid dimethoate and 2) to assess its effect on the cholinergic synapses. To assess the effect on the ChE several sub-steps were needed: a) investigate which ChE forms are present in *F. candid*a, b) ChE activities affected by *in vitro* exposure to dimethoate, c) *in vivo* exposure and effects and finally d) to investigate if there is a relation between avoidance and ChE activities.

2. Material and methods

2.1 Test organism

Cultures of *Folsomia candida* (Collembola) were maintained in laboratory on a moist substrate of plaster of Paris and activated charcoal (8:1 ratio) at 19 ºC, under a photoperiod regime of 16:8 (light:dark). Organisms were fed twice a week with dried baker's yeast (*Saccharomyces cerevisae*). Synchronized cultures were established for the experiments, stimulating egg laying by transferring adults into new breeding substrate. After 48 hours, the eggs were laid and the adults removed. The eggs hatched after approximately 12 days, and 10-12 days old juveniles were used for the avoidance test and the *in vivo* exposure. For cholinesterase (ChE) characterization and the *in vitro* exposure, adults with 26 days old were used, instead of juveniles, due to the high protein content requirements for measurement.

2.2 Test soil, test substance and spiking

The natural standard soil LUFA 2.2 was used. The properties of this soil can be summarized as follows: pH=5.5, organic matter=3.9%, texture=6% clay; 17% silt; 77% sand.

Test substance used was the organophosphate insecticide dimethoate (Sigma Aldrich, 99% purity). Soil spiking was performed using an aqueous solution of dimethoate into pre-moistened (20-40% of the water holding capacity (WHC)) soil batches for each test concentration. Final moisture content was 40-60% WHC. For the *in vitro* test dimethoate was dissolved in the reaction buffer.

2.3. Avoidance test procedures

Tests followed the standard avoidance behaviour guideline for collembolans (ISO, 2011). Soil was spiked in the following nominal concentrations: 0.32-1-3.2-10-32mg dimethoate/kg of soil. Avoidance behaviour tests were performed using the two-section system. Circular plastic boxes were used, along with a removable plastic wall dividing 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. Afterwards, the wall was gently removed and 20 juvenile organisms (10-12 days old) were left at the contact line of the soils. A dual control test was also performed. Five replicates per treatments were done. To reduce water loss by evaporation and to prevent organisms to escape, the test chambers were covered with a lid (containing small holes) and kept, for 48 h, at 20 ± 2 ºC and a photoperiod of 16:8 h (light-dark). At the end of the test period the divider was again inserted in the middle of the two soils and water was added to both sides simultaneously. Both flooded soils were then gently stirred with a spatula, causing the animals to float on the water surface and allowing counting. Missing organisms were considered dead.

2.4. ChE Characterization

Biological sample preparation

Four replicates were used, each containing 400 adult organisms (26 days old). Organisms were collected directly from the culture dishes, frozen in liquid nitrogen and stored at -80ºC until further analysis. Each sample was homogenized in potassium phosphate buffer (0.1 M, pH 7.2) and centrifuged for 10 min at 10000 g (4ºC) to isolate the post-mitochondrial supernatant (PMS).

ChE activity measurements

Enzymatic measurements were performed as described in Howcroft et al. (2011). Briefly, protein content was first measured for each PMS sample and dilutions were made to a final protein concentration of 0.7 mg/ml, minimum possible protein concentration that allows a efficient reaction and with that the use of less organism. The initial and final protein concentrations were determined according to the Bradford method (Bradford 1976), adapted from BioRad's Bradford microassay set up in a 96 well flat bottom plate, using bovine γ-globuline as a standard. Cholinesterase activity was determined in quadruplicate in the PMS of each diluted sample by the Ellman method (Ellman et al. 1961) adapted to microplate (Guilhermino et al. 1996). The absorbance was measured at 414nm during 5min (Thermo scientific, Multiskan Spectrum).

