Universidade de Aveiro Departamento de Biologia
2018

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MEmO: Exposição Multigeracional de organismos em Ecotoxicologia – efeitos, mecanismos e implicações

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Tese apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Doutor em Biologia, realizada sob a orientação científica da Doutora Mónica Amorim, Investigadora Principal do Departamento de Biologia da Universidade de Aveiro, e do Doutor Jörg Römbke, Diretor Geral da ECT Oekotoxikologie GmbH, Alemanha.

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Dedico este trabalho à Ângela e à minha família pelo apoio.

o júri

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palavras-chave

Estádios de vida, longa duração, resposta transgeracional, ciclo de vida, ecotoxicologia de solo, abordagem multiparamétrica, colêmbolos, evitamento, sobrevivência, reprodução, tamanho, biomarcadores, toxicidade de misturas.

resumo

Os poluentes antropogénicos são continuamente libertados para o meio ambiente, o que pode resultar numa exposição de longa duração para os organismos do solo. Atualmente, as normas padrão visam a avaliação de efeitos em apenas um estádio de vida, geralmente juvenis, durante um determinado período de tempo. Além disso, estes métodos avaliam os efeitos nocivos destes compostos p.e. na sobrevivência, reprodução e comportamento de evitamento dos organismos. Os resultados obtidos com estes parâmetros, mesmo quando combinados, podem potencialmente sub/sobrestimar as consequências para a fauna do solo. Assim, o principal objetivo desta tese foi desenvolver e explorar diferentes metodologias para avaliar os efeitos de poluentes, especificamente usando diferentes estádios de vida do organismo modelo ecotoxicológico *Folsomia candida* e a exposição multigeracional. Além disso, objetivou-se integrar uma abordagem multiparamétrica, comparando a sensibilidade dos parâmetros propostos pelos métodos padrão, com outros já testados.

A avaliação dos efeitos de um conhecido e bastante estudado metal, o cádmio, em diferentes estágios de vida da *Folsomia candida*, forneceu novas e valiosas informações para perceber como estes organismos são afetados. O cádmio diminuiu a reprodução após a exposição de adultos, contudo não foram observados efeitos ao nível da eclosão, sobrevivência e reprodução quando os organismos foram expostos a partir de ovos. Assim, os efeitos dos contaminantes podem causar impactos diferentes dependendo da idade dos organismos. Além disso, uma avaliação de diferentes parâmetros permite conclusões mais detalhadas. Após a avaliação do modelo de concentração-adição (CA) para prever a toxicidade de uma mistura (produto biocida) na reprodução e evitamento, dois resultados distintos foram obtidos. Enquanto que o modelo foi capaz de prever os efeitos na reprodução, subestimou fortemente o impacto no evitamento.
A avaliação do impacto dos poluentes após exposição multigeracional mostrou ter consequências imprevisíveis ao longo das gerações. Enquanto que o impacto do fármaco antiparasitário ivermectina na sobrevivência e reprodução de *F. candida* foi similar nas três gerações testadas, o tamanho dos organismos diminuiu. Efeitos no tamanho foram também observados após exposição ao inseticida teflubenzuron, além de uma diminuição na sobrevivência e reprodução com o aumento do tempo de exposição, isto é, ao longo das gerações. Dado que o tamanho é essencial para a capacidade reprodutiva, a continuidade das populações pode estar em risco se estiverem expostas durante longos períodos de tempo. Além disso, foram obtidos diferentes resultados de marcadores celulares e bioquímicos entre gerações, o que contribuiu para a compreensão dos efeitos e mecanismos envolvidos após uma longa exposição.

Esta tese demonstra que as normas atuais podem ser melhoradas com a inclusão de novos parâmetros aos que são requeridos atualmente ou considerados padrão.
A abordagem multiparamétrica usada neste trabalho, que incorporou a medição do tamanho e avaliação de biomarcadores, em combinação com os parâmetros padrão, tais como a sobrevivência, reprodução e evitamento, mostrou a importância da inclusão de uma abordagem mais integrativa no quadro atual de avaliação de risco.

keywords

Life stage, long-term, transgenerational response, full life cycle, soil ecotoxicology, multi-endpoint-approach, collembolans, avoidance behaviour, survival, reproduction, size, biomarkers, mixture toxicity.

abstract

Anthropogenic pollutants are continuously released into the environment, which can result in long term exposure to soil organisms. Currently, standard guidelines focus on the assessment of effects to only one life stage, mostly juveniles, during a fixed exposure time. Additionally, these methods evaluate harmful effects of compounds to e.g. organism's survival, reproduction and avoidance. Results obtained when testing these endpoints, even when combined, can under-/over-estimate potential damage to soil fauna. Therefore, the main aim of this thesis was to develop and explore different methodologies to assess the effects of pollutants, namely using different life stages of the soil ecotoxicological model species *Folsomia candida* and multigenerational exposure. Moreover, it was aimed to integrate a multi-endpoint approach, by comparing the sensitivity of the endpoints proposed in the standard methods, with additional tested ones.

The assessment of the effects of a well-known and studied metal, cadmium, to different life stages of *F. candida* provided new and valuable information to understand how these organisms are affected. Cadmium decreased reproduction after exposure of adults, while no effect on hatching, survival and reproduction was observed when organisms were exposed from eggs. Therefore, effects of contaminants can cause different impact depending on the organism's age. Also, an assessment of different endpoints may result in more detailed conclusions. After evaluation the concentration addition (CA) model to predict the toxicity of a mixture (biocidal product), both to assess reproduction and avoidance behaviour, two distinct results were obtained. While the model was able to predict effects on reproduction, it strongly underestimated the impact on avoidance.
The evaluation of the impact of pollutants after multigenerational exposure showed to have an unpredictable impact over the generations. While the impact of ivermectin (veterinary product) to survival and reproduction of *F. candida* was similar in all three tested generations, the size of the organisms decreased. Effects on size were also observed after exposure to an insect growth regulator – teflubenzuron, in addition to a decrease in survival and reproduction with increasing time of exposure, i.e. along generations. Since size has a crucial role to reproduction, the continuity of the population may be at risk if exposed during long periods of time. Also, different results from cellular and biochemical markers were obtained across generations, which contributed to the understanding of the effects and mechanisms involved after long term exposure.

This thesis shows that the present guidelines can be improved by the incorporation of new parameters in addition to the currently required or standard endpoints.
The multi-endpoint approach used in this work, which incorporated measurement of size and evaluation of cellular and biochemical markers, in combination with standard endpoints such as survival, reproduction and avoidance, showed the added value of the inclusion of a more integrative approach to the current risk assessment framework.

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1. GENERAL INTRODUCTION

The concentrations of chemicals are increasing in all environmental compartments due to human activities.

The toxic effects of pollutants to the organisms can be defined as an alteration in their physiology, when comparing to control conditions (Kooijman et al., 2007), which may lead to consequences on the population and community level.

Ecotoxicology studies the harmful effects of these pollutants on biota by combining approaches from chemistry, ecology, and toxicology (Ardestani et al., 2014). To achieve the main aim of protecting the structure and functioning of ecosystems (van Gestel, 2012), a number of standardized test methods to assess the effects of chemicals on organisms are essential (Römbke and Ahtiainen, 2007). These guidelines recommend the exposure of single species of selected test organisms to pollutants in order to extrapolate the resulting (no) effect concentrations to safe levels for populations and communities (van Gestel, 2012).

Ecological or environmental risk assessment (ERA) uses scientific methodologies to estimate the adverse effects of pollutants and other anthropogenic activities on ecosystems and their compounds (Depledge and Fossi, 1994). Two approaches can be used in ERA: diagnosis and prognosis. The diagnostic approach aims at defining strategies for remediation and risk reduction, i.e. it focuses on chemicals which are already in the environment, meaning that standard test organisms are exposed directly in samples taken from the environment. The prognosis approach try to estimate the effects of pollutants using laboratory tests to regulate or prevent their use before they have been marketed. It is based in the principle that the risk of the pollutants to the environment can be calculated by their toxicity obtained in standard toxicity tests using model species. Potential risk is assessed by comparing the measured or predicted exposure data to the obtained results from these tests (van Gestel, 2012).

1.1 Folsomia candida as a model for ecotoxicology testing

Soil, consisting of a variable mixture of solid anorganic particles, water, gas and organisms and located between atmosphere and lithosphere, is a dynamic and

complex system with constant changes in physical and chemical properties due to environmental processes and anthropogenic pressure (Bur et al., 2012). In favourable conditions, soil supports numerous ecosystem functions or services (e.g. nutrient cycling) which are vital to support, not only crops, but also organisms that relies on these crops (Ockleford et al., 2017).

Besides its function as a habitat and food source for microorganisms, plants, animals and humans (Hund-Rinke et al., 2002), soil is also a sink for organic and inorganic residues (including harmful pollutants) and it acts as a protective layer for groundwater contamination. Therefore, soil contamination has become one of the main concerns due to the potential catastrophic consequences (Sousa et al., 2008). The effects of hazardous compounds depend on the contamination type and the respective soil characteristics (e.g. pH, clay content, cation exchange capacity, amount of organic matter), being responsible for their bioavailability to be absorbed by organisms (Loureiro et al., 2005; Van Gestel and Hensbergen, 1997).

Edaphic invertebrates represent a wide range of life-style characteristics (Diao et al., 2007) and play a crucial role in soil structure and fertility, contribute to decomposition processes and recycling of nutrients, increase aeration and drainage, can constitute an important component of the diet of birds, reptiles or small mammals and increase primary production (Allen, 2001; Quijas and Balvanera, 2013).

Soil bioremediation can be assumed by macro-fauna (>10 mm length, >2 mm width; e.g. earthworms, millipedes, centipedes, woodlice, termites, ants, beetles), meso-fauna (0.2-10 mm length, 0.1-2 mm width; e.g. micro-arthropods, such as collembolans and potworms) and micro-fauna (< 0.1 mm length, < 0.1 mm width; mainly nematodes), hence, soil fauna can help reduce pollutant´s impact (Bur et al., 2012). However, exposure of the organisms to pollutants, from contact or oral uptake routes in the surrounding soil compartment, may induce changes in their physiology, morphology and behaviour (Ockleford et al., 2017), which may disturb populations (e.g. due to mortality of organisms) and thus alter the ecosystem balance (Santorufo et al., 2012).

Collembolans, an integral part of many soil ecosystems and one of the most abundant groups in terrestrial ecosystems, are vulnerable to the effects of soil pollutants (Fountain and Hopkin, 2001). They contribute to soil aggregation (Ockleford et al., 2017) and quality by participating in the soil organic matter dynamics and nutrient mineralization, specially through micro-fragmentation of plant detritus and stimulation of the activity of bacterial and fungi colonies (Buch et al., 2016).

Among Collembola, *Folsomia candida* (Isotomidae) is an anophtalm, unpigmented and parthenogenetic species. It is one of the most tested and sensitive micro arthropod to pesticides (Daam et al., 2011; Frampton et al., 2006), thus has attained the status of a standard test species (OECD 2009). In addition, because they are easy to sample and to culture in laboratory tests (Fountain and Hopkin, 2005) and have a short generation time (Jänsch et al., 2005), are one of the most used in standardized ecotoxicity studies for environmental risk assessment (Hopkin, 1997; Schnug et al., 2014). Actually, it is the only Collembolan species required in EU regulations for pesticides and veterinary pharmaceuticals (European Commission, 2002; VICH, 2004).

1.2 Standard methods improvement with new endpoints

Toxicity in natural ecosystems is frequently a result of the integrated effects of several contaminants instead of single substances (Saxena et al., 2014; Schnug et al., 2015), and can be underestimated or overestimated by ERA methods (Syberg et al., 2008) if each chemical is tested separately (Lock and Janssen, 2002). Even if the individual concentrations of contaminants in the environment are low, the combined concentrations can increase toxicity significantly (Faust et al., 2001). Organisms from contaminated sites are often exposed to several chemicals simultaneously (Lock and Janssen, 2002). Also, the availability and mobility of one contaminant can be affected by others (Van Gestel and Hensbergen, 1997). Hence, it is very important to study the impact of mixtures of chemicals (Schnug et al., 2013).

The possibility of potential toxicity of chemical mixtures to organisms resulted in the development of mathematical models to predict their impact in the environment, such as the Concentration Addition (CA) and Independent Action (IA) models (Loureiro et al., 2009). The observed toxicity can be stronger (synergism) or weaker (antagonism) than the toxicity predicted by the conceptual models (Backhaus et al., 2004) and can be dose-dependent if they occur only in a specific concentration range (Jonker et al., 2005). Both additive and synergistic effects of mixtures are of concern (Phyu et al., 2011; Wang et al., 2015), and, as an example, an EU Council Commission report recommends additional tests of the join effects of pesticides that may be present together in nature (European Commission, 2012).

Wood preservatives are biocidal products used to prevent wood degradation by organisms. They can leach into the environment (e.g. water and soil), becoming a potential risk for soil organisms, their functions and thus for the soil ecosystem. The efficacy of the product formulation is usually improved by a mixture of two or more active substances. Although 3-iodo-2-propynyl N-butylcarbamate (iodocarb, IPBC) and Tebuconazole are some of the most used substances, there is a lack of information about their toxicity, especially for IPBC (Campiche et al., 2015).

1.2.1 Limitations of the current guidelines

Standard tests usually determine a dose–effect response of a specific substance for a certain endpoint (like reproduction or survival) using test organisms with a synchronized age during a fixed exposure time (Broerse and van Gestel, 2010). The results obtained in such tests may not represent the harmful effects of pollutants to other life stages of the organisms. It is therefore important to assess which life stage is more sensitive, e.g. comparing known effects of a given pollutant using the recommended life stage and comparing the effects after exposure of other life stages.

1.2.2 Effects of long-term exposure

One ecological advantage that organisms may present when exposed to unfavourable conditions is the ability to escape (Pereira et al., 2013). Avoidance

tests have already been developed for earthworms and collembolans (ISO 2008, ISO, 2011), as they are easy, fast and sensitive (van Gestel, 2012). However, the ERA process can still benefit from the incorporation of new test designs, test endpoints and additional methods.

Individuals are often exposed to pollutants over extended periods of time, caused by a continuous release of a certain compound to the environment and/or its persistency in soil, such as veterinary medical products and pesticides. The internal effect concentration can vary with different time of exposure (Broerse and van Gestel, 2010), which may affect the organism´s response to chemical stress (Schnug et al., 2013). Toxic effects after exposure to low but continuous doses of a certain substances can occur when it exceeds the excretion/detoxification rate of organisms. Therefore, harmful effects may be underestimated by traditional tests (Broerse and van Gestel, 2010).

To assess the real risks that some pollutants pose to the environment, it is highly recommended to perform multigenerational tests (Schnug et al., 2013). Some authors have already performed multigenerational tests using *F. candida* (Campiche et al., 2007; Ernst et al., 2016) and *Enchytraeus crypticus* (Bicho et al., 2015). They can increase the understanding of adverse effects and cumulative damages to a certain population over extended periods of exposure (Paumen et al., 2008), e.g. related with disrupted trophic interactions or reproductive effects (Ockleford et al., 2017).

The use of pesticides is increasing due to the ongoing growth of world population, being the number one solution to control insect pests in the ever-increasing demand for higher agricultural output (Furlan and Kreutzweiser, 2015). The widespread use of pesticides is of high concern since not only insects (Chagnon et al., 2015; Choung et al., 2011), but also the community structure and ecological functions of soil biota are affected by these compounds (Saha and Joy, 2016).

In recent years, a new approach to control insect pests has been developed with substances that affect insect growth and development, known as “insect growth regulators’’ (IGRs). The IGRs are hormone analogues or bio-rational compounds that are quite selective in their mode of action, presenting low toxicity to non-target

species (Tunaz and Uygun, 2004). By acting on metabolism and affecting growth (El-Aasar et al., 2013), they are not necessarily toxic to organisms but can cause numerous morphological abnormalities that impair populations survival (Siddall, 1976), e.g. reducing adults reproductive potential (Tunaz and Uygun, 2004).

Table 1 shows information regarding the mode-of-action of five IGRs and summarizes reasons why Teflubenzuron (TFB) was considered as a relevant compound to assess multigenerational effects to *F. candida*. The IGR Teflubenzuron is a chitin synthesis inhibitor (CSI) used in numerous important plants (e.g. fruit trees and vegetables, among others) (Campiche et al., 2006; EFSA, 2008a; Oberlander et al., 1997) and is considered persistent due to its adsorption to organic and inorganic matter in the soil (Cycoń et al., 2012). It is highly toxic to *F. candida* and, although its harmfull effects over two generations have been already reported by Campiche et al. (2006), information regarding the impact of TFB after long exposure is limited.

Table 1: Information used for test chemical selection among insect growth regulators, including the mode-of-action (MoA) used as relevance criterion to assess effects to *F. candida* in a multigenerational exposure.

Compound	MoA	Pros	Cons	Reference
Teflubenzuron	Inhibitor of chitin biosynthesis	- Well described - Well known fate - Highly toxic to *F. candida*	- Low solubility in water	Campiche et al., 2006; EFSA, 2008b; EMEA, 1997; FAO, 1996; Scheepmaker, 2008
Fenoxycarb	Juvenile hormone mimics	- Toxic to *F. candida*	- Low solubility in water	Campiche et al., 2006; Marrs, 2012; Smit and Vonk, 2008; Sullivan, 2000
Methoprene	Juvenile hormone mimics	- Toxic to *F. candida*	- Short half-life in soil - Low solubility in water	Campiche et al., 2006; Csondes, 2004; Palma et al., 1993; Thompson, 2003
Tebufenozide	Ecdysone receptor agonist	- Toxic to *F. candida*	- Very low water solubility	Addison, 1996; Campiche et al., 2006; FAO, 1996; Marrs, 2012; Nakagawa, 2005; Sundaram, 1995
Hexaflumoron	Inhibitor of chitin biosynthesis	- Toxic to *F. candida*	- Short half-life in soil - Very low water solubility	Campiche et al., 2006; Coppen and Jepson, 1996; Paranjape et al., 2014; Tan et al., 2014; Wang et al., 2012

1.2.2.1 Size as an additional endpoint in the standard guidelines for risk assessment

The efficiency of the organism's metabolism can be lowered by the exposure to toxic substances, which may lead to a reduction of growth rates (Crouau and Moia, 2006). Because reproduction of *F. candida* is dependent on body size of the adults (Hopkin, 1997), the incorporation of this endpoint in tests may be a sensitive parameter to evaluate the toxicity of chemicals (Crommentuijn et al., 1993; Folker-Hansen et al., 1996; Tranvik et al., 1993). While some authors found growth to be a more sensitive parameter than reproduction (Folker-Hansen et al., 1996), others

found opposite results (Fountain and Hopkin, 2001; Guimarães et al., 2019; Scott-Fordsmand et al., 1999; Smit et al., 2004; van Straalen et al., 1989). Besides the additional work in terms of image treatment (Guimarães et al., 2019), measurement of organisms is easy and does not require expensive equipment (Crouau and Moia, 2006).

1.2.2.2 Biomarkers used as tools to assess effects of a prolonged exposure

Several biochemical studies are available. Oxidative stress, called when there is an imbalance between the production and the neutralization of reactive oxygen species (ROS) by antioxidant mechanisms (Davies, 1995), may be highly relevant to increase mechanistic understanding and should be further studied (Maria et al., 2014).

Biomarkers can be defined by any measurable molecular-genetic, biochemical, cellular, or physiological response to sublethal concentrations of toxicants (Kammenga et al., 2000; Morgan et al., 2007). They can be sensitive indicators for exposure and predict ecological effects (Ockleford et al., 2017), therefore biomarkers may be considered early-warning signals of organisms imbalance (Galloway, 2006; Wu et al., 2005). Results from biomarkers tests can also improve risk assessment, for example, in cases that substances don't affect organisms during the duration of a standard test (Ockleford et al., 2017). A study performed by Duncan et al. (2009) with soil organisms, showed that effects of diazinon on *Porcellionides pruinosus* were only observed five weeks after application, which could be undetected by standard tests.

Also, responses at cellular level can change over long exposure times, therefore biomarkers can increase mechanistic understanding of the effects that pollutants may pose to the organisms (Guimarães et al., 2019).

2. AIMS AND THESIS STRUCTURE

The main aim of this thesis was to explore different approaches to evaluate the effects of pollutants to the ecotoxicological model species *Folsomia candida*, both during different life stages and across multigenerational exposure. In parallel, this

thesis also aimed to integrate a multi parameter assessment combining standard endpoints referred to in the guidelines (e.g. OECD and ISO) with methods already tested, in order to compare their sensibility.

This thesis was structured in 4 publications as follows:

Chapter I: Guimarães, B., Römbke, J., Amorim, M.J.B., 2019. Novel egg life-stage test with *Folsomia candida* – A case study with Cadmium (Cd). Sci. Total Environ. 647, 121–126. This study aimed to develop a test method using different life stages of *Folsomia candida* to evaluate sensitivity of organisms exposed from juveniles (as required by the guidelines) when compared to exposure from eggs and adults.

Chapter II: Guimarães, B., Bandow, C., Amorim, M.J.B., Kehrer, A., Coors, A., 2018. Mixture toxicity assessment of a biocidal product based on reproduction and avoidance behaviour of the collembolan *Folsomia candida*. Ecotoxicol. Environ. Saf. 165, 284–290. This investigation compared the effects obtained by the test of a commercial biocide as a mixture, the two active substances and a solvent with the calculated theoretical mixture toxicity to the collembolan *Folsomia candida*.

Chapter III: Guimarães, B., Maria, V.L., Römbke, J., Amorim, M.J.B., 2019. Multigenerational exposure of *Folsomia candida* to ivermectin – using avoidance, survival, reproduction, size and cellular markers as endpoints. Geoderma 337, 273–279. This investigation aimed to study the effects of a veterinary product to *Folsomia candida* during 3 generations using a multi endpoint approach.

Chapter IV: Guimarães, B., Maria, V.L., Römbke, J., Amorim, M.J.B. Exposure of *Folsomia candida* (Willem 1902) to teflubenzuron over three generations – Increase of toxicity in the third generation. Appl. Soil Ecol. (*in press).* This study aimed to assess the effects of the exposure of 3 generations of the collembolan *Folsomia candida* to the insect growth regulator (insecticide) teflubenzuron, using

a multi endpoint approach, which included survival, reproduction, avoidance, size and analysis of cellular and biochemical markers.

Chapter V: Final remarks.

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Chapter I

Novel egg life-stage test with *Folsomia candida* – a case study with Cadmium (Cd)

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HIGHLIGHTS

- The standard collembolan test is based on exposure of one life stage (juveniles).
- An egg stage test has been here developed and optimized.
- Lower Cd impact on reproduction from pre-exposed eggs (compared to juveniles).
- Cd seems to affect reproduction via exposure of adults.
- It is recommended to test different life stages in a combined approach.

ABSTRACT

Toxicity of pollutants is known to have a different impact depending on the organisms' life stage. Standard tests are often based on one life stage, i.e. effects could be underestimated. We aimed here to develop and optimize a test system using eggs of *Folsomia candida* (4-5 days) instead of the juveniles (10-12 days old) required by the OECD standard test guideline No. 232 (2009). Accordingly, the exposure time and thus the test duration was extended. Tests with "standard" juveniles (10-12 days old) and, adults (21 and 28 days old) were also performed. Cadmium (Cd) was used as test substance. The extension to the test guideline starts as follows: 1) synchronization of eggs in a thin soil layer on plaster of Paris, 2) selection of viable eggs, 3) burying these eggs in groups of 5 in soil. Afterwards, the test procedure will follow the standard procedure as described in the OECD standard test. Cadmium caused ca. 50% effects on reproduction at 60 mg Cd/kg soil dry weight (d.w.) when exposing juveniles or adults. There was no significant impact of Cd on the eggs, the hatching process or the latter life stages until ca. 250 mg Cd/kg d.w. (Cd is stable during this exposure period). Hence, Cd seems to affect reproduction before egg laying, i.e., during egg formation or during juvenile-adult stages. In order to clarify whether other chemicals do act in a similar way, testing with different chemicals is highly recommended. Exposure of different life stages does provide understanding of the mechanisms and effects of contaminants and offers important insight.

Keywords: life-stage; full-life-cycle; long term; cadmium; Collembola.

1. INTRODUCTION

Pollutants have a different impact depending on the life stage during which the tested organisms are exposed (Belanger et al., 2010; Bicho et al., 2015). Testing for hazard assessment has been based on standard guidelines where organisms' life stage or age is often optimized and synchronized, e.g. for *Folsomia candida* (Willem 1902; Collembola), 10-12 day old juveniles are used (OECD 232, 2009).

This exposure regime may cover potential effects on more than one life stage, but the way the test is designed does not allow for the identification of effects at individual life stages (which can reveal increased sensitivity, among others, see e.g. Bicho et al., 2016), hence the need for refinement. The implications are currently not covered in the Environmental Risk Assessment (ERA) procedures of the European Union (EU). In detail, the sensitivity of organisms to contaminants may be higher in certain periods of their life cycle, especially early life stages (Tarazona et al., 2014). For instance, eggs of the springtail *F. candida* are not mobile but show high physiological activity due to embryo growth and development, whereas hatched juveniles are in active contact with pollutants, e.g. can avoid it in patchiness field contamination. However, juveniles are not effective migrators and cannot avoid toxic conditions, especially in the first days of life. For oligochaete worms, different sensitivity between juveniles and adults is known, e.g. regarding survival in earthworms (Kwak and An, 2015; Van Der Ploeg et al. 2011) or reproduction of enchytraeids (Bicho et al., 2015, 2017; Santos et al., 2017). Few studies testing alternative exposure regimes have been published, including different multigenerational approaches with collembolans (Amorim et al., 2017; Campiche et al., 2007; Ernst et al., 2016). For instance, Filser et al. (2013) exposed collembolan eggs to chemicals and reported preliminary results, but hatching success was relatively low. Besides, differences in sensitivity of juvenile *F. candida* differing in age by just one day are known (Crouau and Cazes, 2003) and show the importance and inherent variability with age. Therefore, the optimization of a test to expose *F. candida* from egg stage is highly relevant for chemical risk assessment in the soil compartment.

Hence, the aim of this study was to optimize and develop a test system using eggs of *F. candida* (4-5 days) instead of the standard juvenile stage (10-12 days old). In addition, it was investigated which exposure regime would be relevant to cover the life cycle of the collembolan, from the egg stage till the reproduction phase. Further, in order to compare the recorded sensitivity of the egg stage with those from older life stages, adults with an age of 21 and 28 days were also tested. Cadmium was used as test substance since: 1. its toxicity to collembola in standard tests is well-known (Amorim et al., 2017); 2. it has embryotoxic

(Stummann et al., 2008); 3. it is not an essential metal and is widespread in the environment due to anthropogenic activities (Son et al., 2011); 4. it is quite bioavailable to living organisms (Crommentuijn et al., 1997; van Gestel & Mol, 2003; Vig, 2003).

2. MATERIAL AND METHODS

2.1. Test organisms

The test organisms used belong to the standard species *Folsomia candida* (Collembola). Individuals were cultured on a moist substrate of plaster of Paris and activated charcoal (8:1 ratio), at 20±1 °C, under a photoperiod regime of 16:8 (light:dark). The organisms were fed weekly with dried baker's yeast (*Saccharomyces cerevisiae*). Cultures were synchronized in order to obtain four different life-stages: eggs (4-5 days old), juveniles (10-12 days), young adults (21 days) and adults (28 days).

2.2. Test substance, soil and spiking procedures

As test substance, Cadmium chloride anhydrous ($CdCl_2$, 99% purity, Fluka) was used.

As substrate during the test phase the natural standard LUFA 2.2 soil (Speyer, Germany) was used. The main characteristics were as from the supplier: pH (0.01 M $CaCl_2$, ratio 1:5 w/v)=5.5, organic matter=10.1%, cation exchange capacity (CEC)=1.77 meq/100g, water holding capacity (WHC)=41.8%, grain size distribution of 7.3% clay, 13.8% silt, and 78.9% sand.

The soil was spiked by mixing $CdCl_2$ as an aqueous solution into the pre-moistened soil. A full concentration range was tested with eggs and juveniles (10-12 days old) at 0-32-60-128-256 mg Cd/kg soil dry weight (d.w.). A reduced test regime was used for the assays with 21 and 28 days old organisms, corresponding to 0 and 60 mg Cd/kg soil d.w., also included in the full test range with eggs and juveniles. A soil concentration of 60 mg Cd/kg soil d.w. was determined as the EC50 for reproduction in a standard OECD test (van Gestel and Mol, 2003). The moisture content of the soil was adjusted to 40–60% of the

maximum water holding capacity (WHC). Soil was left to equilibrate for 3-4 days prior to test start.

2.3. Experimental procedures

Optimization steps for egg culture synchronization and egg hatching success

Optimization procedures included a suite of alternatives to investigate the best testing option. For details of the tested alternatives please see the Supplementary Information (Fig. S1).

2.4. Egg stage test

Exposure of eggs to Cd was performed in test vessels (Ø 5.5 cm, 250 mL volume), filled with 30 g WW (wet weight) of soil.

The experimental procedure followed the standard guideline (OECD 232, 2009) with adaptations (as optimized in the previous step, see SI for details): twenty eggs, synchronized in cultures of Plaster of Paris with a thin layer of soil were introduced as groups of 5 and buried in a small pre-made hole partially covered with soil. Test conditions were 20±1°C and 16 h: 8 h photoperiod. Food and water loss were replenished on the soil surface weekly. Sampling was performed on day 7, 14, 21, 28, 35, and 42 days; 4 replicates per treatment, i.e. control and 60 mg Cd/kg soil d.w. (a total of 48). At test end, each test vessel was flooded with water, the content was transferred to a crystallizer dish and the surface was photographed for further automatic counting using the software ImageJ (Schneider et al., 2012).

2.5. Other life stages test

Following the standard guideline OECD 232 (2009), organisms of synchronized age 10-12 days were used, as well as organisms with 21 and 28 days old. Ten organisms were introduced in each test vessel and the test ran under the same conditions as described above (Fig. 3). Sampling days included day 7, 14, 21, 28 and 35. The same procedure was done in parallel but using petri dishes (∅ 60 mm) with 50 g of compacted and levelled soil. Two replicates per treatment were

done. Every two days the boxes were checked under the binocular and the number of adults, eggs and juveniles were monitored until test end.

2.6. Data analysis

Significant differences between treatments and exposure time were determined using One-way ANOVA (ANalysis Of VAriance). Whenever significant differences were obtained, the Post-Hoc Dunnett's was used ($p<0.05$). All analyses were done using (SigmaPlot 12.0, 2011). The Effect Concentrations (ECx) were calculated using the logistic regression model (Erickson, 2010).

3. RESULTS

The results from optimization steps are shown in the supplementary (Fig. S3).

3.1. Life stage comparison: eggs, juveniles and adults – limit test

When eggs were exposed to Cd, their hatching success and the survival of the adults at day 42 was >80% (Fig. 1). Juveniles can be found from day 35 onwards (50-150 juveniles) although a few juveniles (<10) were observed already on day 28. The coefficient of variation (CV) for reproduction at day 42 was 14%, also below the 30% value required in the 28 day standard guideline for tests with 10-12 days juveniles. At day 42, survival and reproduction of the adult organisms was not affected, in fact, it was significantly higher in the Cd exposed organisms.

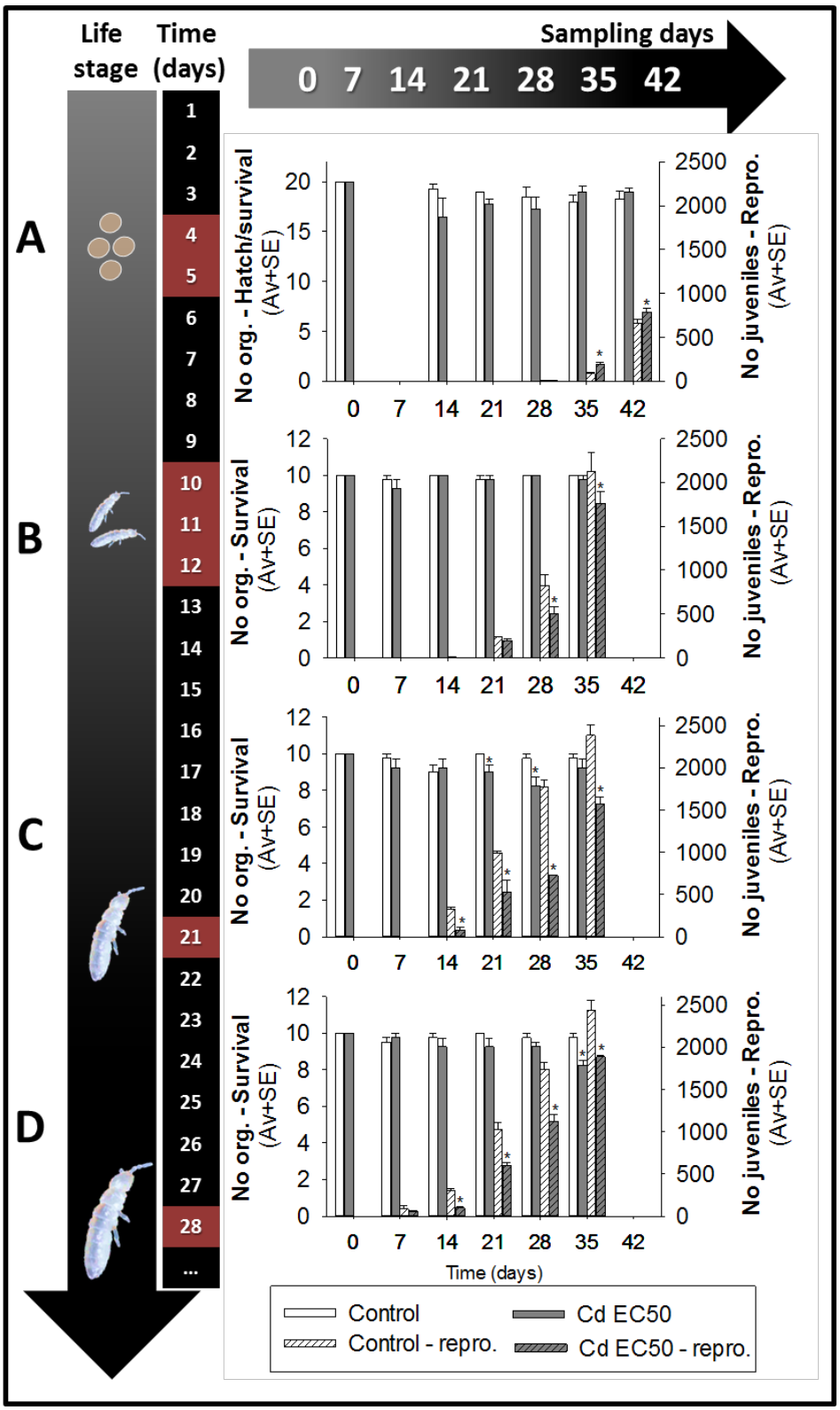

Figure 1: Hatching success, survival and reproduction of *Folsomia candida* when organisms were exposed as A) eggs, B) 10-12d C) 21d and D) 28d old organisms to 60 mg Cadmium /kg d.w., which has been determined to be the EC50

reproduction for *F. candida*) in LUFA 2.2 soil. Open columns refer to hatching/survival numbers and striped columns to the number of juveniles. For day 0 columns represent the number of introduced eggs/organisms. Values are expressed as average ± standard error (AV±SE). $p<0.05$*: corresponds to statistically significant differences between the control and the treatment (Dunnetts'; $p<0.05$).

Results from exposure of 10-12 days old juveniles confirm that the validity criteria were fulfilled. In addition, the ca. 50% effect of Cd on reproduction (tested EC50) was confirmed at day 28. Reproductive outcome from first clutches were observed already at day 21 but no significant effects (toxicity) was observed until day 28. For adults, the validity criteria were fulfilled too. Reproduction could be observed at days 14 and 7 after starting the tests with 21d old adults and 28d old adults, respectively. Further, for 28d old adults, unhatched eggs were observed at day 7, with higher numbers in the control soil (although no quantitative record was done at the time). Reproduction of *Folsomia candida* exposed at different life stages are summarised in Table 1. The equivalent time to reach similar life stages and reproduce differs with the start age, thus please note that the equivalent time is 42, 28, 21 and 14 days for the test started with eggs (4-5d), juveniles (10-12d), 21 and 28 days' adults, respectively.

Table 1: Effects of cadmium (60 mg Cd/kg) on the reproduction of *Folsomia candida* in LUFA 2.2 natural soil based on tests using different life stages. Results are expressed as average ± standard error the number of organisms and the relative percentage of effects on organisms found in spiked soil compared to control soil.

Life stage	**Eggs**		**Juveniles** (standard)		**Adults**		**Adults**	
Age (days)	4-5		10-12		21		28	
Cd (mg/kg)	0	60	0	60	0	60	0	60
Exposure time (days)								
14	0	0	0	0	(0%) 322±25	(75±10%) 81±33	(0%) 303±29	(67±4%) 101±10
21	0	0	(0%) 238±8	(16±9%) 199±21	(0%) 989±22	(47±15%) 523±151	(0%) 1027±80	(41±3%) 605±29
28	0	0	(0%) 829±118	(39±10%) 502±82	(0%) 1780±83	(59±0%) 726±5	(0%) 1738±84	(35±5%) 1123±78
35	(0%) 85±22	(-132±25%) 197±21	(0%) 2124±210	(17±6%) 1763±130	(0%) 2388±119	(34±4%) 1570±83	(0%) 2435±123	(23±1%) 1886±16
42	(0%) 658±47	(-20±6%) 789±42	-	-	-	-	-	-

3.2. Life stage comparison: Eggs and juveniles – full range test

The validity criteria were fulfilled for the standard test. Adult *F. candida* that were exposed as eggs to Cd had higher reproductive output than those specimens that had been exposed as juveniles (Fig. 2). In the assays where eggs were exposed, the no observed effect concentration was 128 mg Cd/kg soil d.w., while for exposed juveniles the EC50 reproduction was determined to be 95 mg/kg soil d.w.. In terms of survival, there was an exceptional decrease at 128 mg/kg soil d.w. in the egg test with unknown reasons. If discarding that, effects on adults were similar.

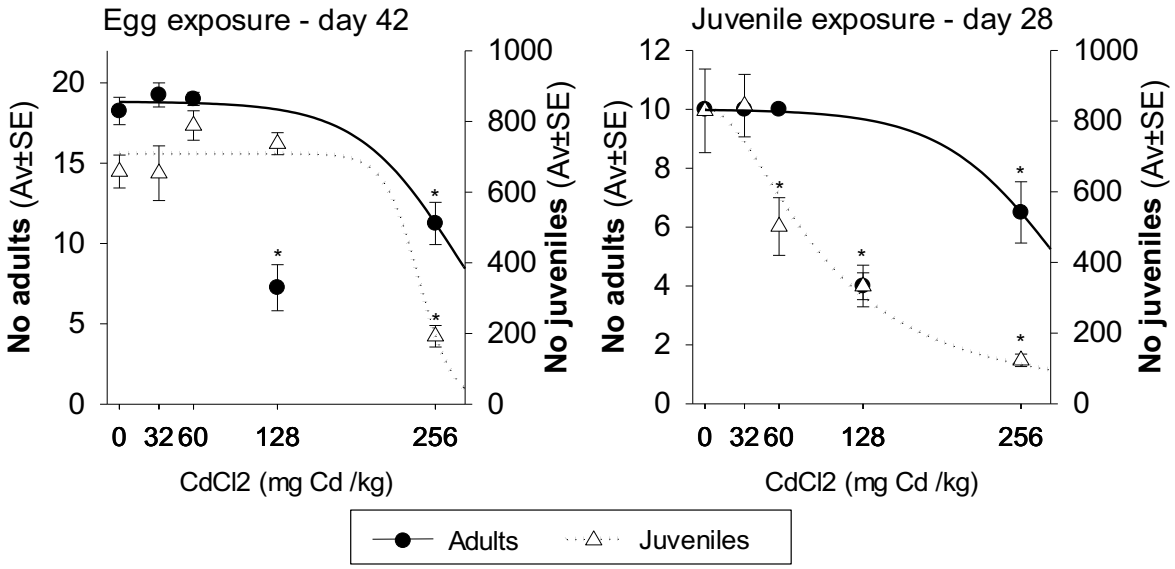

Figure 2: Hatching/survival and reproduction of *Folsomia candida* started from eggs (4-5 days) and juveniles (10-12 days), when exposed to $CdCl_2$ in LUFA 2.2 soil, during 42 and 28 days respectively. Values are expressed as average ± standard error (AV±SE). Lines represent model fit do data. *p<0.05 (Dunnetts').