Substrates

The enzyme activity was determined in the four replicates, in quadruplicate, at 12 increasing concentrations from 0.01 to 20.48 mmol of the substrates acetylthiocholine iodide (ATCh), s-butyrylthiocholine iodide (BTCh) and propionylthiocholine iodide (PTCh). Cholinesterase activity was determined in the presence of these substrates with the different substrate concentrations dissolved in the reaction buffer. Blank reactions were made for each substrate concentration using the same volume of potassium-phosphate homogenization buffer (0.1 M, pH 7.2) instead of the sample.

Inhibitors

Eserine, tetraisopropyl pyrophosphoramide (iso-OMPA) and 1,5-bis-(4-allyldimethylammoniumphenyl)-pentan-3-one dibromide (BW284C51) were used as selective inhibitors of ChE, PChE and AChE, respectively. Inhibitors were tested in 6 increasing concentrations from 0.781 to 800 µM for eserine and BW284C51, and from 0.016 to 16 mM for iso-OMPA, dissolved in the reaction buffer. Cholinesterase activities of the four samples were measured in quadruplicate, using two different substrates: ATCh 0.075 M and PTCh 0.075 M. Blank reactions were made for each inhibitor concentration using the same volume of potassium-phosphate homogenization buffer (0.1 M, pH 7.2) instead of the sample. Controls without the inhibitors were also performed.

2.5. ChE effect assessment *in vitro*

The remaining PMS after the ChE characterization was used to determine the in vitro effects of dimethoate on ChE activity. This determination was done as described in 2.3, with dimethoate being dissolved in the reaction buffer (ATCh used as substrate) in order to obtain the final concentrations: 0.003, 0.006, 0.012, 0.024, 0.048, 0.0975, 0.195, 0.39, 0.78, 1.56, 3.12, 6.25, 12.5, 25, 50, 100, 200, 400, 800, 1600, 3200, 6400, 12800 µM. Blank reactions were made for each dimethoate concentration using the same volume of potassium-phosphate homogenization buffer (0.1 M, pH 7.2) instead of the sample. A control ChE activity determination, without dimethoate in the reaction buffer, was also performed.

2.6. ChE effect assessment *in vivo*

Exposure

Exposure of organisms was done according to the standardized procedures with few adaptations. In short, fifty (50) organisms with 10 to 12 days old were introduced in the test containers with the test soil (moistened to ca 50% of the maximum water holding capacity, WHC). Concentration range was 0-0.1-0.32-1mg/Kg (test 1) and 0-3.2-10mg/Kg (test 2). Exposure time was 48 hours. Five replicates per treatment were used. Test conditions were 20ºC and 16:8 light/dark photoperiod. At the end of the exposure period, organisms were recovered by flotation. The process consisted in pouring water into the test vessel, which was transferred into a 500ml glass beaker and gently stirred with a spatula. Using a teaspoon, organisms were recovered from the water surface to a recipient with a layer of mixed plaster of Paris and activated charcoal, absorbing the water. The organisms were then collected, placed in eppendorfs, frozen in liquid nitrogen and stored at -80ºC until further analysis.

ChE activity measurement

Organisms from each replicate were homogenized in 200 µl potassium-phosphate buffer (0.1 M, pH 7.2) and centrifuged for 10 min at 10000 g (4ºC). Cholinesterase activity was determined as described in 2.3 with the substrates ATCh and PTCh (for *test 2* only ATCh was used as substrate).

2.7. Data analysis

The avoidance results were expressed as the percentage of collembolans that avoided the treated soil in relation to the total number of collembolan in that container. The avoidance response (A) was calculated according to following equation:

$$A = ((C - T)/N) \times 100$$

Where C = number of collembolans observed in the control soil; T = number of collembolans observed in the test soil; N = total number of springtails per replicate. A positive A indicates avoidance and a negative A indicates a non-response (or attraction) to the chemical, being considered as no avoidance response. The habitat function of soils is

considered to be limited if, on average, more than 70% of springtails avoid the test soil and are found in the control soil (indication of an impact on behaviour) (ISO 2011).

Data for substrate affinity were analyzed by fitting experimental curves using the Michaelis–Menten equation, in order to determine the ChE kinetic parameters: maximal velocity (Vmax), Michaelis–Menten constant (Km) and their ratio (Vmax/Km) that indicates the catalytic efficiency of the enzyme (SPSS 1997).