Corresponding LC/ECx values can be found in Table 2. There was an apparent lower sensitivity for eggs compared to juveniles (EC10/50), although not significant. Further, the EC50 values in the egg test did not differ between survival and reproduction, in contrast to the juvenile test, which showed higher toxicity for reproduction.

Table 2: Estimated median Effect Concentrations (ECx) for *Folsomia candida* when exposed to Cadmium (mg/kg) in LUFA 2.2 soil from eggs (4-5 days old) and juveniles (10-12 days old) after 42 and 28 days, the equivalent time for same endpoint. ECx were calculated with a logistic regression model (Log2 or Log3 parameters). Exposure was log scaled. For survival data the 128 mg Cd/kg was excluded to fit model. S: Slope. Y0: top point. CI: 95% Confidence Intervals.

	EC10	**EC50**	**Model parameters**
Egg test – d42			
Survival	185 -65<CI<434	272 214<CI<329	Log 2 par S: 0.63E-02; Y0: 18.3.
Reproduction	n.d.	242 -782<CI<1267	Log 3 par S: 0.18E-01; Y0: 709.9.
Juvenile test – d28			
Survival	182 -4<CI<368	285 209<CI<361	Log 2 par S: 0.54E-02; Y0: 10.0.
Reproduction	30 15<CI<60	95 69<CI<130	Log 2 par S: 1.08; Y0: 836.4.

4. DISCUSSION

4.1. Egg hatching success/survival and reproduction test

Optimization steps for egg culture synchronization and egg hatching success had the highest success when a synchronization of eggs in plaster of Paris with a soil layer (SI) was performed. One possible explanation for the increased hatching performance could be related to the fact that soil particles form a protective layer around the eggs (e.g. decrease moisture changes such as desiccation and impacts of direct manipulation), hence also less damage is inflicted in the eggs when transferring them to the test vessels.

The selection of viable eggs performed as proposed by Hafer and Pike (2010) resulted in higher egg hatching rates (ca. 93%) in our study compared to the results obtained in other studies. For example, Filser et al. (2013) obtained 60%

hatching success possibly because a pre-exposure observation step was missing. Moreover, we find this is feasible to include in the test set-up after gained experience, similarly the collected juveniles are observed under the binocular for healthy status before introducing them in the test vessels.

Introducing the eggs as groups of 5 and burying them into the soil, i.e. covered with soil, also improved hatching success, and will ensure exposure. Based on our daily monitoring, we postulate that some kind of stimuli promoted synchronous hatching when eggs were used in groups. *F. candida* eggs introduced individually in the test soil hatched at different times and the variability in the observed endpoints later in the test was higher than when introduced as a group. Eggs introduced in a small hole and partially covered with soil will be better protected from dehydration.

The present design (as described in section 2.4, please see fig.3) can be implemented in the standard guideline to assess the effects of stressors from eggs life stage of *Folsomia candida*.

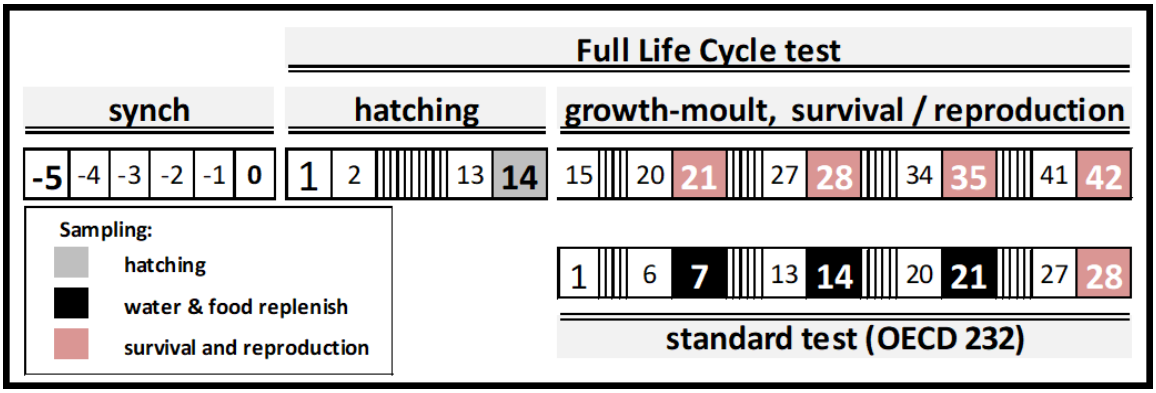

Figure 3: Schematic calendar representing the proposal for the “egg test” with additional endpoints and test duration compared to the standard (OECD 232) as developed for *Folsomia candida*.

The primary aspect will refer to the hatching success of the eggs as an added endpoint, but also the survival and reproduction of these organisms will be an important follow up result.

4.2. Ecotoxicity of Cd in different life stages

When using the OECD standard method, (starting with juveniles 10-12 days old) showed that Cd median effect concentrations for reproduction were within a similar concentration range (69<EC50<130) – as reported in the literature (Amorim et al., 2017; van Gestel and Mol, 2003).

The impact of Cd on the reproduction of exposed adults (i.e. 21 and 28 days old) was not significantly different to the one obtained for exposed juveniles in the standard test for the one same concentration. Cadmium seems to act in adults and affects reproduction, but not hatchability or juvenile survival.

The higher numbers of juveniles observed at the sampling dates on the 21-28d organism test are due to more cumulative egg laying clutches as naturally expected if starting with later life stage animals. This has not changed the relative effect of Cd compared to control.

Results from tests with exposed eggs showed no significant impact of Cd on hatching/survival or reproduction of *F. candida*; hence Cd seems to affect reproduction before egg laying, i.e., during egg formation. This result of lower reproduction sensitivity to Cd when exposing collembolan eggs should not be generalised, since, for instance, for Cu, Pb and Zn there was a reduction on hatching success (Xu et al., 2009), indicating an effect on the egg stage at concentrations above 200 mg/kg soil d.w.. Other studies suggest that also the soil microbial community may influence egg viability (Hafer and Pike, 2010). In the case of Cd, concentrations under 70 mg/kg soil d.w. do not affect the soil C:N ratio (stable microbial index) (Khan and Scullion, 2002), hence this factor is unlikely. Lower sensitivity of different life stages has been reported for other organisms. For example, in tests with *E. crypticus* CuO nanomaterials were more toxic (reproduction) when exposing juveniles rather than cocoons (Bicho et al., 2017).

An additional hypothesis for the reduced effect observed when exposing eggs of *F. candida* to Cd could be the activation of different defence mechanisms in the eggs which are then kept in the adult influencing the reproduction outcome. This could involve epigenetic mechanisms, i.e., changes in the genes that can be transferred between generations but without changing the genome (e.g. via

methylation of DNA) (Marczylo et al. 2016). Although there is apparently no global methylation for *F. candida* (Noordhoek et al., 2017), other epigenetic mechanisms (e.g. histone modifications or RNAi) cannot be excluded. Besides, epigenetics is also life stage dependent (Lyko 2001) which requires further in-depth studies.

The exposure of eggs (instead of juveniles) allowed to assess the impact of cadmium on hatching and survival of young juveniles, and the reproduction thereafter. This provided a better insight on life stage specific effects and mechanisms in *Folsomia candida*. Moreover, the new test set-up offers increased flexibility regarding the testing of different life stages, and the probability of identifying effects of chemicals with different mode-of-actions is more likely. For reasons of comparability of sensitivity of different life stages, this method adds on the importance of testing the standard age as established. Regarding the differences in sensitivity, regarding the endpoint reproduction, between exposure to Cd from eggs and juveniles, we recommend that more tests with additional chemicals are done before drawing any general conclusions, especially before proposing additions to the existing OECD/ISO guideline.

5. CONCLUSIONS

The exposure from egg stage test methodology was optimized and the proposed design can be implemented as an annex to the standard OECD/ISO guidelines. The suggested improvements ensure a high hatchability success and survival in controls, as required in the standard guideline. It is also possible to implement a similar procedure to start the testing by using adult organisms (21-28 days old) and assess survival and reproduction. However, the use of adults seems to have less advantages compared to the standard guideline starting with juveniles.

Cadmium affected *F. candida* reproduction possibly via the exposure of adults, not affecting the hatching success or survival of the hatched juveniles and reproduction thereafter. This would not be possible to interpret from the standard juvenile life stage test.

It is recommended to test different life stages in a combined approach in order to provide a better insight in the mechanisms and effects of contaminants on

collembolans. However, before proposing an extension of the standard guideline to include the egg stage further validation studies (using different soils and contaminants) are necessary.

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SUPPLEMENTARY INFORMATION

Novel egg life-stage test with *Folsomia candida* – a case study with Cadmium (Cd)

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MATERIALS AND METHODS

Optimization steps for egg culture synchronization and egg hatching success

Synchronization of eggs was performed in 2 culture media in petri dishes (∅ 90 mm): 1) Plaster of Paris (PP) and 2) Plaster of Paris with a thin layer of sieved (<0.125 mm) LUFA 2.2 soil (PP+S) (Fig. S1).

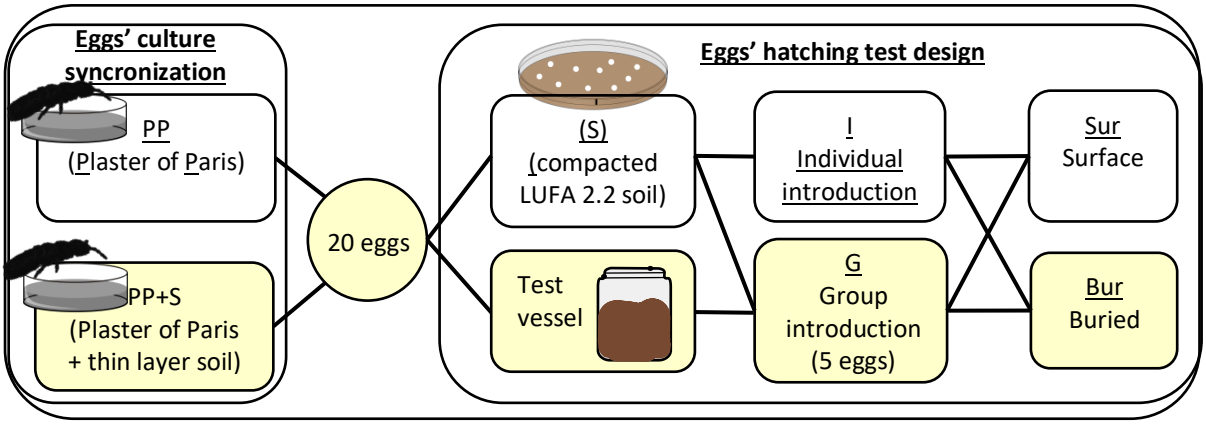

Figure S1: Schematic representation of the tested alternatives for *Folsomia candida* eggs' culture synchronization and the hatchability of the eggs introduced in pre-test. Eggs were synchronized in Plaster of Paris (PP) and Plaster of Paris with a thin layer of soil (PP+S), then introduced in a Petri dish with 20 g of compacted LUFA 2.2 soil (S) as 1) Group of 5 (G) and individually (I), and in each of these a) laid on the surface (Sur) or b) buried (Bur) in soil. Yellow background indicates the optimized method where eggs were synchronized in PP+S, buried in

groups of 5 (G_Bur) in test vessels (Ø 5.5 cm, 250 mL volume) containing 30 g of LUFA 2.2 soil.

The hatchability of the eggs laid in option 1 and 2 was compared. First, they were transferred with a thin brush from the synchronized petri dishes to the test containers. Eggs from option 2 had soil particles adhered to the eggs surface. These consisted of petri dishes, filled with 20g of moistened soil (40-60% WHC). The soil was compacted and levelled until a smooth surface was achieved. Prior to that, egg visualization under the microscope was done to assess the good health (shape, size and smooth surface, according to (Hafer and Pike, 2010)) to improve viability success (Fig. S2).

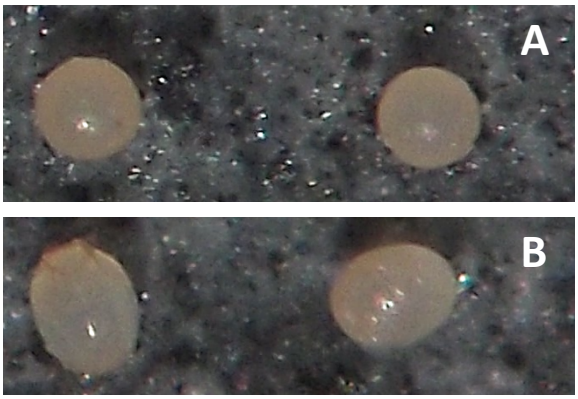

Figure S2: Visualization of *Folsomia candida* eggs with 4-5 days (50 x amplification). A: Inviable eggs (round shape, smaller and/or irregular size); B: Viable eggs (oval shape, bigger size and smooth surface). Selected based on Hafer and Pike (2010).

Eggs were introduced as 1) Groups of five (G) and 2) Individually (I), and in each of these a) laid in the surface (Sur) and b) buried (Bur) in soil. In the latter method, a small hole (1-2 mm depth) was made, and the eggs were carefully introduced inside, being partially covered with soil with a thin brush. This allowed the eggs to be inside a small “cave”, i.e. they were protected from direct light and dehydration. Four replicates and 20 eggs per replicate were used. Hatching and survival were monitored daily under the binocular for 25 days.

Egg stage test optimization

Additionally, and only as extra controls, 4 replicates using Petri dishes (∅ 60 mm), filled with 20 g of compacted and levelled soil were used. The Petri dishes test was used as a comparison and control to check daily under a binocular microscope, and hence evaluate, the progress of eggs development and hatching. On day 9, juveniles were transferred to the test vessels, following the same procedure as described above. Test conditions were 20±1°C and 16h:8h photoperiod. Food and water loss were replenished on the soil surface weekly. Sampling was performed on day 10, 21, 28, 35, 42 and 49 days. At test end, each test vessel was flooded with water, the content was transferred to a crystallizer dish and the surface was photographed for further automatic counting using the software ImageJ (Schneider et al., 2012).

RESULTS

Optimization steps for egg culture synchronization and egg hatching success

A maximum hatchability of 80% (16 out of 20) was observed for PP_I after 14 days (Fig S3). The different treatments showed a similar pattern in terms of number of organisms with time. Although, e.g. from day 6 to 9, PP+S_I showed higher hatching success than PP_I, this being significant at day 6. Mortality of juveniles started to increase after day 12 and was almost 100% at day 22 (Fig. S3 A).

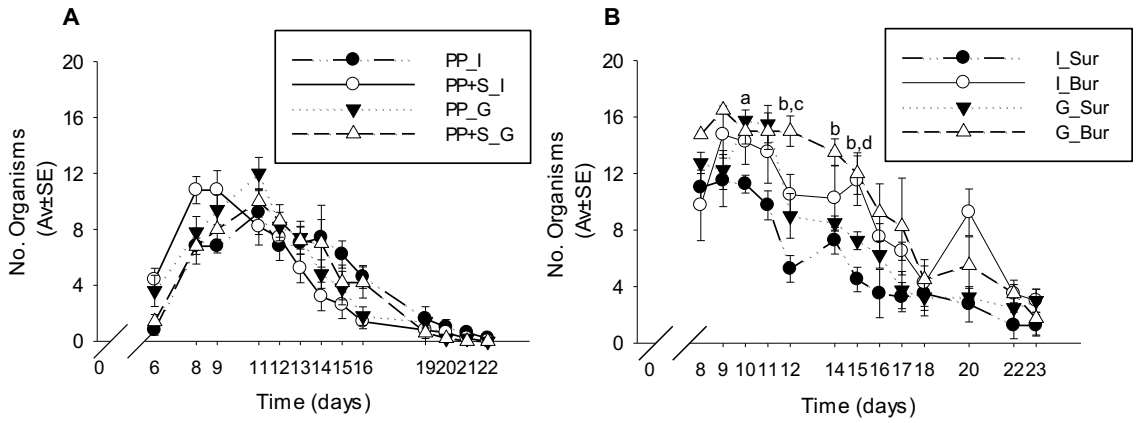

Figure S3: Results of hatching success and survival of the organisms from the optimization tests as starting with eggs of *Folsomia candida*. Tests were conducted for 22 days (A) and 23 days (B). A: Synchronized in Plaster of Paris (PP) and in Plaster of Paris with thin soil layer (PP+S), either introduced individually (I) or as a group (G); B: Synchronized in PP+S and introduced individually (I) or as a group (G) and buried in (Bur) or laying on the surface (Sur) of control LUFA 2.2 soil. N=20. Values are expressed as average ± standard error (AV±SE). $p<0.05$ (Tukey). a) between G_Sur and I_Sur; b) between G_Bur and I_Sur; c) between G_Sur and G_Sur; d) between I_Bur and I_Sur.

Results of the second optimization step, where eggs were synchronized in plaster of Paris with a thin layer of soil (PP+S) (Fig. S3 B) show that at day 8 and 9 there was a higher hatching success of G_Bur. This difference was significant from day 12 to 15. After day 12 mortality of the juveniles increased for all treatments and on day 23 the survival was less than 20%.

Egg stage test optimization

Results of the egg stage test, i.e. hatching, survival and reproduction of *Folsomia candida* when exposed from eggs to 60 mg Cd/kg soil d.w and control, can be observed in figure S4.

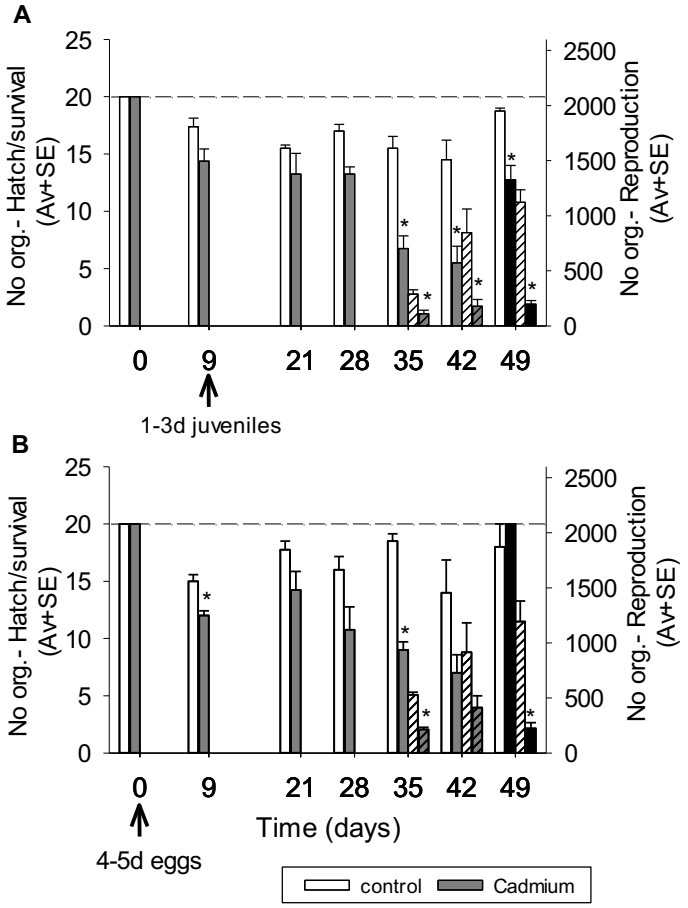

Figure S4: Hatching success, survival and reproduction of *Folsomia candida* when organisms were exposed to 0-60 mg Cadmium/kg d.w. in LUFA 2.2 soil, from A) eggs that hatched and grew in petri-dishes, being transferred to test vessels with 1-3 days old at day 9 and B) eggs with 4-5 days old, transferred to test vessels at day 0. Open columns refer to hatching/survival numbers and striped columns to the number of juveniles produced. Arrows indicate the age and time of insertion for eggs, juveniles or adults in the test vessels. The dashed line indicates the average starting number of eggs/organisms. Note that the black colour bar at day 49 is due to the uncertainty of number of adults due to lack of size discrimination. Values are expressed as average ± standard error (AV±SE). $p<0.05$*: corresponds to statistically significant differences between the control and the treatment (T-test; $p<0.05$, one-sided).