One way analysis of variance (ANOVA) was performed to assess differences between treatments. The post hoc Dunnett's method for multiple comparisons was used to discriminate statistical significant differences relatively to the control group, using 95% confidence level. The data of in vitro ChE activity was square root transformed for normal distribution and homogeneity of variance. The inhibition concentration (IC_x) was calculated using a 4 parameter logistic regression model. For the *in vivo* results, the pool of the AChE controls from test 1 and 2 was used for the calculation. Pearson correlation test was used to calculate correlation between ChE activity and avoidance behaviour.

3. Results

3.1. ChE characterization

Substrates

The highest rate of substrate hydrolysis was obtained with acetylthiocholine iodide (ATCh), followed by propionylthiocholine iodide (PTCh) and s-butyrylthiocholine iodide (BTCh) (Fig. 1).

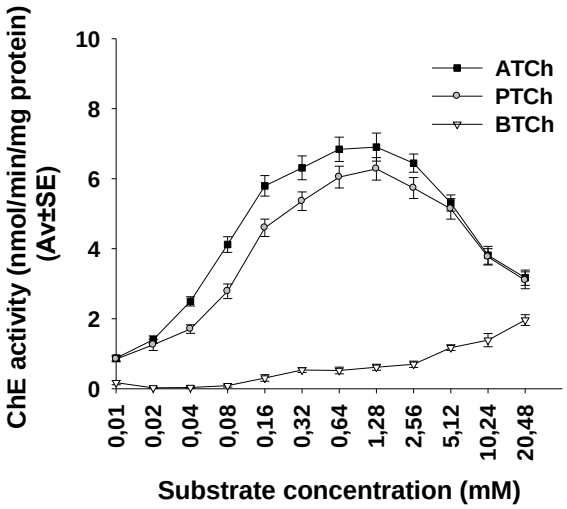

Figure 1: Results for the cholinesterase substrate preferences in *Folsomia candida.* Cholinesterase activity is expressed as mean values ± standard error (Av±SE).

The highest cholinesterase (ChE) activity value was measured at 1.28 mmol of ATCh (6.9 ± 0.4 (Av±SE) nmol/min/mg protein), after which there was a reduction of activity both with ATCh and PTCh as substrates. These results show that there was a ChE inhibition by excess of both ATCh and PTCh substrates. Cholinesterase kinetic parameters for each of the substrates used, Michaelis–Menten constant (Km) and maximal velocity (Vmax) are presented in Table 1.

Table 1: Values of the Michaelis–Menten constant (Km), maximal velocity (Vmax) and the catalytic efficiency of *Folsomia candida* ChEs' (ratio Km/Vmax) for the three tested substrates.

Substrate	**Vmax (nmol/min/mg protein)**	**Km (mM)**	**Vmax/Km (µl/min/mg protein)**
Acetylthiocholine (ATCh)	5,7 ± 0,16	0,03 ± 0,01	167,1
Propionylthiocoline (PTCh)	5,2 ± 0,15	0,05 ± 0,01	107,2
Butyrylthiocholine (BTCh)	2,1 ± 0,14	3,29 ± 0,66	0,6

The ratio Km/Vmax indicates the enzymatic catalytic efficiency and the enzyme affinity, i.e. the following preference order was observed: ATCh (167.1) > PTCh (107.2) > BTCh (0.6).

Inhibitors

The results on the cholinesterases activities obtained with the addition of inhibitors, in the presence of ATCh and PTCh as substrates can be seen in figure 2.

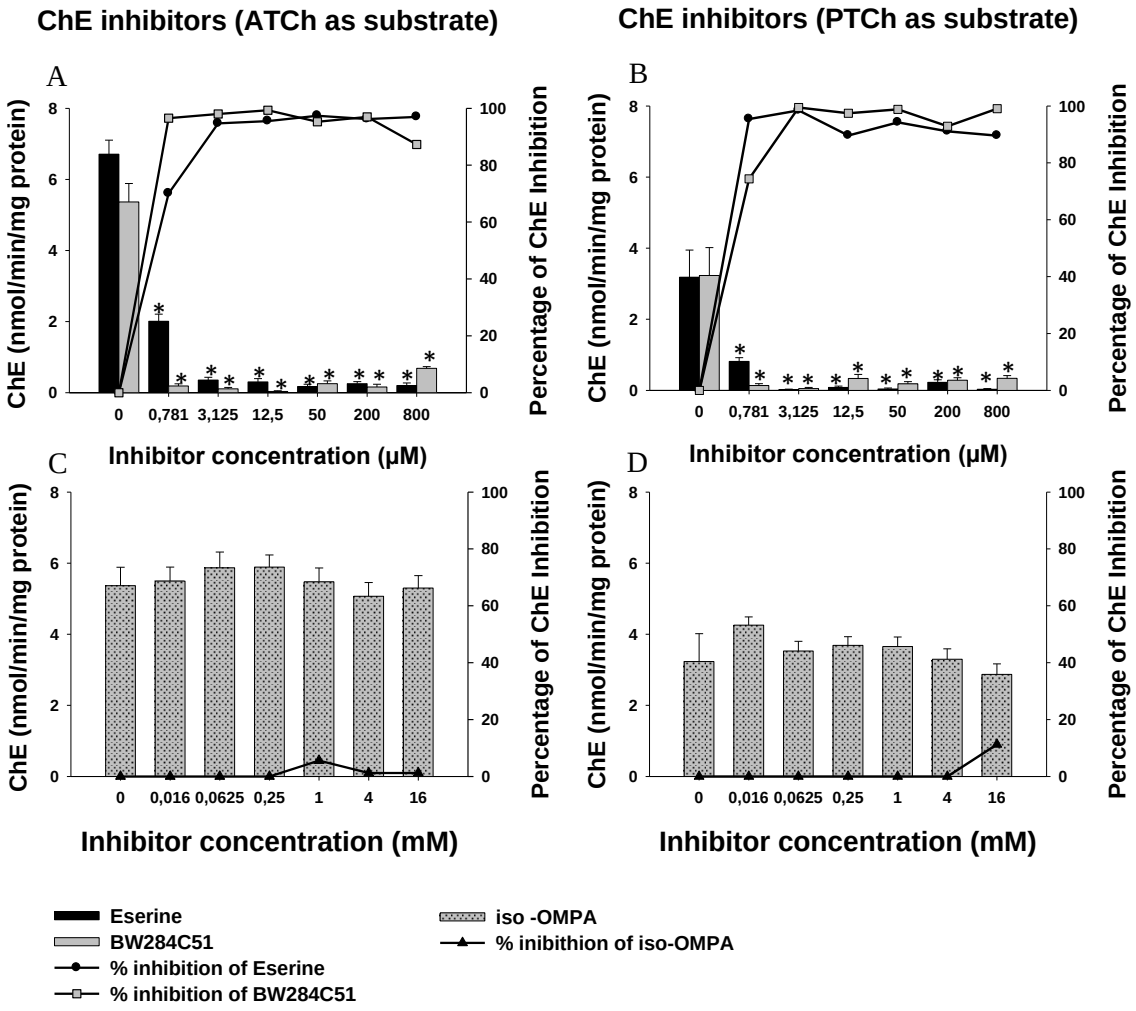

Figure 2: Effects of the inhibitors eserine (black bars – A, B), BW284C51 (grey bars – A, B) and iso-OMPA (grey dotted bars – C, D), on *Folsomia candida* ChE activities (expressed as mean values ± standard error) with acetylthiocholine (ATCh – left side) or propionilthiocholine (PTCh – right side) as substrates. Bars correspond to ChE activities and lines correspond to the percentage of ChE inhibition (asterisk indicates statistically significant differences, Dunnett's test $p < 0.05$).