In terms of validity criteria, compared to the reference standard guideline values, here at day 49 mortality was <20% (10%±4) and the coefficient of variation for

reproduction was >30%. Reproduction could be assessed from day 35 onwards, with ca. 400-600 juveniles.

Overall, the organisms hatched from the eggs placed in the Petri dishes and transferred to the soil after 9 days were more sensitive to Cd than those already introduced in the test vessels from day 0. This may be due to the transfer process from one container to the other in such a sensitive stage of the young juveniles' life.

Chapter II

Mixture toxicity assessment of a biocidal product based on reproduction and avoidance behaviour of the collembolan *Folsomia candida*

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HIGHLIGHTS

- Mixture toxicity of a biocidal product studied in collembolans.
- Concentration-Addition model predicted effect of product on reproduction well.
- Mixture effect on avoidance behaviour was strongly underestimated by this model.
- Underestimation not reduced by including known formulation additive in prediction.

ABSTRACT

Biocidal products represent mixtures that might be released into the environment at application and continuously during service life. Concentration addition (CA) has been proposed as default model to calculate theoretical mixture toxicity. However, the suitability of CA for chronic toxicity towards soil organisms has so far rarely been evaluated and therefore needs further experimental evidence. The present study investigated the toxicity of a wood preservative product and the individual active substances (tebuconazole and IPBC) therein with the aim to evaluate the compliance with the CA prediction for the product. *Folsomia candida* was selected as test organism for this purpose using the endpoints reproduction and avoidance behaviour. Both endpoints were increasingly impacted by increasing concentrations of the wood preservative product as well as its active substances tested individually. The chronic effects of the product could be predicted by CA with less than 4-fold deviation, while the assessment for avoidance behaviour indicated a strong underestimation. This underestimation could not be attributed to the one known formulation additive, an organic solvent. Overall, the present study provides some more evidence that CA could be applied as default model for standard endpoints of soil organisms but warns against using CA for behavioural responses.

Keywords: fungicide; concentration addition; formulation additives; wood preservative; mixture assessment.

1. INTRODUCTION

Wood preservative products are used to protect wood in service from decay and destruction by fungi, algae, and insects. Such products may contain one or more biocidal active substances (e.g., fungicides and insecticides) together with a number of formulation additives. Hence, wood preservative products represent mixtures that are released as such into the environment during application, and mixtures of the product ingredients may reach the environment by continuous leaching from wood in service. In the European Union, wood preservative products are subject to an authorization process as biocides according to the Biocidal

Product Regulation (BPR, EU 2012). Recent guidance from the European Chemicals Agency (ECHA 2017) recommends theoretical mixture toxicity calculations based on the concept of concentration addition (CA) as one approach for the environmental risk assessment of biocidal products, which can render an experimental testing of the product unnecessary. For the aquatic compartment, the suitability of CA for the prediction of toxicity of mixtures has been demonstrated in general (Deneer 2000, Belden et al. 2007, Cedergreen 2014) and in particular for formulated biocidal and pesticidal products (Coors & Frische 2011, Coors et al., 2012, 2014, 2018).

For the terrestrial compartment, however, evidence for the suitability of CA for mixture toxicity prediction is still scarce. The joint effects of the three active substances esfenvalerate, picoxystrobin, and triclosan were reported to be additive with regard to survival and reproduction of earthworms (Schnug et al. 2013), but synergistic at higher concentration levels with regard to reproduction of collembolans (Schnug et al. 2014). Yet, the deviation between the CA-predicted and experimentally observed response in that study was less than 2-fold. Binary mixtures of the insecticides atrazine, dimethoate, lindane, and the metals zinc and cadmium exhibited concentration-dependent deviations from predicted additive effects on survival and reproduction of the collembolan *Folsomia candida* (Amorim et al. 2012) as well as on avoidance behaviour of the enchytraeid *Enchytraeus albidus* and the isopod *Porcellionides pruinosus* (Loureiro et al. 2009). Binary mixtures of formulated plant protection products showed, with one exception, no deviation from CA predicted effects on avoidance behaviour of *P. pruinosus* as well as survival and reproduction of *F. candida* (Santos et al. 2010). Unfortunately, the influence of the formulation additives in these products could not be assessed, because the active substances were only tested in formulations but not individually, i.e. separately from formulation additives.

The present study aimed to investigate whether the mixture toxicity of a formulated biocidal product could be reliably predicted for the terrestrial compartment in view of the recommended CA-based approach (ECHA 2017). The collembolan soil species *Folsomia candida* was selected for this purpose as one soil standard test species for which data may occasionally be required in the regulatory environmental risk assessment of biocidal products. In addition to the standard reproduction endpoint, the present study investigated avoidance behaviour and its

mixture predictability in the same collembolan species. While testing for avoidance behaviour is not required for the environmental risk assessment of biocides and not deemed a suitable replacement for a reproduction test (ISO 2011), it may nevertheless help interpreting the environmental impact of wood preservative products. This is because treated wood such as fence posts represent a point source for soil contamination with the potential impact being restricted to a small area that might be effectively avoided, reducing impacts at the population level. Previous studies with waste indicated that collembolans may respond to toxicants by avoidance behaviour at lower concentrations than those exhibiting effects on the population relevant endpoint reproduction (Natal-da-Luz et al. 2009).

2. MATERIAL AND METHODS

2.1 Test organisms

A commercially available wood preservative product as well as individual substances contained in this product were tested in standard collembolan reproduction studies (OECD 2009) and in collembolan avoidance tests (ISO 2011). The test organism was in all studies *Folsomia candida* Willem 1902 (Isotomidae, Collembola). Organisms were obtained from an in-house culture kept at 20±2 °C on a mixture of plaster of Paris and activated charcoal in a ratio of 8:1 (w/w), in constant darkness, and fed weekly with dried baker's yeast (*S. cerevisiae*). Reference tests conducted in regular intervals confirmed the required sensitivity of the test organisms (data not shown). All tests were conducted in the same laboratory under similar climatic conditions.

2.2 Test chemicals

The wood preservative product selected for the present study is authorized in Europe for use class 3, i.e., for outdoor use on wood exposed to weathering but without permanent contact to water or soil. There were two fungicidal active substances (a.s.) in the product: tebuconazole and 3-iodo-2-propynyl N-butylcarbamate (iodocarb, IPBC) at weight proportions of 0.40 and 0.70%, respectively. In addition, the product contained one known additive that was labelled as hazardous, though not as hazardous to the environment. This additive (the solvent dimethylcapramide, DCM) was contained at a weight proportion of

1.64%. The product was obtained from a commercial online shop together with its material safety data sheet providing this information. Single substance tests were conducted with IPBC (CAS 55406-53-6), obtained from Sigma-Aldrich at a purity of 97%, tebuconazole (CAS 107534-96-3) obtained from Fluka (99% purity), and DCM (CAS 14433-76-2) obtained from TCI Chemicals (purity of 99.5%). DCM can be considered a non-volatile organic solvent due to the high boiling point (110°C according to the material safety data sheet from TCI).

2.3 Test soil and spiking

All tests used artificial soil composed according to OECD guideline 232 (OECD 2009) (20% kaolin, 74.81% quartz sand, and 0.19% $CaCO_3$) with a peat content of 5%. The product was dissolved in water for spiking the soil, while acetone was used in the tests of the single substances. Accordingly, there were controls with deionised water and, in the tests with the single substances, additional solvent controls with acetone spiked at concentrations identical to those in the treatments. The tested concentration ranges were selected based on previous range finding tests.

2.4 Reproduction and avoidance tests

The reproduction tests were performed according to OECD guideline 232 (OECD 2009) over an exposure period of 28 days. All tests were conducted with eight replicate vessels for the water control (or solvent control, if applicable), and four replicate vessels for each test concentration level. Each replicate vessel contained 30 g soil (fresh weight, f.w.) and 7-10 mg of granulated dry yeast as food. The tests were started with juvenile *F. candida* (9-12 days old, ten individuals per replicate) obtained from synchronized breeding cultures. After 28 days, the number of adults and juveniles was determined by counting under a stereoscope using ink as dye. The test conditions were temperature between 14.6 and 21.9°C, light intensity between 416 and 796 lux, and a light:dark cycle of 16:8 h. The pH of the soil in the test vessels was between 5.4 and 6.8 during the tests, the soil moisture ranged from 38.9 to 58.7% of the maximum water holding capacity (WHC_{max}).

The avoidance tests with *F. candida* were performed according to ISO guideline 17512-2 (ISO 2011) over an exposure period of 48 h. All tests were conducted with five replicate vessels per treatment, each containing 20 juvenile *F. candida* (9 – 12 days old) obtained from synchronized breeding cultures. Each replicate contained 30 g soil (f.w.) spiked with the test item in one half of the test vessel (Ø 5.5 cm, 250 ml volume). The other half of each test vessel was filled with 30 g soil f.w. representing the (solvent) control. Test conditions were temperature between 18.2-21.5°C, light intensity between 470 and 736 lux, and a light:dark cycle of 16:8 h. The pH of the soil in the test vessels was between 5.6 and 6.8 during the tests, the soil moisture ranged from 44.9 to 54.8% of the WHC_{max}. After 48 h of exposure, living and dead (=missing) collembolans were counted separately on each side of each test vessel.

Details on temperature and pH are provided for individual tests in the supplement Table S1.

2.5 Data analysis

The response variables reproduction (number of juveniles as well as number of surviving adults after 28 days) and avoidance were evaluated statistically. Avoidance (%) was calculated for each replicate vessel according to the guideline (ISO 2011) as

$$Avoidance\ (\%) = \frac{n_C - n_t}{N} * 100$$

With n_c and n_t being the counted live collembolans on the control and the treated soil, respectively, and N being the total number of counted live collembolans.

Average negative avoidance per treatment (i.e., attraction to the test item-spiked soil) was set to zero as prescribed by the guideline (ISO 2011), but negative avoidance in an individual vessel was in no case set to 0% avoidance. Effect concentrations (ECx), i.e., the estimated concentration causing x% effect, were determined by fitting non-linear concentration-response curves. Concentration-response modelling was done in the free software R, version 3.2.2 (R Development Core Team 2015) using the most recent version of the package “drc” (Ritz & Streibig 2005). A three-parameter log-logistic model was fitted to

reproduction data with the lower limit fixed at 0, according to the function LL.3 given as

$$f(x) = \frac{d}{1+e^{(b*(\log(x)-\log(EC_{50})))}}$$

with *b* describing the steepness of the curve and the EC_{50} being directly modelled as inflection point of the curve. The model was reduced to two parameters by fixing the upper limit *d* at 1 for fitting avoidance and survival data. EC_{10} and EC_{50} values (concentrations resulting in 10 and 50% effect, respectively) and their 95% confidence intervals were obtained with the implemented function “ED” of the “drc” package using the delta method and the t-distribution.

In addition, Lowest Observed Effect Concentrations (LOECs) and the resulting No Observed Effect Concentrations (NOECs) were determined for the standard endpoint reproduction by William’s multiple t-test or, in case of variance heterogeneity, by Welch t-test (both: alpha=0.05; one-sided greater) in the software ToxRat Professional, version 2.10, release 20.02.2010 (ToxRat Solutions GmbH, Alsdorf, Germany).

Based on the relative nominal proportions (P_i) of the considered *i* components in the product and the individual toxicity estimates of each component ($EC_{x,i}$) the predicted toxicity estimates for the mixture ($EC_{x,mix}$) were calculated according to the concept of concentration addition (CA) as

$$EC_{x,mix} = \frac{1}{\sum \frac{P_i}{EC_{x,i}}}$$

These calculations were done either considering only the two a.s. in the product (i.e., P_i=0.364 for tebuconazole and P_i=0.636 for IPBC) or considering the a.s. and additionally the one known additive (P_i=0.146 for tebuconazole, P_i=0.255 for IPBC, and P_i=0.599 for dimethylcapramide). The alternative concept of independent action (IA) was not applied in the present project, because CA is usually more conservative and the difference between the two is rather small, particularly in the case of only a few mixture components (Junghans et al. 2006).

To quantify the compliance of the observed product toxicity with the CA prediction, the Model Deviation Ratio (MDR) was calculated according to Belden et al. (2007) for each toxicity estimate as

$$MDR = \frac{predicted\ toxicity\ estimate}{observed\ toxicity\ estimate}$$

An MDR well above 1 indicates that the effect of the product was underestimated by the CA prediction, while an MDR well below 1 indicates that it is overestimated.

3 RESULTS

The control treatments of all conducted reproduction tests met the validity criteria of the guideline (mean mortality at maximum 11.3%, at least 250 juveniles per vessel, and coefficient of variation (CV) of numbers of juvenile between 23.6 and 27.1%). Since all validity criteria have been fulfilled in the conducted tests, the slight deviations in temperature and pH from the conditions described in the guideline are not deemed to invalidate the results. The conducted avoidance tests were also valid according to the guideline criteria, because the mean mortality (including missing individuals) of collembolans did not exceed 20% in most treatments as well as in all controls, and the mean number of collembolans in each section of the control combination treatments was between 40 to 60%. Only in the highest two test concentrations of DCM as well as in the highest test concentration of the product more than 20% mean mortality was observed; these treatments were hence excluded from the evaluation of avoidance.

3.1 Reproduction tests

Reproduction of the collembolan *F. candida* was increasingly inhibited by increasing concentrations of the wood preservative product and its active substances tested individually (Figure 1). Mortality in the reproduction tests (Figure 1) was below 20% at all tebuconazole treatments but reached in the test with the product 45 and 100% mortality at 1000 and 3160 mg product/kg soil d.w., respectively. Mortality was also above 20% in the three highest tested concentrations of IPBC in the reproduction test. Although not the standard endpoint, survival of adult collembolans was statistically evaluated as well deriving a median lethal concentration (LC_{50}) for 28 days of exposure (Table 1). However, mixture effects for the product could only be predicted as greater-than value due to the lack of lethal effects of tebuconazole in the reproduction test. The LC_{50} of the

product when based solely on the IPBC concentration equals 5.7 mg/kg d.w., which is about half of the LC_{50} of IPBC.

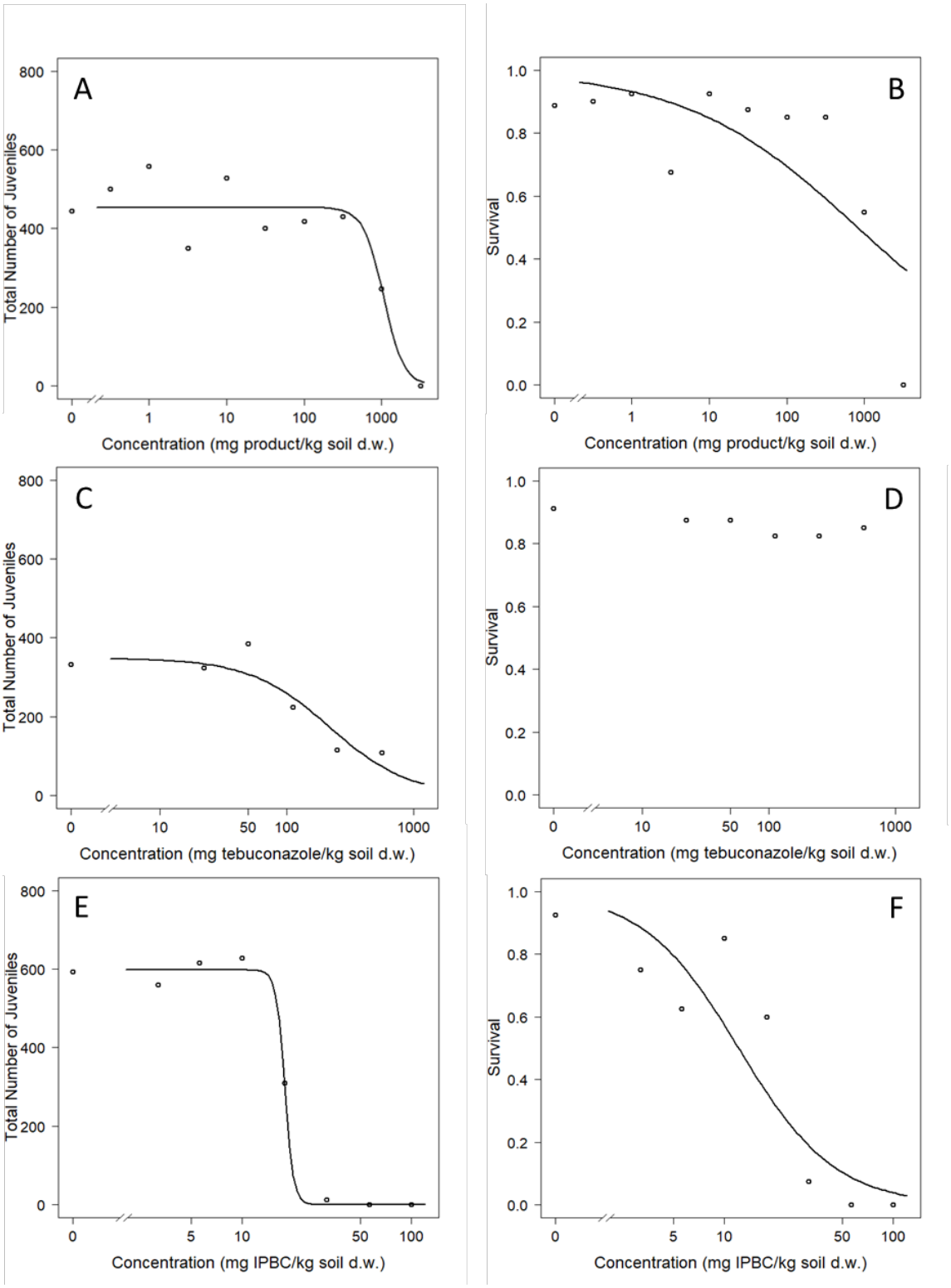

Figure 1: Total number of offspring (left) and survival of introduced collembolans (right) in the 28 day reproduction tests with *F. candida.* Shown are means per treatment and the fitted 3-parameter log-logistic regressions in dependence of nominal concentrations of the product (A, B), tebuconazole (C, D), and IPBC (E, F).

Table 1: Estimated Effect Concentrations (ECx), No Observed Effect Concentration (NOEC) and Lowest Observed Effect Concentration (LOEC) for *Folsomia candida* exposed to a commercial wood preservative formulation and to the individual active substances of this product. Reported endpoints are number of juveniles (reproduction) and survival of adults in the standard reproduction test and avoidance behaviour. Additionally, product toxicity as predicted by the CA model and the resulting Model Deviation Ratio (MDR) are provided for NOEC as well as EC_x values.

	Reproduction (28 days)		**Survival (28 days)**	**Avoidance (48 h)**
	NOEC (LOEC)	EC_{10} (95% CI)	LC_{50} (95% CI)	EC_{50}
Product (mg product/kg soil d.w.)	316.0 (1000)	544.9 (0-1487.4)	820.7 (139.2-1502.2)	67.6 (0-198.1)
Product (mg sum active substances/kg soil d.w.)	3.48 (11.0)	5.99 (0-16.35)	9.03	0.74
Tebuconazole (mg/kg soil d.w.)	50.0 (111.8)	44.5 (6.2-82.8)	>1250	14.2 (0-56.3)
IPBC (mg/kg soil d.w.)	10.0 (17.8)	15.70 (0-54.6)	12.17 (9.71-14.63)	260.9 (0-2596.4)
DCM (mg/kg soil d.w.)		not tested		>10
Mixture, predicted (mg sum active substances/kg soil d.w.)	14.10	20.53	>19.0	35.6
MDR	4.06	3.43	>2.11	48.0

In all three tests, LOECs, NOECs and 10% effect concentrations (EC_{10}) could be derived for the endpoint reproduction (Table 1). For tebuconazole, a NOEC of 50 mg/kg soil d.w. was determined, which is considerably below the NOEC of 250

mg/kg listed as endpoint in the regulatory dossier (EC 2007). For the product and IPBC, no data on chronic collembolan toxicity were available so far. Calculation of mixture toxicity considering only the active substances in the product (Table 1) indicated an about 3-fold deviation between prediction and observation based on the EC_{10}, while the deviation was slightly higher when based on the NOEC.