The ChE activity was almost completely inhibited by eserine**,** with significant differences already with the first inhibitor concentration tested (0.781 µM) where 70% and 95% inhibition was observed with the substrates ATCh and PTCh, respectively (Fig. 2 – A, B). BW284C51 decreased ChE activity, showing significant differences also with the first inhibitor concentration tested (0.781 µM) with 97% and 74% of inhibition with the substrates ATCh and PTCh, respectively (Fig. 2 – A, B). No significant inhibition of the

ChE activity occurred in the presence of tetraisopropyl pyrophosphoramide (iso-OMPA), with either substrates, even at higher concentrations of this inhibitor (Fig. 2 – C, D).

3.2. ChE activity *measurements*

In vitro

Values of in vitro ChE activity in the PMS, measured in adults, were around 7.0 nmol/min/mg protein (ATCh as substrate). A significant decrease in the ChE activity was observed at concentrations of dimethoate of 800 µM (Fig. 3).

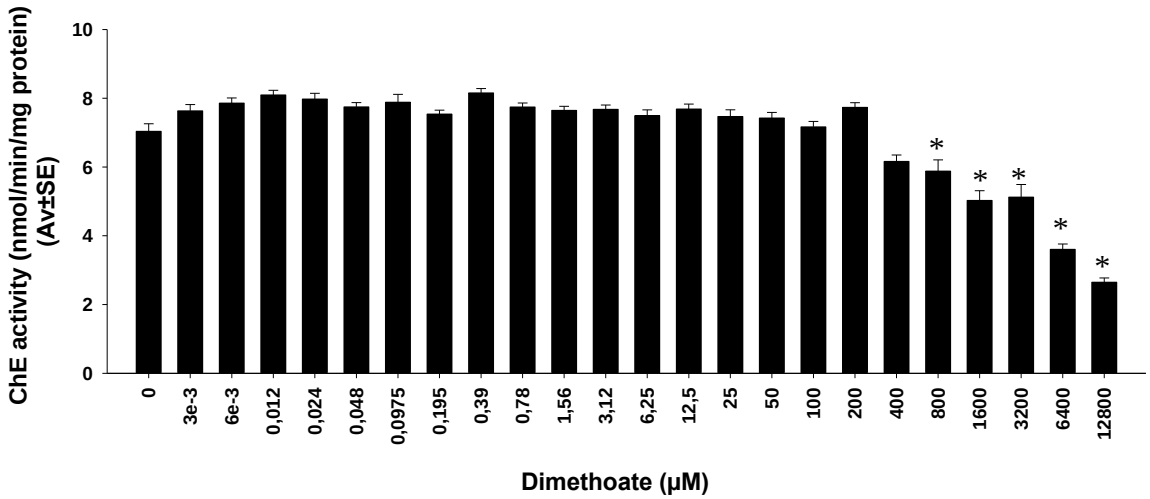

Figure 3: Cholinesterases activity expressed as mean values (± standard error) in *Folsomia candida* exposed *in vitro* to dimethoate (* Dunnett's test, $p < 0.05$).

The inhibition concentration that reduce in 50 % (IC_{50}) ChE activity was 7205µM (99% Confidence limits: 5792 – 9177) and no total ChE inhibition was observed even at 12800 µM.

In vivo

Values of *in vivo* ChE activity in the post-mitochondrial supernatant of control juveniles were around 25.4 (and 32.7 in *test 2*) and 21.5 nmol/min/mg protein with ATCh and PTCh as substrates, respectively (Fig. 4). A higher ChE activity was observed when ATCh was used as substrate, in all treatments.

Cholinesterases activity in *F. candida* exposed to dimethoate for 48 hours showed similar activities within the tested concentrations (0-1 mg/Kg) and with both ATCh and PTCh as substrates, (Fig. 4 – *test 1*). Because affinity for both substrates was similar and higher for ATCh, in test 2 only AChE was measured. At the higher dimethoate concentrations tested (*test 2*), significant decreases in the ChE activity were observed. At concentrations 3.2 and 10 mg/Kg of dimethoate, comparing with the control treatment, 90% and 97% of ChE inhibition was observed, respectively (Fig. 4). The IC_{50} value determined for the *in vivo* exposure to dimethoate was 1.4mg/Kg (6.2 µM (99% Confidence limits: not determinate).

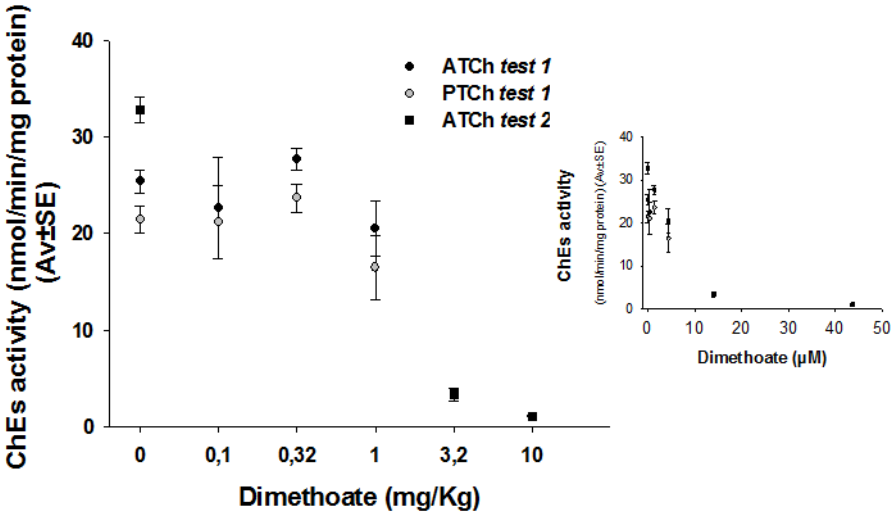

Figure 4: Results of cholinesterase activity expressed as mean values (±standard error) of *Folsomia candida* when exposed to dimethoate for 48 h in soil LUFA 2.2 (* Dunnett's test, $p < 0.05$). Test 1 was represented by black circles when the substrate ATCh was used and grey circles for the substrate PTCh. Test 2 was represented by black squares. (Graph on the right side is presenting concentrations in terms of µM and in linear scale).

Additionally, video record is provided for observation of the organisms behaviour (supplemental material): at test concentrations of 10 mg/kg collected animals were clearly immobilized, laying sideways with discoordinated movement. At 3.2mg/kg, the same effect was observed but in less organisms.

3.3. Avoidance test

Test validity criteria were fulfilled, i.e. less than 20% mortality per treatment. In the dual control test, no significant difference was observed in the distribution of the organisms between the two sides of the containers. No avoidance response was observed in any of the treatments and even an increasing "attraction" was observed with increasing concentration (Fig. 5).

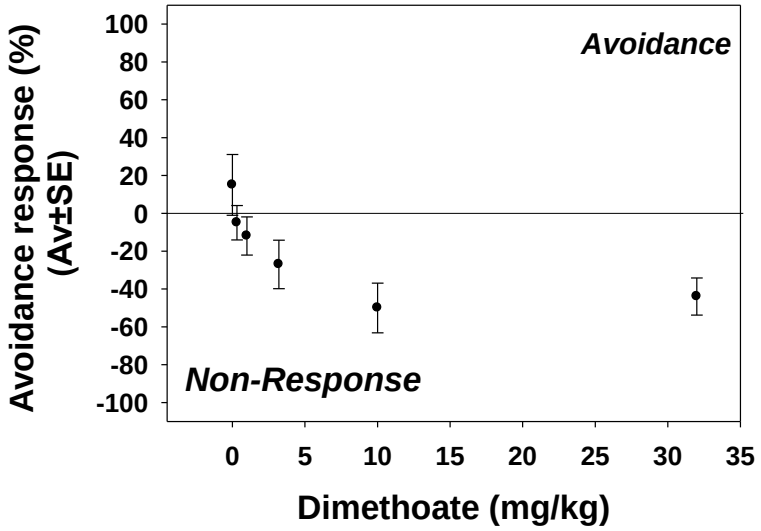

Figure 5: Results of the avoidance test with *Folsomia candida* when exposed to dimethoate in LUFA 2.2 soil. Avoidance responses are expressed as mean values ± standard error.