3.2 Reproduction tests

Collembolans increasingly avoided spiked soil with increasing concentrations of the test item in the tests with the product, tebuconazole and IPBC (Figure 2). Average mortality per treatment was below 20% in the avoidance tests with tebuconazole and IPBC, while mortality increased with increasing product concentrations up to 33% in the avoidance test with the product (Figure 2). An avoidance test with the solvent additive DCM revealed high mortality at concentrations of 100 and 1000 mg/kg soil with an estimated LC_{50} (48 h) for mortality of 302.9 mg/kg soil. At the highest valid treatment (i.e., with mortality below 20%), no avoidance response was observed, resulting in an EC_{50} for avoidance of >10 mg/kg soil (Table 1).

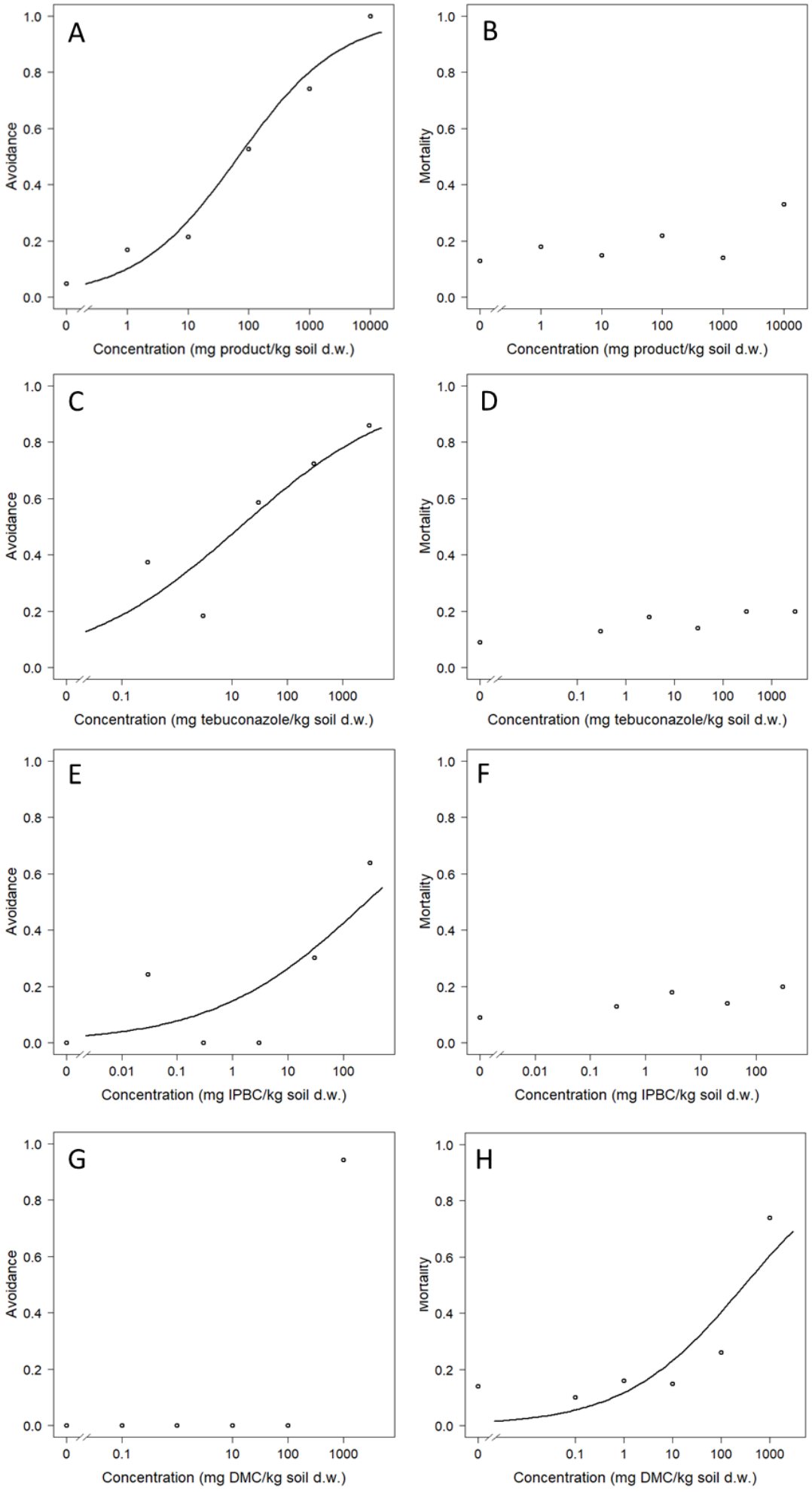
A
Avoidance
Concentration (mg product/kg soil d.w.)
B
Mortality
Concentration (mg product/kg soil d.w.)
C
Avoidance
Concentration (mg tebuconazole/kg soil d.w.)
D
Mortality
Concentration (mg tebuconazole/kg soil d.w.)
E
Avoidance
Concentration (mg IPBC/kg soil d.w.)
F
Mortality
Concentration (mg IPBC/kg soil d.w.)
G
Avoidance
Concentration (mg DMC/kg soil d.w.)
H
Mortality
Concentration (mg DMC/kg soil d.w.)

Figure 2: Avoidance behaviour (left) and mortality (right) in the 48 h avoidance tests with *F. candida.* Shown are means per treatment and the fitted 2-parameter log-logistic regressions in dependence of nominal concentrations of the product (A, B), tebuconazole (C, D), IPBC (E, F), and DCM (G, H).

The mixture assessment for avoidance behaviour indicated a strong underestimation by CA for the product when considering only the active substances: the avoidance response of collembolans for the product was almost 50-fold greater than predicted. Including the solvent DCM in the mixture calculation (with a value of 10 mg/kg soil as EC_{50}) reduced the underestimation to an MDR of 7.59. However, at the product EC_{50} for avoidance, the concentration of DCM was 1.11 mg/kg d.w., which is too low to cause on its own any avoidance response or mortality.

4 DISCUSSION

Reproduction was a more sensitive endpoint than survival in the chronic tests both with the single active substances as well as with the mixture. This is similar to a study that tested esfenvalerate, picoxystrobin and triclosan with *Folsomia fimetaria* (Schnug et al. 2014). The lethal effect of the product was most likely dominated by IPBC, as the LC_{50} for the product when related solely to the IPBC concentration differed approximately 2-fold from the LC_{50} determined for IPBC (Table 1).

Earlier studies with mixtures of copper and pesticides or metals, respectively, reported conflicting results regarding the predictability of mixture toxicity in other soil organisms: mixture effects on chronic endpoints were underestimated in *Caenorhabditis elegans* (Jonker et al. 2004), overestimated in *Enchytraeus albidus* (Lock & Janssen 2002), or concentration-dependent in the collembolan *Paronychiurus kimi* (Son et al. 2016). Chronic mixture toxicity of organic insecticides and herbicides was also mostly overestimated by CA for *F. candida* (Santos et al. 2010). However, these studies did not provide a quantitative estimate for the degree of deviation between predicted and observed mixture toxicity that could be compared to the results of the present study.

In the present study, the effect of the biocidal product on *F. candida* reproduction was fairly well predicted by CA, given a less than 4-fold underestimation of toxicity

based on the EC_{10} and a less than 5-fold underestimation based on the NOEC (Table 1). This deviation is only slightly larger than what has been considered an acceptable deviation of mixture predictions in the aquatic compartment (see e.g. Coors et al. 2018). Hence, these results support the application of CA in the regulatory context when it comes to chronic toxicity to soil invertebrates. However, the supporting evidence is rather limited given that only one formulated product was tested in one species and that at least some literature data do not support the use of CA. In addition, the tendency for underestimation of product toxicity indicates that a contribution of formulation additives to the overall effect on reproduction could have occurred in the reproduction test. Whether this can be attributed to the known hazardous additive, DCM, or other (unknown) additives remains unclear. An influence of environmental conditions on the mixture toxicity prediction is deemed highly unlikely, because toxicity is improbable to change much within the temperature range of 15-22°C and dependence on pH will also be rather limited for the three chemicals, which are not ionizable in the relevant pH range.

The effect of tebuconazole on avoidance behaviour was stronger than that of IPBC, while the effect on reproduction was weaker than that of IPBC (Table 1). Consequently, no consistent correlation was found between the two endpoints reproduction and avoidance. Yet, the endpoint reproduction is usually found to be more sensitive than avoidance in *F. candida*, as reported e.g. for two insecticides (Santos et al. 2012) as well as dredged sediments (Cesar et al. 2015). Hence, an avoidance response may be predictive or serve as warning for effects on reproduction in *F. candida* (Natal-da-Luz et al. 2009) but based on the results of the present study not necessarily for all types of chemicals.

In contrast to the endpoint reproduction, the avoidance response towards the product was strongly underestimated by the CA prediction when based only on the active substances contained in the product. This could be related to a greater uncertainty in the toxicity estimates for avoidance behaviour compared to those for reproduction. On a relative basis, the confidence intervals of the avoidance EC_{50} values were similarly broad as those of the reproduction EC_{10} values for the product, but up to 2-fold larger for the single substances. This difference renders the predicted mixture toxicity for avoidance somewhat more uncertain than that for reproduction, but it is deemed rather unlikely to cause the large observed

underestimation in the case of avoidance. DCM as the only known additive in the product could not explain the strong avoidance response to the product, since the additional test with this organic solvent alone detected no avoidance response at the relevant concentration range. Yet, the tested commercial product may have contained other additives, e.g. solvents, emulsifiers, surfactants and/or preservatives. One or several of them may have triggered an avoidance response, which could not have been included in the prediction due to the lack of information on ingredients.

Formulation additives such as solvents (including DCM) or emulsifiers may also interact with the bioavailability of chemicals or their uptake into organisms. This has been described as mechanism of toxicokinetic synergistic interaction (Spurgeon et al. 2010). When assuming such a synergistic interaction between formulation additives and the active substances it remains open, however, why it occurred only in the avoidance test but not similarly strong in the reproduction test. Time-dependence of mixture effects as discussed by Broerse & van Gestel (2010) may explain the lack of synergistic interaction in the chronic reproduction test. Yet, a simple explanation could also be a general inability of the CA model to predict behavioural responses. Few studies on avoidance behaviour of soil organisms tested the predictability of mixture toxicity (Santos et al. 2010; Loureiro et al. 2009), with the results being species- and contaminant-specific, but generally not showing indication for synergistic interaction. Hence, the most likely explanation for the unexpected high avoidance response towards the product appears to be the presence of unknown additives in this product that triggered avoidance behaviour but did not induce toxic effects on reproduction.

5 CONCLUSIONS

The present study showed that reproduction and avoidance behaviour of the collembolan *F. candida* were affected by a wood preservative product and its active substances tested individually. The CA model was able to predict the toxicity of the product with less than 4-fold underestimation with regard to the standard endpoint reproduction, but strongly (>40-fold) underestimated the behavioural avoidance response. Hence with regard to regulatory risk assessments, the application of the CA model as default for a theoretical mixture

toxicity assessment of the standard endpoint reproduction is supported by these results, although more studies are needed to confirm this predictability for other types of chemicals and other soil organisms. Using CA as default for behavioural responses such as avoidance, however, is not supported by the present study.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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SUPPLEMENTS

Table S1: Climatic conditions (pH and temperature) during exposure of *Folsomia candida* to a commercial formulation of a biocide (product), to the individual active substances (tebuconazole and IPBC) and to an organic solvent (dimethylcapramide) in OECD artificial soil, following the standard guidelines. Given ranges relate to parameters measured continuously during the test (temperature) or determined at test start and test end in control and treated samples (pH).

Test item	Test	pH soil	Temperature range (°C)
Product	Reproduction	5.7-5.9	14.6-21.9
Tebuconazole	Reproduction	6.7-6.8	17.9-21.1
IPBC	Reproduction	5.4-5.6	17.8-21.3
Product	Avoidance	5.6-5.9	18.2-20.9
Tebuconazole	Avoidance	5.7-5.9	19.1-20.8
IPBC	Avoidance	6.1-6.2	18.4-21.5
Dimethylcapramide	Avoidance	6.6-6.8	18.3-19.5
Required conditions according to both test guidelines		5.5-6.5	18.0-22.0

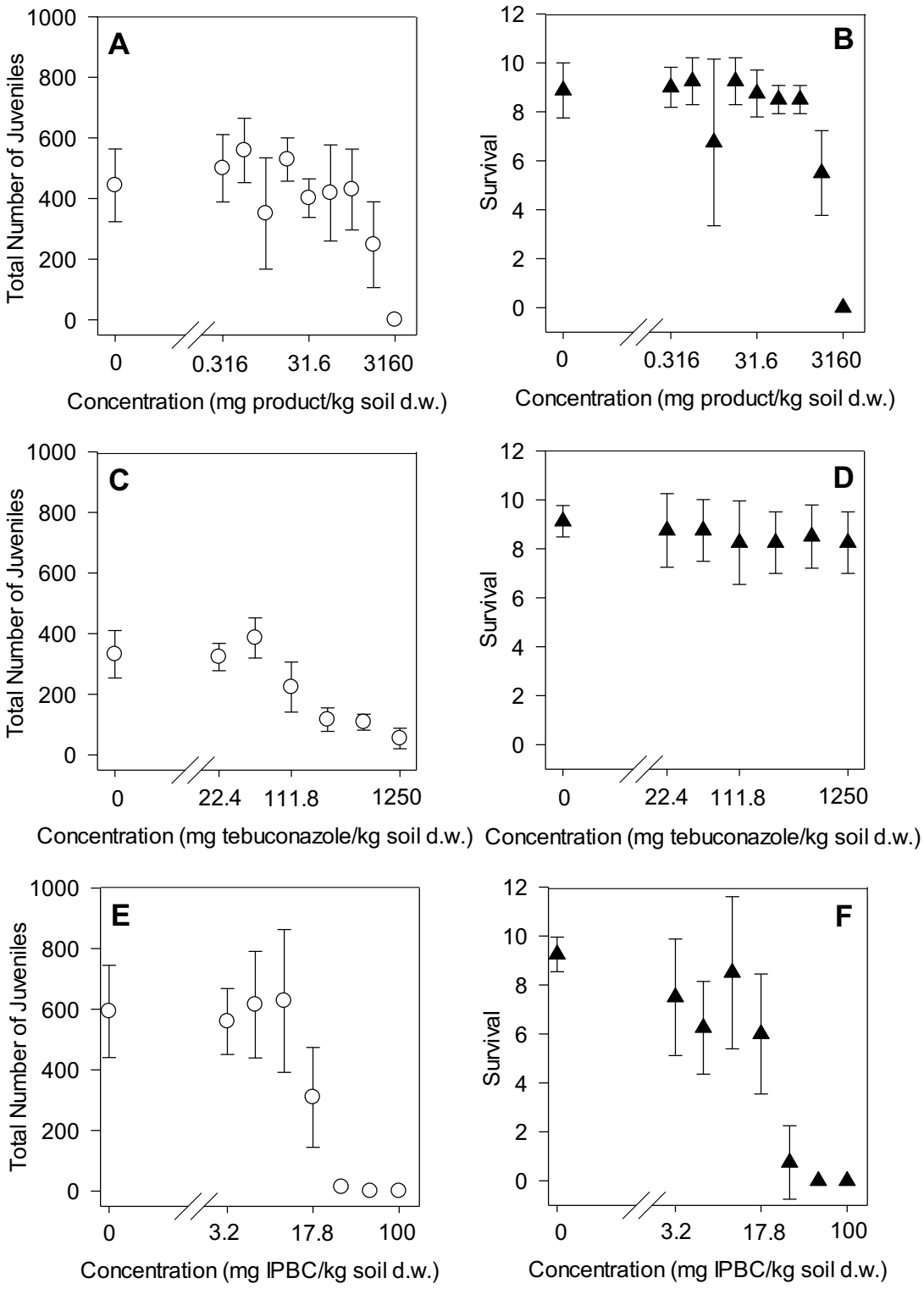

Figure S1: Total number of offspring (left) and survival of introduced collembolans (right) in the 28 day reproduction tests with *F. candida.* Shown are means per treatment ± standard deviation in dependence of nominal concentrations (log-scale) of the product (A, B), tebuconazole (C, D), and IPBC (E, F).

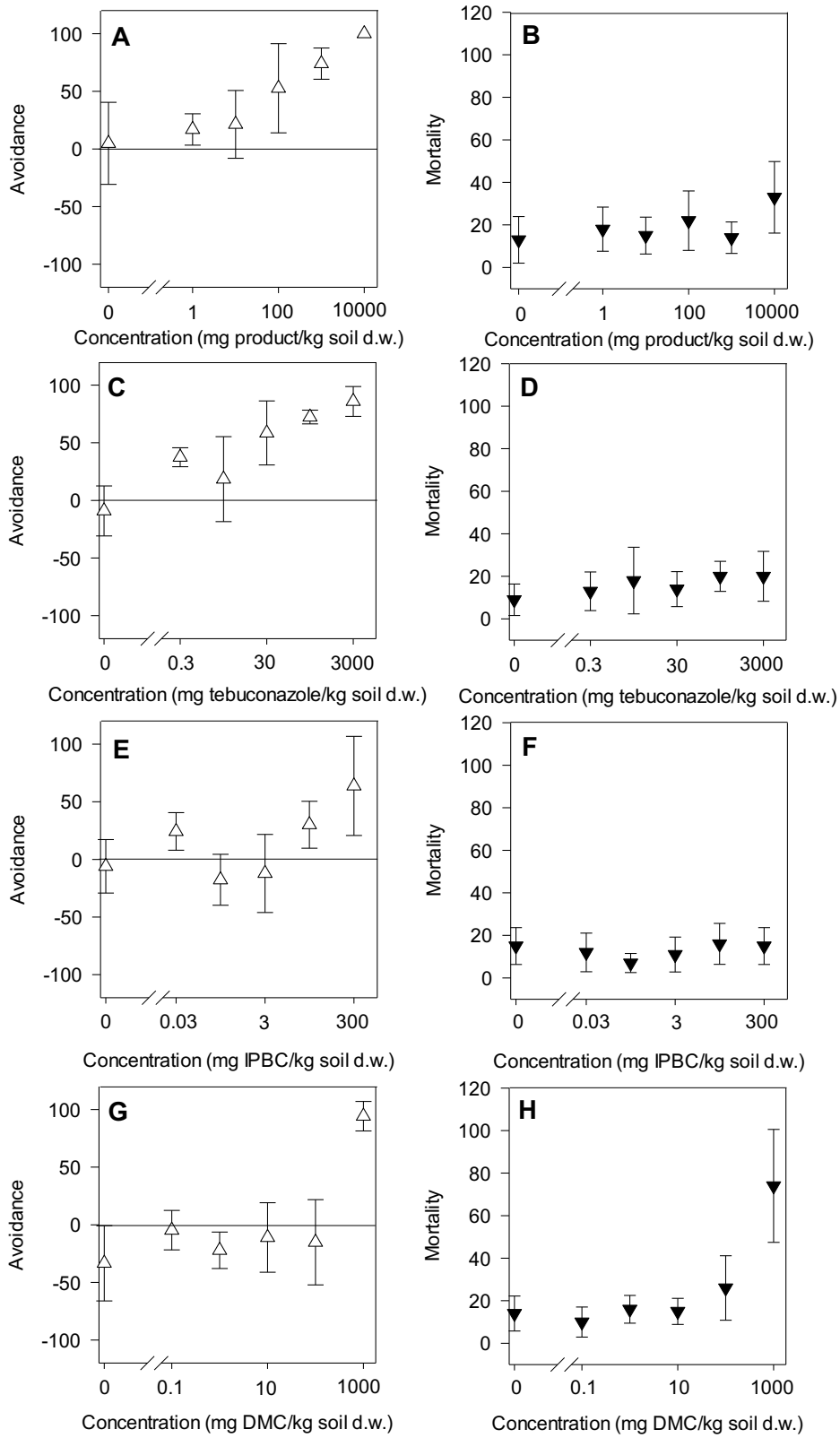

Figure S2: Avoidance behaviour (left) and mortality (right) in the 48 h avoidance tests with *F. candida.* Shown are means per treatment ± standard deviation in dependence of nominal concentrations (log-scale) of the product (A, B), tebuconazole (C, D), IPBC (E, F), and DCM (G, H). Note that average responses were not set to zero.