Additionally, as organisms were collected at test end, we observed that at 10mg/kg they were clearly immobilized (see section 3.2 description and supplemental material).

A significant correlation (Pearson Product Moment Correlation) between ChE activity and avoidance was observed: r = 0,586, p = 0,003. Decreasing levels of ChE activity

corresponded to increasing non-avoidance response, i.e., increasing attraction to the spiked soil.

4. Discussion

ChE characterisation

Results indicated that the enzymatic activity measured was mainly due to cholinesterases (ChEs) and not to other esterases (97% and 90% inhibition by eserine with the substrates acetylthiocholine iodide (ATCh) and propionylthiocholine iodide (PTCh), respectively). AChE is known to typically hydrolyse the substrate ATCh at a higher rate than PTCh and to a very low rate the substrate s-butyrylthiocholine iodide. In insects, e.g. the cockroach *Periplaneta americana* (Edwards 1980) and the carabid beetle *Pterostichus cupreus* (Jensen et al. 1997), as observed here for *F. candida* (Fig. 1, Table 1), they also contain ChEs with characteristics of typical AChE.

ChEs of several invertebrates such as the crustacean *Daphnia magna* (Diamantino et al. 2003) or the oligochaeta species *Allolobophora chlorotica* (Rault et al. 2007) and *Enchytraeus albidus* (Howcroft et al. 2011) have been shown to display mixed properties of acetylcholinesterase (AChE) and pseudocholinesterase (PChE). Studies on other invertebrates like the crustacean *Porcellionides pruinosus* or the earthworm *Eisenia andrei* showed only one form, AChE (Caselli et al. 2006, Ferreira et al. 2010).

The catalytic efficiency (ratio Km/Vmax) of ChEs present in *F. candida* showed the enzyme preference for the ATCh substrate. Moreover, an inhibition by excess of substrate was verified at concentrations higher than 1,28 mmol with both substrates ATCh and PTCh (Fig. 1), matching another characteristic of typical AChE – increased activities at low substrate concentrations and inhibited at higher concentrations (Toutant 1989). This AChE enzymatic kinetics behaviour of inhibition by excess of substrate has been reported in vertebrates (Garcia et al. 2000, Monteiro et al. 2005) and in some invertebrate species as well, like *Drosophila melanogaster* and *Caenorhabditis elegans* (Marcel et al. 1998). Also, as typical AChE, *F. candida* ChEs' were highly sensitive to BW284C5, a selective inhibitor of AChE, with 97% of inhibition even at the lowest concentration of 0.781 µM (Fig. 2 – A, B), as well as insensitive to iso-OMPA, a selective inhibitor of PChE (Fig. 2 – C, D) with less than 15% reduction in activity even at the very high concentration of 16

mM. Therefore, according with these results, the soluble post-mitochondrial fraction of *F. candida* seems to contain mainly AChE, being able to hydrolyze both substrates ATCh and PTCh.

Cholinesterases activity values extracted from control adults (*in vitro*) and juveniles (*in vivo*) were different, suggesting that it is dependent on their developmental stage. Adults (26 days old) had ChE activity in the range of 7 nmol/min/mg protein (±0.21SE), whereas juveniles (10-12 days old) had 25 nmol/min/mg protein (±1.2SE), a three-fold difference. A similar pattern was seen in the fish species *Pleuronectes vetulus* where older/larger fish had lower catalytic activities than younger/smaller fish, with ChE activities being significantly correlated with fish length (Rodríguez-Fuentes et al. 2008). One cannot completely exclude the existence of different ChE compositions in *F. candida* juveniles and adults. Nevertheless, given the fact that in test 1 both AChE and PThE, were measured in the juveniles (Fig. 4) indicated that likely the same ChE must be present at both life stages.