Chapter III

Multigenerational exposure of *Folsomia candida* to ivermectin – using avoidance, survival, reproduction, size and cellular markers as endpoints

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HIGHLIGHTS

- Multigenerational (MG) exposure for *F. candida* is not covered by the standard test.
- Impact of ivermectin was assessed during three generations (F1-F3).
- Similar toxicity for survival and reproduction between MG.
- Impact on size: organisms were smaller and more in F2 and larger and less in F3.
- The multi-endpoint strategy was beneficial to interpret long-term exposure.

ABSTRACT

In standard toxicity tests one generation of test organisms is used, and they are usually exposed only during a fraction of their life-cycle. This approach is very important but does not cover the potential effects of multigenerational (MG) exposure and may underestimate risks. Hence, the main aim of this study was to assess the MG impact of the veterinary pharmaceutical ivermectin (IVM) on *Folsomia candida* during three generations (F1-F3). Ivermectin is a veterinary medicine, persistent in the environment and toxic to non-target soil invertebrates. A suite of different endpoints was used including avoidance, survival, reproduction, size and other biomarkers (catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GR), glutathione S-transferase (GST), acetylcholinesterase (AChE) and lipid peroxidation (LPO)). Survival and reproduction were affected (LC50: 40 mg/kg; EC50: 5 mg/kg), but no avoidance occurred, which poses additional ecological concern. Exposure throughout the generations showed similar toxicity in terms of survival and reproduction. Regarding size there was an impact, e.g., organisms were smaller and more abundant in F2 and larger and less abundant in F3. This can have implications in terms of risk as e.g. smaller organisms can respond differently to stress compared to larger organisms in future generations. The antioxidant mechanisms were dynamically activated along the generations, e.g. in F1 CAT was increased whereas in F3 there was increased GST activity, which resulted in damage (LPO) for F1 and F2 organisms but not for the F3 generation. The multi-endpoint approach proved to be beneficial for the interpretation of results and we recommend it, especially for persistent and/or highly adsorptive chemicals, but also endocrine disruptors. Moreover, the evaluation of size as an additional sub-lethal endpoint has significantly added to the relevance of this test. The relative proportion of small, medium and large animals may be an even more relevant aspect of this endpoint. This does not require guideline modifications and is hence easily implementable.

Keywords: Antiparasitic drug; collembolans; transgenerational responses; soil ecotoxicity; multi-endpoint-approach.

1. INTRODUCTION

Organisms are often exposed to contaminants during several generations although standard toxicity tests (e.g., OECD 2009, ISO 2004) are based on one generation, and usually exposure occurs during a fraction of the life-cycle. This is of course a good compromise for feasibility purposes but does not cover the potential effects of multigenerational (MG) exposure and may underestimate risks. Long term exposure in soils is of high concern because persistent chemicals can be deposited for long periods of time, accumulate in soil, undergo transformation, etc., while the organisms can be continuously exposed. There are still very few studies among terrestrial species that cover MG exposure, examples include the oligochaete species *Enchytraeus crypticus* (Bicho et al., 2017; Menezes-Oliveira et al., 2013), *Eisenia fetida* (Schnug et al., 2013), and the collembolan species *Folsomia candida* (Amorim et al., 2017; Campiche et al., 2007; Paumen et al., 2008). Results differed, and this is not surprising since effects of multigenerational exposure of chemicals cannot be extrapolated from one endpoint to another due to biological and chemical differences

In the present study we assessed the multigenerational effect of ivermectin (IVM), a high environmental concern parasiticide widely used in veterinary medicine. IVM is partly metabolized by cattle, pigs and sheep and considerable amounts (up to 80% depending on the route of application and the treated farm animal) of the parent drug are excreted via faeces (Hennessy & Alvinerie 2002), finally reaching the soil. IVM is persistent in the environment (Kövecses and Marcogliese, 2005) and has been shown to be highly toxic to dung- (Madsen et al., 1990; Römbke et al., 2009) and soil-inhabiting invertebrates (Jensen & Scott-Fordsmand 2012; Jensen et al. 2003; Römbke et al. 2010). From standard laboratory as well as microcosm tests with IVM, it is assumed that collembolans are among the most sensitive soil organisms (Jensen & Scott-Fordsmand 2012; Jensen et al. 2003; Römbke et al. 2010). IVM causes neurotransmission failure because of neuromuscular synapses interference (Õmura, 2008), and is known to act by the interaction with glutamate-gated or γ-aminobutyric acid related chloride channels

in synapse membranes (Campbell, 1985; Duce and Scott, 1985), hence behavioural effects, e.g., avoidance, are a relevant endpoint.

Therefore, we aimed to assess the effects of multigenerational exposure to ivermectin using the soil ecotoxicity model species *Folsomia candida* (Collembola) (OECD 2009; ISO 2004), in terms of survival and reproduction, along 3 generations. In order to increase mechanistic understanding and thus the relevance of this study, avoidance behaviour and biomarkers involved in neurotransmission (AChE-acetylcholinesterase), biotransformation (GST-glutathione S-transferase), antioxidant defence (CAT-catalase, GPx-glutathione peroxidase, GR-glutathione reductase) and oxidative damage (LPO-Lipid Peroxidation) were also measured.

2. MATERIALS AND METHODS

2.1. Test organisms

The standard test species *Folsomia candida* (Collembola) was used. Cultures were kept on a moist substrate of plaster of Paris and activated charcoal (8:1 ratio), at 20±1°C, under a photoperiod of 16:8 (light:dark). Food consisted of dried baker's yeast (*Saccharomyces cerevisiae*) provided weekly. Age-synchronized juveniles (10-12 days) were used for the test.

2.2 Test substance, soil and spiking procedures

Ivermectin (IVM) (≥90% purity; Sigma-Aldrich) and the natural standard LUFA 2.2 soil (Speyer, Germany) were used. Soil characteristics are summarised as follows: pH (0.01 M $CaCl_2$) of 5.5±0.1, 1.61±0.15% organic carbon, 7.9±1.8% clay, 16.3±2.5% silt, and 75.8±3.9% sand.

Ivermectin is not water soluble, therefore acetone (100% purity; VWR Chemicals) was used as a solvent. Nominal test concentrations were 0-0.32-1-3.2-10-32-100 mg/kg soil dry weight (d.w.) for the survival, reproduction and avoidance tests and 0-1-3.2 mg/kg soil d.w. for the multigenerational test. The latter were selected based on the reproduction effect concentrations (0-EC10-EC50). Solutions were prepared and serially diluted and thoroughly homogenized with the soil. Acetone

was left to evaporate overnight. Water was added to the soil in order to achieve 40–60% of the maximum water holding capacity (WHC). In addition to a water control, a solvent control was used in all tests, resembling the maximum added volume of solvent with the ivermectin spiking.

2.3. Experimental procedure

2.3.1. Avoidance test

The avoidance test guideline ISO 17512-2 (2011) was followed, using the 2 chamber option. Circular plastic boxes (Ø 8 cm x 4.5 cm) divided in the middle by a removable plastic barrier were used. Five replicates were done. Half of each of the containers were filled with 30g of the control soil and the other half with 30g of the spiked soil. After removal of the plastic barrier, 20 juveniles (10-12 days old) were placed in the middle. The test was conducted for 48 h, at 20±2 ºC, under a photoperiod of 16:8 h (light:dark). At the end of the test, the plastic wall was placed in the middle section of each box and the soil from each half of the container was separated and put into new vessels, flooded with water and the number of floating individuals was counted directly.

2.3.2. Reproduction tests

The standard guideline OECD 232 (2009) was followed. In short, 10 organisms were introduced into each test vessel, containing 30g of moist soil. Five replicates were done. The test ran for 28 days at 20±2ºC, under a photoperiod of 16:8 h (light:dark). Food and water loss were replenished weekly. At test end, test vessels were flooded with water, the content was transferred to a crystallizer dish and the surface was photographed for further automatic counting using the software ImageJ (Schneider et al., 2012). Two endpoints were evaluated: survival and reproductive output.

2.3.3. Multigenerational test

Each multigeneration test was conducted following of the same OECD guideline 232 (2009), except that at test end the juveniles were sampled and further exposed. In short, at test end, the similar flooding and photographing procedure

for counting and measuring was done, both using the functions available in software ImageJ, and juveniles were transferred with a spoon to a box with a layer of Plaster of Paris (culture medium). For the exposure of the next generation, ten of the biggest juveniles (ca. 11 days old) were transferred to new test vessels, with freshly spiked soil. Additionally, 300 plus 150 juveniles were sampled in 2 microtubes, snap frozen in liquid nitrogen and stored at -80ºC, until further analysis. This was repeated for all 3 generations, i.e. 28, 56 and 84 days exposure for the three consecutive generations of juvenile collembolans. Five replicates were used for the controls and ten for each treatment, in order to ensure enough organisms to start the next generation tests and analysis. Three endpoints were evaluated: survival, reproductive output and size (area, mm^2).

2.3.4. Cellular and biochemical markers analysis

Procedures followed the previously optimized methodology as detailed by Maria et al. (2014). The selected biomarkers were catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GR), acetylcholinesterase (AChE), glutathione S-transferase (GST) and lipid peroxidation (LPO). In short, pools of 300 juveniles were homogenized in potassium phosphate buffer (0.1 mM, pH 7.4). For LPO, 4% BHT (2,6-dieter- butyl-4-metylphenol) in methanol was added to 150 µL of the homogenate and stored at -80ºC. The remaining 850 µL of the homogenate were centrifuged and the PMS (Post Mitochondrial Supernatant) was stored at -80ºC. Protein concentration was assayed using bovine γ - globuline as a standard adapted from literature (Bradford, 1976) in a 96-well flat bottom plate. For CAT, Clairborne (1985) was followed, as described by Giri et al. (1996). GPx, GR and GST activities were determined according to Mohandas et al. (1984), Carlberg & Mannervik (1975) and Habig et al. (1974), respectively, and as detailed in Maria et al. (2014). Lipid peroxidation (LPO) was determined according to Ohkawa et al. (1979) and Bird & Draper (1984), adapted by Filho et al. (2001). Acetylcholinesterase (AChE) activity was determined according to Ellman et al. (1961), adapted by Guilhermino et al. (1996).

2.4. Data analysis

Avoidance response (A) was calculated as the percentage of organisms that avoided the treated soil compared to the total number of organisms in the vessel, calculated as follows:

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

where C=number of organisms observed in the control soil; T=number of organisms observed in the test soil; N=total number of organisms per replicate. No avoidance or a non-response to the compound is considered when A is negative (ISO, 2011).

The Effect Concentrations (ECx) were calculated, based on nominal concentrations, using a logistic and threshold 2 parameters regression model (Toxicity Relationship Analysis Program (TRAP) – version 1.20, US EPA).

One-way analysis of variance (ANOVA), followed by the Post-Hoc test (Dunnett's or Holm-Sidak, $p<0.05$) was used to assess differences between control and treatments or between generations (SigmaPlot 12.0, 2011).

Results in terms of size were obtained for the various types of measurement, including length and area (mm^2). Area was selected as the most representative, although results followed the same pattern with the other size measure. In terms of size range distribution, the 3 main area size classes (mm^2) were: 1) Small (S): $S<0.05$, 2) Medium (M): $0.1>M>0.05$ and 3) Large (L): $0.8>L>0.1$.

3. RESULTS

3.1. Avoidance Test

Results for the avoidance behaviour test are presented in Figure 1.

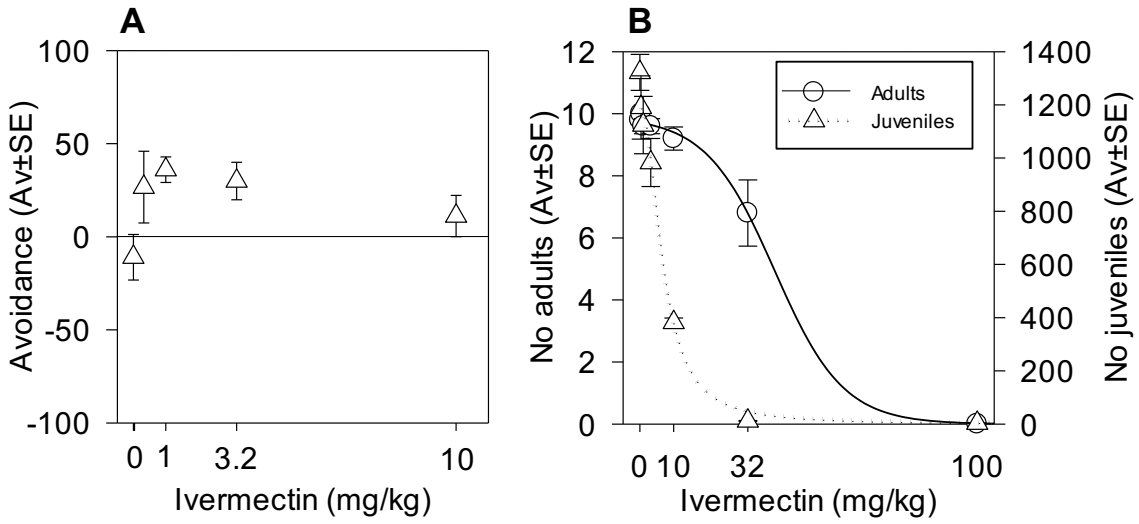

Figure 1: Results of *Folsomia candida* A) avoidance and B) reproduction test after exposure to ivermectin in LUFA 2.2. Values are expressed as average ± standard error (AV±SE). Lines represent model fit to data.

The test validity criteria were fulfilled (mortality <20% in all treatments). Avoidance was not significant in any treatment, although there was a tendency of increasing avoidance up to 1 mg/kg. Afterwards, a slight decrease did occur.

3.2. Reproduction Test

Validity criteria was fulfilled according to the guideline (mortality <20% and number of juveniles >100, coefficient of variation <30%), the pH showed a normal variation of 6.0±0.5 between treatments at test start and end.

Effects in the control and the solvent control were not significantly different, hence the control data is presented.

A dose-response effect was observed for both survival and reproduction for the tested range of ivermectin (Fig. 1). The estimated effect concentrations (ECx) are presented in Table 1.

Table 1: Estimated Effect Concentrations (ECx) for *Folsomia candida* exposed to ivermectin (mg/kg) in LUFA 2.2 soil following the standard guideline (exposure logged scale). Results from the multigenerational exposure estimates are shown

for relative comparison in terms of % reduction. Log. 2 par: logistic 2 parameters; Thresh 2 par: threshold sigmoid 2 parameters. S: Slope. Y0: top point. CI: 95% Confidence Intervals. n.d.: not determined.

	EC10 (mg/kg)	**EC20** (mg/kg)	**EC50** (mg/kg)	**Model parameters**
Standard test				
Survival (F0)	17.5 11<CI<25	25.8 22<CI<30	39.9 34<CI<46	S: 0.019; Y0: 9.8
Reproduction (F1)	0.4 0.2<CI<1	1.1 0.6<CI<2	5.1 3<CI<7	S: 0.945; Y0: 1190.2
Multigenerational test				
F1	10% reduction	20% reduction	50% reduction	
Reproduction	0.1 0.04<CI<0.5	0.3 0.1<CI<0.6	1.3 1<CI<1.6	S: 0.576; Y0: 840.44
Size juveniles	n.d.	1.9 0.2<CI<3.6	3.2 1.9<CI<4.6	S: 0.259; Y0: 9.47E-02
F2				
Reproduction	0.7 0.4<CI<1.2	1 0.7<CI<1.5	2.3 1.9<CI<2.9	S: 1.036; Y0: 811.8
Size juveniles	n.d.	1.5 -0.5<CI<3.6	4.3 0.8<CI<7.7	S: 0.135; Y0: 0.128
F3				
Reproduction	0.9 0.8<CI<1	1.1 1<CI<1.2	1.7 1.6<CI<1.9	S: 1.994; Y0: 1444.6
Size juveniles	n.d.	2.7 0.4<CI<4.8	4.6 -0.2<CI<9.3	S: 0.182; Y0: 0.1195

3.3. Multigeneration test

Results of the multigenerational test (3 generations) in terms of survival, reproduction and the size of adults and juveniles are presented in Figure 2.

Differences between control and solvent control were significant in F3 (with more animals in the solvent control). Comparison between generations showed that the survival of the organisms was similar in all generations. In terms of reproduction there was a relatively higher number of juveniles in F2 and F3, this being significantly higher for exposure to 1 mg/kg in F3.

There was a decrease in the average size with increasing ivermectin concentration, both for adults and juveniles, although not significant (Fig. 2).

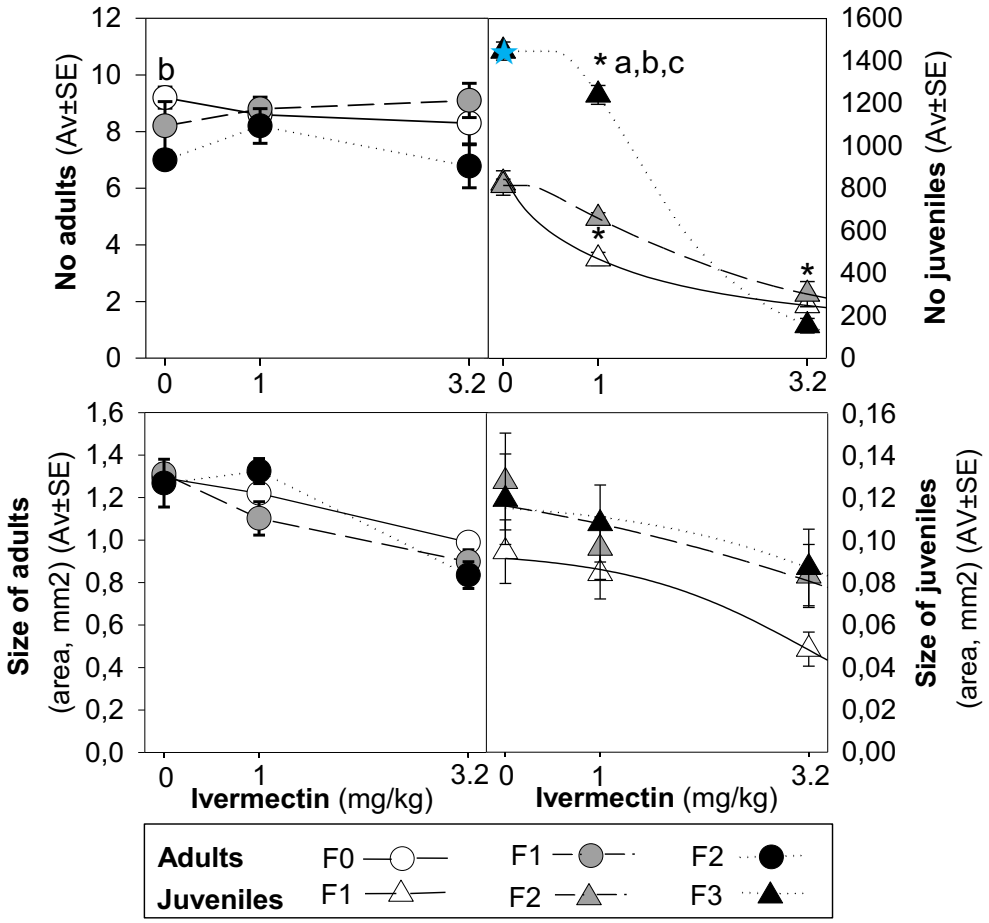

Figure 2: Results of the *Folsomia candida* multigenerational test (endpoints: survival, reproduction and size) after exposure to ivermectin (0-1-3.2 mg/kg, i.e. ca. 0-EC10-EC50) in LUFA 2.2 soil for 3 generations (F1, F2, F3). Values are expressed as average ± standard error (AV±SE). $p<0.05$*: between control and treatments, a: between F1-F2, b: between F1-F3 and c: between F2-F3. ★: solvent control data used (control=647±70; solvent control=1445±43 juveniles).

The detail of the different size classes and numbers of individuals (Fig. 3) showed e.g. that after the first exposure to ivermectin, juveniles are more but smaller (size: F2<F1) when exposed to 1 mg/kg (ca. EC10) and as many but smaller (size: F2<F1) when exposed to 3.2 mg/kg (ca. EC50).

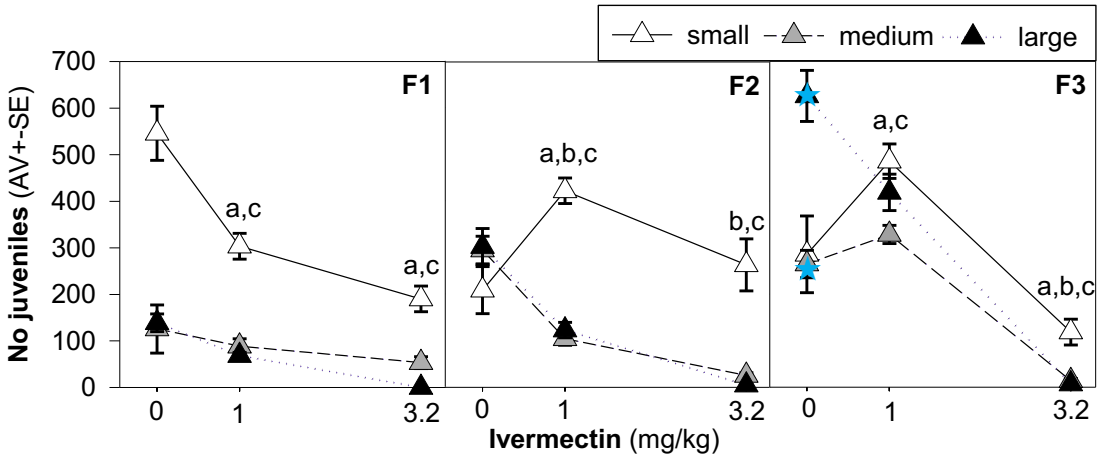

Figure 3: Results of the *Folsomia candida* multigeneration test (endpoint: size as area) after exposure to ivermectin (0-1-3.2 mg/kg, i.e., ca. 0-EC10-EC50) in LUFA 2.2 soil for 3 generations (F1, F2, F3). Values are expressed as average ± standard error (AV±SE). Small (S): S<0.05, Medium (M): 0.1>M>0.05 and Large (L): 0.8>L>0.1. p<0.05: a, b, c- comparison between control and treatments (t-test) in small, medium and large size groups, respectively. ★: solvent control data used: control: (L=224±18; M: 119±6), solvent control (L=626±55; M: 265±30).

In F3 differences between control and solvent control were observed, with significantly higher numbers of large and medium juveniles in the solvent control. Hence this seems to confirm that the solvent acetone also affected this endpoint, in this case showing a shift towards larger animals (size: F3>F2).

3.4. Cellular and biochemical markers

Due to mortality it was not possible to analyse results for 3.2 mg/kg (fig. 4).

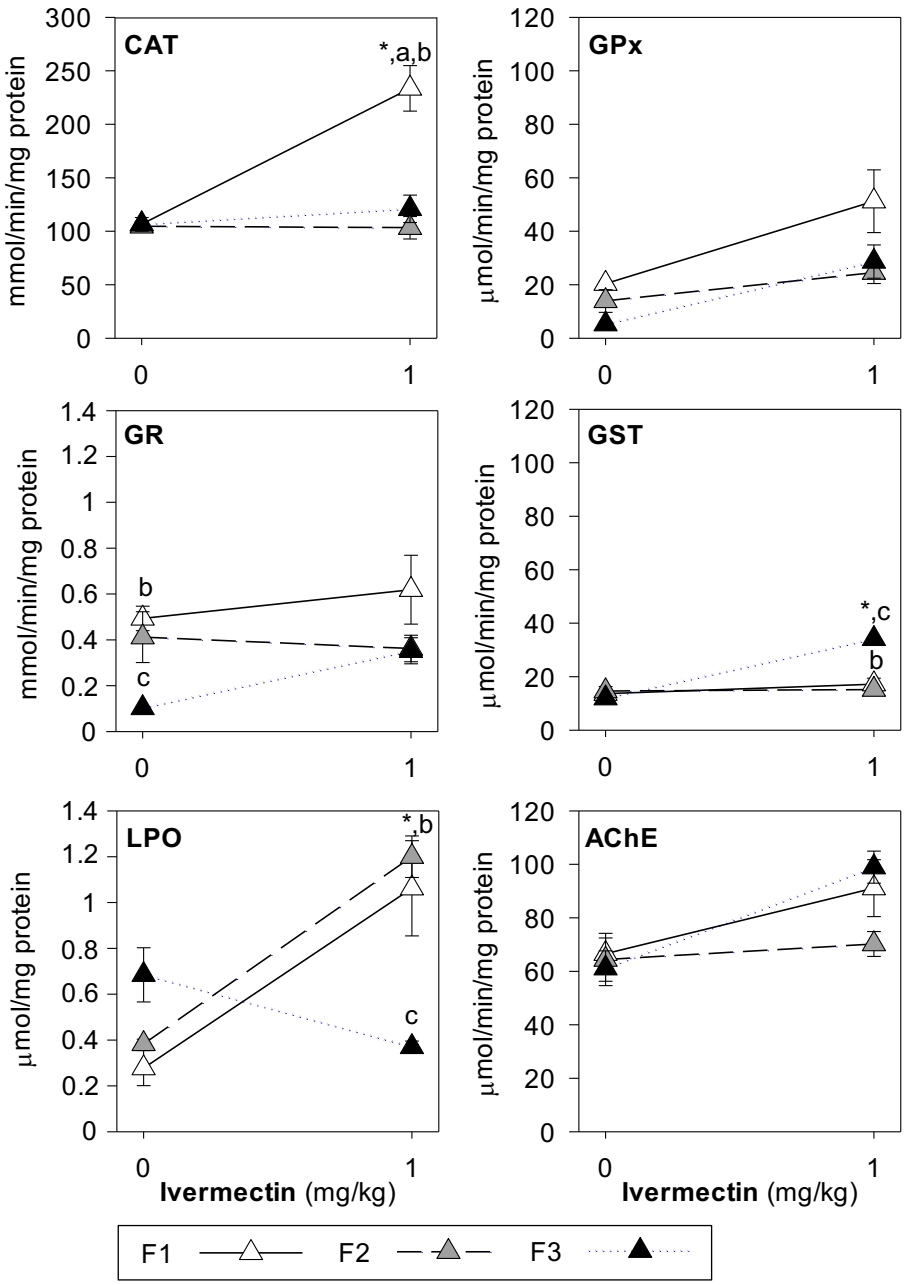

Figure 4: Results of *Folsomia candida* multigeneration test (endpoint: cellular and biochemical markers) after exposure to ivermectin (0-1 mg/kg) in LUFA 2.2 soil for 3 generations (F1, F2, F3, i.e. 28, 56, 84 days). Values are expressed as average ± standard error. Acetylcholinesterase (AChE), glutathione peroxidase (GPx), catalase (CAT), glutathione S-transferase (GST), glutathione reductase (GR) activities and lipid peroxidation (LPO). $p<0.05$ *: comparison between control and treatments (t-test); a: between F1-F2, b: between F1-F3 and c: between F2-F3 (ANOVA; Holm-Sidak).

Results showed an increase of CAT for F1 (after one ivermectin exposure cycle), which was absent in the next generations (F2-F3) (Fig. 4). For GST, the activity increased at F3. An increase in GPx and GR observed in F1 was not significant. For AChE, there was an overall increase except for F2. LPO shows that damage occurred for the F1 and F2 organisms ($p<0.001$), with a clear shift to decreased damage in F3, resembling a recovery scenario.

4. DISCUSSION

Results showed that *F. candida* did not avoid ivermectin contaminated soil in a dose response pattern, i.e., although there was a tendency to avoid concentrations up to 1 mg/kg, exposure to higher concentrations caused similar or lower avoidance, meaning that this endpoint is difficult to assess. Similarly, other compounds have been shown to interfere with the ability to avoid in *Folsomia candida*, e.g. boric acid (Amorim et al., 2012) or dimethoate (Pereira et al., 2013). For dimethoate exposure, a correlation between non-avoidance and AChE inhibition was shown. In the present study with ivermectin the measurements of AChE (which could indicate if the lack of avoidance would be related with the neurotransmitter blockage), were inconclusive due to the high mortality in higher concentrations of no avoidance and longer exposure duration compared to the avoidance setup. Nevertheless, Torkhani et al. (2011) has reported that *Eisenia fetida* is "attracted" to IVM (8-256 mg/kg) in an avoidance test setup exposure. Other examples include decreased locomotor capacities in ivermectin exposed beetles (Verdú et al. 2015). In fact, there are good reasons that IVM affects organism groups depending on their ancient phylogenetic patterns, meaning that sensitivity to ivermectin is compatible with recent phylogenomic hypotheses grouping the Nematoida with the Arthropoda as Ecdysozoa (moulting animals), in contrast to, among others, Oligochaeta (Puniamoorthy et al., 2014), i.e., the effects observed by *F. candida* (Arthropoda), which are comparable to those observed by beetles (and nematodes) can be in part explained by the recent phylogenetic hypothesis proposed by Pumiamoorthy et al (2014).

Such avoidance inhibition has been observed also in other species and compounds (e.g. in enchytraeids for boric acid (Amorim et al., 2012), LAS (Linear Alkylbenzene Sulfonate) and TBTO (Tributyltin oxide) (Amorim et al., 2008)). One of the main concerns are the ecological implications of this kind of endpoint: if organisms are not able to avoid a certain compound in the field (where soil patchiness is known to still offer an escape for, in particular, small mesofauna species such as collembolans) then the risk on the population level is probably much higher in comparison to an assessment based solely on effects on survival and, especially, reproduction (Ockleford et al., 2017).

Reproduction

As recorded, effects on reproduction showed an EC50 of ca. 4 mg IVM/kg. This is higher than effect levels observed by Römbke et al. (2010) in an OECD soil with similar total organic content.

The apparent difference of reproduction EC values can be due to relatively steep dose-response curve found for the effects of ivermectin. This is one of the chemicals where *Folsomia candida* is a particularly sensitive species compared to other soil organisms (Jensen and Scott-Fordsmand, 2012) including oligochaetes (Jensen et al., 2003). Other results available show variations in different soils, e.g. a natural loamy sand (Förster et al., 2011), a natural sandy clay loam (Jensen et al., 2009), or a tropical artificial soil (Zortea et al., 2017).

Common across these studies is also the lower impact on the adults, showing that the effects on the endpoint reproduction are not due to adult mortality, hence it is not a narcotic type of effect. An increase in reproduction after exposure to acetone has been observed before (Römbke et al. 2010). Further, it seems that multigenerational exposure to acetone exponentiates the effects in F3.

Results from the multigenerational exposure showed that the reproduction effect was similar to the comparable F1 in literature (Römbke et al. 2010) and within generations (F1-F3). Interestingly, adding an additional endpoint size, we could quantify that ivermectin affects body size. This has not been shown before for ivermectin. The model for size is merely indicative (as there are too few data points) but shows that size (average) and reproduction were similarly affected. Although, the relative proportion of small, medium and large animals may be an

even more relevant aspect of this endpoint. After the first exposure to ivermectin, the number of juveniles were greater, but smaller (size: F2<F1) when exposed to 1 mg/kg (ca. EC10) indicating a stress mechanism activation. This type of R strategy – the ability to reproduce rapidly (exponentially) – is usually linked to relatively little investment in other individual assets, i.e. they are typically weak or smaller and, thus, subject to predation and stress. The exposure to higher ivermectin concentration of 3.2 mg/kg (ca. EC50) also caused a decrease in size (size: F2<F1), although reproduction did not increase in F2 compared to F1 (as occurred for the lower concentration). Evidences are that there is a compromise between energy allocation for size and reproduction, which is dependent on the concentration. At least for *Folsomia candida* this has also been observed in a long multigenerational exposure to cadmium (Amorim et al., 2017). The authors suggested that among the reasons for the extended survival to continuous exposure to the EC50 (and not to the EC10) was an investment in terms of optimal size for survival. For ivermectin multigenerational exposed *Folsomia candida* this response mechanism seems to be more transient than for Cd, as based on F3 observations of larger animals. These results would indicate a shift to the opposite K strategy, with a significantly higher number of large and medium juveniles (size: F3>F2). The effects of the solvent acetone itself in the MG is not possible to disregard in F3, as observed by the significant difference between control and solvent control alone. Therefore, we recommend to add the measurement size as an additional endpoint of the collembolan reproduction test (OECD, 2009). Overall, the fitness of the organisms may be assessed by their growth (Fountain and Hopkin, 2001; Hopkin, 1997; Scheu and Simmerling, 2004), since a minimum size is required to be able to reproduce (Crommentuijn et al., 1993). So, small reproduction rates can be related to a decrease in growth, probably by a reduction in the metabolic activity (Crouau and Moia, 2006; Smit and Van Gestel, 1997).

From the recorded cellular and biochemical markers there are indications of activation of stress response mechanisms after exposure to IVM during one and two generations, e.g. CAT increased in F1 juveniles, and LPO was measured in F1 and F2 but not in F3 hence there was an activation of a mechanism towards “homeostasis”, also with the antioxidant levels returning to basal levels (e.g. GPx,

GR). This is also in agreement with the apparent change in strategy from F1 to F2 and then F3, where more energy would be required to activate these antioxidant enzymes in the first generation(s), establishing homeostasis, after which the opposite occurs, no damage is measured in F3 and less energy was required.

Other studies have shown the activation of this mechanism after exposure to ivermectin, e.g. GPx in the aquatic vertebrate *Clarias gariepinus* (Ezenwaji et al., 2017) or CAT and GST in *Danio rerio* (Domingues et al., 2016). The "renovation" of GSH by GR activity seems to be occurring in F3, this combined with GST that was significantly increased. The positive interaction between these complementary enzymes is well known (Meister 1995a; Saint-Denis et al. 1999; Saint-Denis et al. 2001). To summarise, it seems that the initial effort made by the antioxidant system was not successful to prevent oxidative damage (LPO increased in F1 and F2), which could indicate inefficacy of the activation of CAT and GPx. On the other hand, the "joint work" of GST/GR enzymes seemed more efficient given no oxidative damage (decreased LPO in F3). This could be the result of a re-iteration of the antioxidant system, starting to respond with CAT and feedback after to GST activation.

The half-lives of IVM in 3 natural soils (sandy loam) under aerobic conditions have been reported as 16, 37 and 67 days, while under anaerobic conditions no significant dissipation up to 120 days occurred (Krogh et al., 2009). Using the main soil properties (e.g. pH, CEC, OC and texture) of LUFA 2.2 for comparison with the York soil used by Krogh et al. (2009) the half-life of IVM is at least as high as 67 days (York soil). Hence in the present multigenerational study where soil was freshly spiked with 1 and 3.2 mg/kg soil d.w. every 28 days these concentration levels were probably maintained during the test period.

In the following, we used the EC10 values determined in this study in comparison to the NOEC (No Observed Effect Concentrations) values used by Liebig et al. (2010) – a practice agreed-on by these authors in cases where NOEC values were not available. Liebig et al. (2010) published the most comprehensive risk assessment done for ivermectin so far, meaning that we can discuss whether, and if yes, how our results would modify the outcome presented in the literature (Table 2). Using always the worst-case assumptions for the determination of the PEC

(Predicted Effect Concentration) listed by Liebig et al. (2010) it becomes clear that a relatively small increase in sensitivity (the NOEC decreases from 300 µg/kg soil d.w. to 100 µg/kg soil d.w.) changes the outcome of the risk assessment. Interestingly, an even stronger change was found by Jensen et al. (2009), who studied ivermectin in a two-species laboratory test consisting of the Collembolan *Folsomia fimetaria* (a species closely related to *Folsomia candida*) and the predatory mite *Hypoaspis aculeifer*. However, when comparing similar endpoints such complex multi-species tests require higher efforts than multigeneration tests, in particular if effects occur already in the F2 generation.

Table 2: Worst case risk assessment according to the rules of the European Union (VICH, 2004, 2000) for the effects of ivermectin on collembolans (*Folsomia candida*), comparing our results with the data from (Liebig et al. (2010). All data are given in µg/kg soil d.w..

Test method	**Effect concentration (NOEC/EC10)**	**Assessment factor**	**PNEC**	**PEC (worst case)**	**RQ (worst case)**
Initial risk assessment					
OECD	300	10	30	6.08	0.20
New method	100	10	10	6.08	0.61
Refined risk assessment					
OECD	300	10	30	11.4	0.38
New method	100	10	10	11.4	**1.14**

NOEC: No Observed Effect Concentration; EC: effect concentration; PNEC: Predicted No Effect Concentration; PEC: Predicted Effect Concentration; RQ: risk quotient. RQ values in bold indicate a risk of ivermectin to *Folsomia candida*.

5. CONCLUSIONS

Exposure to ivermectin in *Folsomia candida* showed that (almost) no avoidance behaviour occurred, although survival and reproduction were highly affected. The multigenerational exposure showed no variation in terms of the EC values for survival and reproduction along three generations. Nevertheless, there were shifts in energy allocation between size and reproduction within the three generations, i.e., more organisms were smaller in F2 and more were larger in F3. This can have implications in terms of the associated risk for the next generations. The antioxidant mechanisms were activated with updated activities along the generations, e.g. in F1, CAT was increased whereas in F3 there was an increased activity of GST, which resulted in damage (LPO) for F1 and F2 but not for F3. The multi-endpoint approach proved to be beneficial for the interpretation of results and we recommend it. Moreover, the evaluation of size as an endpoint from the standard test with *Folsomia candida* has significant added value. This does not require any modifications on the protocol, except for the additional work in terms of image treatment, thus, it is highly recommended.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ETHICAL APPROVAL

This article does not contain any studies with animals performed by any of the authors.

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Chapter IV

Exposure of *Folsomia candida* (Willem 1902) to teflubenzuron over three generations – Increase of toxicity in the third generation

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HIGHLIGHTS

- The standard collembolan test is based on exposure of one generation.
- *F. candida* did not avoid soil spiked with teflubenzuron.
- Toxicity to *F. candida* survival and reproduction increased in the 3rd generation.
- Juveniles were smaller after exposure to teflubenzuron.

ABSTRACT

The potential implications of long-term exposure to contaminants are not covered by current standard toxicity guidelines, usually referring to one generation and a fraction of the life cycle of the test species. Hence, in the present study, we aimed to assess the effects of the multigenerational exposure (generation 1-3: F1-F3) of *Folsomia candida* to an insect growth regulator (IGR) compound: teflubenzuron (TFB). The selected endpoints included both the standard ones as in the OECD and ISO guidelines (survival, reproduction, and avoidance) as well as additional ones (organisms' size and cellular/biochemical markers: acetylcholinesterase, glutathione S-transferase, catalase, glutathione peroxidase, lipid peroxidation). Although no avoidance behaviour was recorded at field-relevant concentrations (PEC (Predicted Environmental Concentration)=0.06 mg/kg soil dry weigh (d.w), survival and reproduction were impacted (LC50=0.1 mg/kg soil d.w; EC50=0.05 mg/kg soil d.w.). Multigenerational exposure to TFB caused increased toxicity in *F. candida* in F3 in terms of survival and reproduction. This could be related to the mode of action of TFB which does not seem to activate some of the general stress mechanisms of response like oxidative stress. In addition, TFB causes a reduction of the organisms' size, with a reduction of the number of large-sized juveniles, which has potential adverse consequences in terms of organisms' performance, e.g. change in age structure and hence population dynamics. Hence, both observations may increase the environmental concern and associated risk of this insecticide.

Keywords: Insect growth regulator, size, collembolans, soil ecotoxicity, biomarkers, behaviour.