Effects of dimethoate on ChEs *in vitro* caused inhibition only at considerably high concentrations and no total inhibition (LOEC=800 µmol, IC_{50}=7205µM) (Fig. 3). This difference when compared to the in vivo effects (IC_{50}=6.2µM) is not surprising given that dimethoate inhibits ChE after metabolisation (IPCS 1989), which occur *in vivo* exposure conditions. This is in agreement with results of the study by Mdegela et al. (2010) with the catfish *Clarias gariepinus*, where *in vitro* effects of dimethoate showed lower ChE inhibition than other organophosphate insecticides like diazinon or fenitrothion which inhibited ChE activities at much lower concentrations than dimethoate (1000 times lower), under the same incubation periods (Mdegela et al. 2010).

Avoidance and ChE in vivo

F. candida was not able to avoid the soil spiked with dimethoate, actually an "attraction-like" was observed in all concentrations tested. The tested range is known to cause severe effects on their reproduction, hence populations in the field will likely be very affected given the limited ability to escape. To notice that the reproduction EC_{50} is 1.6 mg/Kg (Amorim et al. 2012), very similar to the obtained ChE IC_{50} in vivo in the present study

(1.4 mg/Kg). It would be too naive to link these two effects but there may be a relation in the case of this particular chemical and group of organisms.

The significant inhibition of ChE activity started to occur at 3.2 mg/kg. It is known that this inhibition leads to the overstimulation of cholinergic receptors and ultimately neuromuscular paralysis and uncoordinated movements, as shown via video record (supplemental info). Hence this could explain the incapacity to move away from the spiked soil once the animals "step" on it. But this inability to avoid was already occurring < 1mg/kg i.e., before ChE levels were significantly affected. This could mean that either minute decreases in ChE cause this or that we are still under normal unaffected behaviour.

Several studies have reported relationships between alteration in behaviour and inhibition of ChE activity (Garcia-de la Parra et al. 2006, Vieira et al. 2009, Xuereb et al. 2009, Kumar et al. 2010), namely with dimethoate exposures (Jensen et al. 1997, Engenheiro et al. 2005). In these latter mentioned studies, significant correlations were found between AChE inhibition and alterations in locomotor behavior of the isopod *Porcellio dilatatus* (Engenheiro et al. 2005) and the carabid beetle *Pterostichus cupreus* (Jensen et al. 1997). Similarly, in our study we in fact observed the immobilisation of the organisms, hence the inability to avoid was an indirect effect. A previous study with *Folsomia candida* had indicated a decrease in velocity and coordination in locomotion behaviour with increase in exposure time (up to 48h) to dimethoate (Petersen and Gjelstrup 1998). Very interestingly, the authors highlighted the fact that the total walked distance would be a better estimate of effect [which integrates the reduced speed] than the occurrence in a particular side of the test arena, as it is presently used in an avoidance test. In a study with *Folsomia fimetaria* a decline of locomotion behaviour was also observed with increasing dimethoate concentrations (Petersen and Gjelstrup 1998). In that study, authors observed that with increasing concentrations of dimethoate the organisms gradually lost their mobility. The authors described that [via continuous observations] the organisms were for example turning or backing when passing the borderline to the treated soil and that the gas phase of dimethoate can have an effect on the behaviour.

In our study, we inferred that organisms were virtually able to avoid (at least at 0.32-1mg/kg) but the effect on locomotion prevails over avoidance. This was confirmed to be mainly related with AChE inhibition.

5. Conclusions

F. candida contains mainly a typical acetylcholinesterase form, able to hydrolyze both substrates acetylthiocholine iodide and propionylthiocholine iodide. Dimethoate (*in vitro* exposure) is not a very strong inhibitor of the ChE activity in *F. candida* at least when short incubation periods are applied. Organisms were not able to avoid the spiked soil, showing signals of paralysis with increasing concentrations. This was correlated with the decrease in the cholinesterases activities. Because e.g. reproduction EC50 is 1.6mg/kg, dimethoate poses a serious ecological problem in field populations as they will not escape from contaminated to clean soil patches.

Observations indicated that organisms were virtually able to avoid (at least at 0.32-1mg dimethoate / kg) but the effect on locomotion prevailed over avoidance. This constitutes a confounding factor in an avoidance behaviour test and consequent interpretation which is not accounted for at present.

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