1. INTRODUCTION

Organisms can be exposed to contaminants for long periods of time during several generations, especially in the case of persistent chemicals that accumulate in the soil. The potential effects of long-term exposure of organisms to contaminants can

be underestimated by current standard toxicity tests (e.g., OECD, 2009; ISO, 2014), which are based on the exposure during a fraction of the organisms' life-cycle. A few studies have focused on the effects of multigenerational exposure, e.g. with collembolans (Amorim et al., 2017; Campiche et al., 2007; Ernst et al., 2016; Mendes et al., 2018; Paumen et al., 2008; van Gestel et al., 2017) and oligochaetes (Bicho et al., 2017; Menezes-Oliveira et al., 2013; Schnug et al., 2013). Studies indicate for instance that effects on *Folsomia candida* (Willem, 1902) reproduction can occur even after transferring the organisms to clean media during two subsequent generations (Campiche et al. 2007). This was the case for insect growth regulators (IGR) like methoprene, fenoxycarb, precocene II and teflubenzuron.

Teflubenzuron (TFB) is an IGR that belongs to the benzoylureas group and acts by inhibition of chitin synthesis and moulting processes. Female fertility of insects may be reduced after contact or ingestion with TFB (EFSA, 2008a). It has been widely used in agriculture (EFSA, 2008b), and has moderate to high persistency in soil (Cycoń et al., 2012), with a DT50 of 30-152 days (EFSA, 2008a). Its predicted environmental concentration (PEC) in soil is 0.06 mg/kg soil d.w. (Campiche et al., 2006).

Since the information regarding the effects of TFB on soil organisms is limited, we aimed to assess the effects of multigenerational exposure to TFB over 3 generations on survival and reproduction of *F. candida* (Collembola), standard soil ecotoxicity test species (OECD, 2009; ISO, 2014). Additional endpoints included organisms' size and biomarkers involved in neurotransmission (acetylcholinesterase), biotransformation (glutathione S-transferase), antioxidant defence (catalase and glutathione peroxidase) and oxidative damage (lipid peroxidation) were also measured to help understanding the mechanisms involved after long exposure.

2. MATERIALS AND METHODS

2.1 Test organisms

Tests were performed with the standard test species *F. candida* (Collembola). Organisms were cultured on a moist substrate of plaster of Paris and activated charcoal (8:1 ratio), under a photoperiod of 16:8 (light:dark), at 20±1°C. Individuals were fed once a week with dried baker's yeast (*Saccharomyces cerevisiae*). Cultures were synchronized to obtain 10-12 days old juveniles.

2.2 Test substance, soil and spiking procedures

The insecticide teflubenzuron (TFB) (≥98% purity; Sigma-Aldrich) was tested. TFB (molecular weight (M)=381.11 g/mol; n-octanol-water partition coefficient (K_{OW})=2×10^4 (pH7, 20ºC); adsorption coefficient (K_d): 169-944 ml/g) has low solubility in water (0.01 mg/L), therefore acetone (100% purity; VWR Chemicals) was used as a solvent. The natural standard LUFA 2.2 soil (Speyer, Germany) is characterized as follows: pH (0.01 M $CaCl_2$); 5.5±0.1; organic carbon: 1.61±0.15%; texture: 7.9±1.8% clay, 16.3±2.5% silt, and 75.8±3.9% sand content.

The TFB concentrations used in the survival, reproduction and avoidance tests were 0, 0.0032, 0.01, 0.032, 0.1, 0.32 mg/kg soil dry weight (d.w.). They were selected based on information from literature (Campiche et al., 2006). For the multigenerational test, the concentrations used were 0, 0.027, 0.064 mg/kg soil d.w., which corresponded to the sub-lethal effective concentrations of EC10 and EC50 on reproduction, respectively. Solutions were prepared with acetone, serially diluted and thoroughly homogenized with the soil. Freshly spiked soil was used in each generation. After acetone evaporation overnight, water was added to the soil until 50% of the maximum water holding capacity (WHC) was achieved. In addition to water control, a solvent control spiked with the maximum amount of solvent was prepared in all tests.

2.3 Experimental procedure

2.3.1 Avoidance test

The two-chamber option of the avoidance test guideline ISO 17512-2 (2011) was followed, using circular plastic boxes (Ø 8 cm × 4.5 cm) divided in the middle by a plastic card. In short, half of each container was filled with 30 g of the control soil and the other half with 30 g of the spiked soil. Twenty juveniles (10-12 days old) were placed in the middle, after removal of the plastic card. Five replicates were used. The test duration was 48 h, at 20±2ºC, under a photoperiod of 16:8h (light:dark). At the test end, the plastic barrier was inserted in the middle section of the containers. The soil of each half of the boxes was separated into new vessels and then flooded. The number of floating springtails was counted directly.

2.3.2 Reproduction tests

Tests followed the standard guideline OECD 232 (2009). In short, each test vessel contained 30 g of moist soil with food (baker's yeast) after which 10 juveniles were introduced, and the vessel was covered with parafilm with holes to allow aeration. Five replicates were done. Test conditions were 20±2ºC and 16:8h (light:dark) photoperiod. Food and water loss were replenished weekly. After 28 days, test vessels were flooded with water and the content was transferred to a crystallizer dish. The surface was photographed for further automatic analyses (count and measure) using the software ImageJ (Schneider et al., 2012). The survival and reproductive output were evaluated.

2.3.3 Multigenerational test

Each multigeneration test was conducted following a modified version of OECD 232 (2009). All procedures were the same and at test end, after the similar flooding and photographing procedure for counting, juveniles were transferred with a spoon to a box with a layer of Plaster of Paris (culture medium) which adsorbed extra water from the spoon. For the exposure of the next generation, ten of the biggest juveniles (ca. 11 days old) were transferred to new test vessels, with freshly spiked soil. Additionally, 300 plus 150 juveniles were pooled per replicate and sampled in 2 microtubes, snap frozen in liquid nitrogen and stored at -80ºC, till

further analysis. This was repeated for all 3 generations, i.e. 28, 56 and 84 days exposure for the three consecutive generations of juvenile springtails. It was not possible to analyse results for the concentration of 0.064 mg/kg soil d.w. due to an insufficient number of juveniles. Five replicates were used for the controls (water and solvent) and ten for each treatment, in order to ensure enough organisms to start the next generation tests. Three endpoints were evaluated: survival, reproductive output, and organisms' size (area, mm^2).

2.3.4 Cellular and biochemical markers analysis

Procedures followed the methodology previously optimized by Maria et al. (2014). The selected biomarkers were catalase (CAT), glutathione peroxidase (GPx), acetylcholinesterase (AChE), glutathione S-transferase (GST) and lipid peroxidation (LPO). To summarize, pools of 300 juveniles per replicate were homogenized in potassium phosphate buffer (0.1 mM, pH 7.4). For LPO, 2.5 µL of 4% 2,6-di-tert-butyl-4-methylphenol in methanol was added to 150 µL of the homogenate and stored at -80ºC. The remaining 850 µL of the homogenate was centrifuged and the post-mitochondrial supernatant was stored at -80ºC. Protein concentration was assayed using bovine γ - globuline as a standard adapted from literature (Bradford, 1976) in a 96-well flat bottom plate. For CAT, Clairborne (1985) was followed GPx, GR and GST activities were determined according to Mohandas et al. (1984), Carlberg and Mannervik (1975), and Habig et al. (1974), respectively, and as detailed in Maria et al. (2014). Lipid peroxidation (LPO) was determined according to Ohkawa et al. (1979) and Bird and Draper (1984), as adapted by Filho et al. (2001). AChE activity was determined according to Ellman et al. (1961), as adapted by Guilhermino et al. (1996).

2.4 Data analysis

Avoidance response (A) was calculated as the percentage of organisms that avoided the treated soil compared to the total number of organisms in the vessel, calculated as follows:

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

where C=number of organisms observed in the control soil; T=number of

organisms observed in the test soil; N=total number of organisms per replicate. No avoidance or a non-response to the compound is considered when A is negative (ISO, 2011).

The effect concentrations (ECx) were calculated using a logistic and threshold 2 parameters regression model (Toxicity Relationship Analysis Program (TRAP) – version 1.20, US EPA).

One-way analysis of variance (ANOVA), followed by the Dunnett's post-Hoc test was used to assess differences of survival and reproduction between control and treatments, and differences between different generations within the same treatment (SigmaPlot 12.0, 2011). For biomarkers data, a t-test was used to assess differences between control and the one treatment.

Results in terms of size were obtained for the various types of measurement, including organism's length and area (mm^2). Area was selected as the most representative, although results followed the same pattern with the other size measure. In terms of size range distribution, the 3 main organisms area size classes (mm^2) were: 1) Small (S): $S<0.05$, 2) Medium (M): $0.1>M>0.05$ and 3) Large (L): $0.8>L>0.1$.

3. RESULTS

3.1 Avoidance Test

Validity criteria were fulfilled (mortality <20% in all treatments). There was no avoidance behaviour pattern (Fig. 1) within the tested range (0, 0.0032, 0.01, 0.032, 0.1 mg/kg soil d.w.).

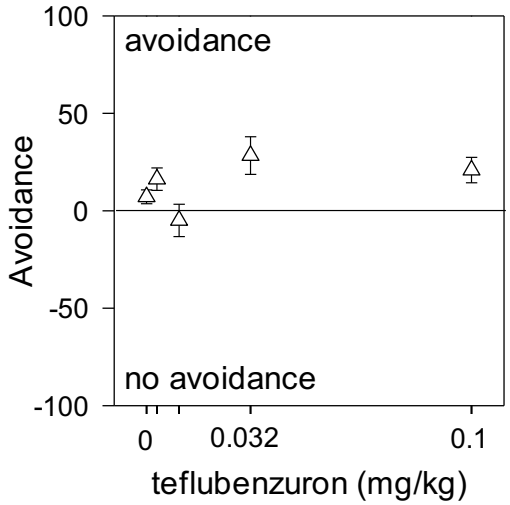

Figure 1: Results of *Folsomia candida* test after exposure to teflubenzuron in LUFA 2.2 soil in terms of avoidance behaviour. Values are expressed as % average ± standard error.

3.2 Reproduction Test

Validity criteria were fulfilled (mortality <20%, number of juveniles >100, coefficient of variation <30%), and the pH was 6.0±0.5 between treatments and test start and end.

The results show the water control data since effects in the measured endpoints were similar between the water control and the solvent control.

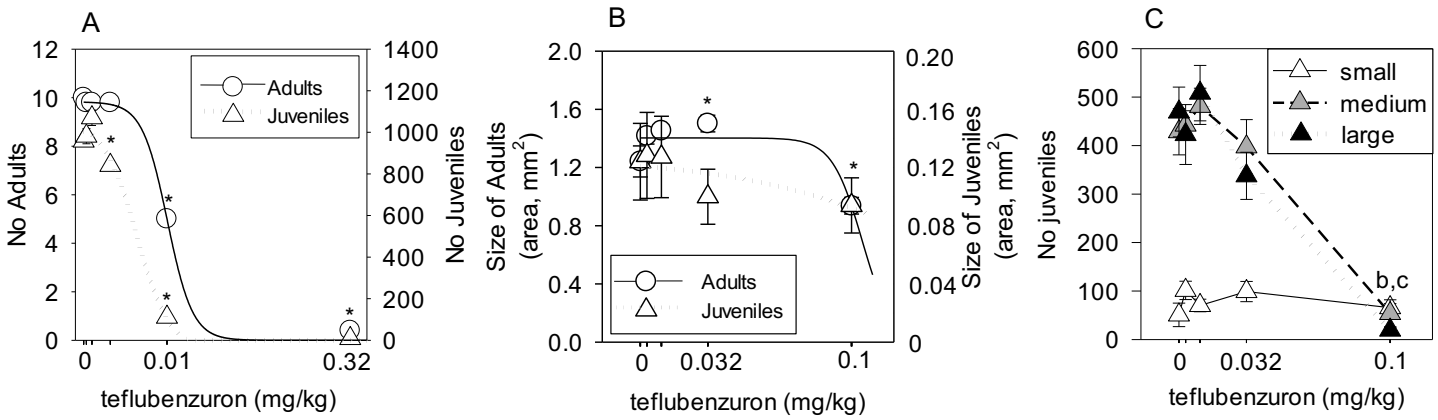

Figure 2: Results of *Folsomia candida* test after exposure to teflubenzuron in LUFA 2.2 soil in terms of A) survival and reproduction, B) organisms' size, and C) size classes distribution for juveniles. Values are expressed as average ± standard error. Lines (A and B) represent model fit to data. Small (S): S<0.05 mm^2, Medium (M): 0.1>M>0.05 mm^2 and Large (L): 0.8>L>0.1 mm^2. $p<0.05$ (Dunnetts' post-Hoc test) *: between control and treatments; b, c: between control and treatment in each size group: (b) Medium and (c) Large.

A TFB dose-response effect was observed for both endpoints, survival and reproduction of adults and juveniles (Fig. 2). Size of adults decreased significantly from 0.032 mg TFB /kg soil d.w.. The size class distribution of juveniles showed a decrease ($p<0.05$, Fig. 2 C) in the number of medium and large juveniles for 0.1 mg TFB /kg soil d.w.. Table 1 shows the estimated effect concentrations (ECx).

Table 1: Estimated effect concentrations (ECx) for *Folsomia candida* exposed to teflubenzuron (mg/kg soil d.w.) in LUFA 2.2 soil following the standard guideline. Results from the multigenerational exposure are shown in terms of % reduction for relative comparison.

	EC10	EC20	EC50	Estimated slope; Maximum response
Standard test				
Survival (F0)	0.07 0.02<CI<0.1	0.08 0.05<CI<0.1	0.10 0.09<CI<0.1	Sl: 16.5; Y0: 9.8
Reproduction (F1)	0.03 0.02<CI<0.03	0.04 0.03<CI<0.05	0.06 0.06<CI<0.07	Sl: 14.9; Y0: 1005.5
Size juveniles (F1)	0.04 -0.08<CI<0.2	0.08 0.001<CI<0.2	0.10 -0.03<CI<0.3	Sl: 5.177; Y0: 0.1
Multigenerational test				
F1	10% reduction	20% reduction	50% reduction	
Reproduction	0.01 0.001<CI<0.02	0.03 0.02<CI<0.03	0.05 0.05<CI<0.06	Sl: 13.8; Y0: 1088.6
Size juveniles	0.06 -4.4<CI<4.5	0.06 -1.9<CI<2	0.07 -2.2<CI<2.3	Sl: 63.8; Y0: 0.1
F2				
Reproduction	0.02 0.01<CI<0.04	0.04 0.03<CI<0.04	0.06 0.05<CI<0.06	Sl: 15.9; Y0: 901.4
Size juveniles	0.03 -0.02<CI<0.08	0.05 0.02<CI<0.08	0.09 0.01<CI<0.17	Sl: 8.8; Y0: 0.1
F3				
Reproduction	0.01 -0.02<CI<0.04	0.01 -0.005<CI<0.03	0.02 0.02<CI<0.03	Sl: 38.6; Y0: 984.4
Size juveniles	n.e.	n.e.	n.e.	-

Sl: Slope. Y0: top point. CI: 95% Confidence Intervals. n.e.: no effect.

3.3 Multigeneration test

Results of the multigenerational exposure to TFB showed an increase in toxicity for survival and reproduction (Fig. 3) in F3 compared to the previous generations.

A higher number of juveniles ($p<0.05$, Dunnett's post-Hoc test) was observed in F1 in the solvent control compared to the water control (see Fig. 3).

Size of adults was smaller ($p<0.05$, Dunnett's post-Hoc test) in F3 when exposed to the TFB EC50. The size of juveniles did not vary significantly with increasing concentration or generation (Fig. 2), although there was an overall decrease of their number.

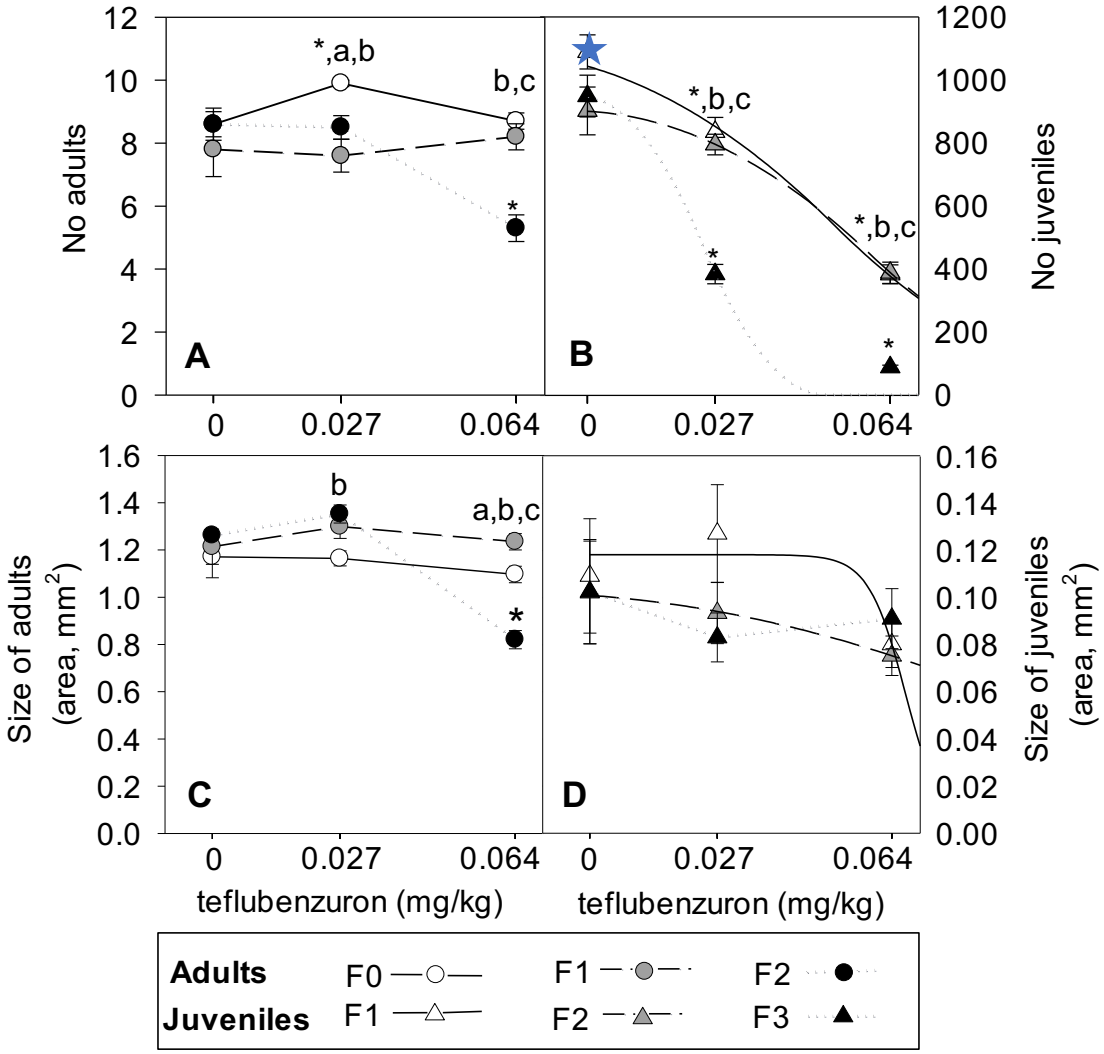

Figure 3: Results of *Folsomia candida* multigenerational test (endpoints: A) survival, B) reproduction, C) adults' size, and D) juveniles' size) after exposure to teflubenzuron (0, 0.027, 0.064 mg/kg soil d.w., i.e. ca. 0, EC10, EC50) in LUFA 2.2 soil for 3 generations (F1, F2, F3). Values are expressed as average ± standard error. $p<0.05$ (Dunnetts' post-Hoc test) *: between control and treatments in the

same generation; a: between F1-F2, b: between F1-F3 and c: between F2-F3. ★: solvent control data used (control=793±118; solvent control=1089±121 juveniles).

The relative differences between small, medium, and large animals size showed that there is a shift from large to less large animals in F1 due to TFB exposure (EC50) (Fig. 4), which persists in F2 and F3. Differences between control and solvent control occurred only in F1 as detailed in Figure 4.

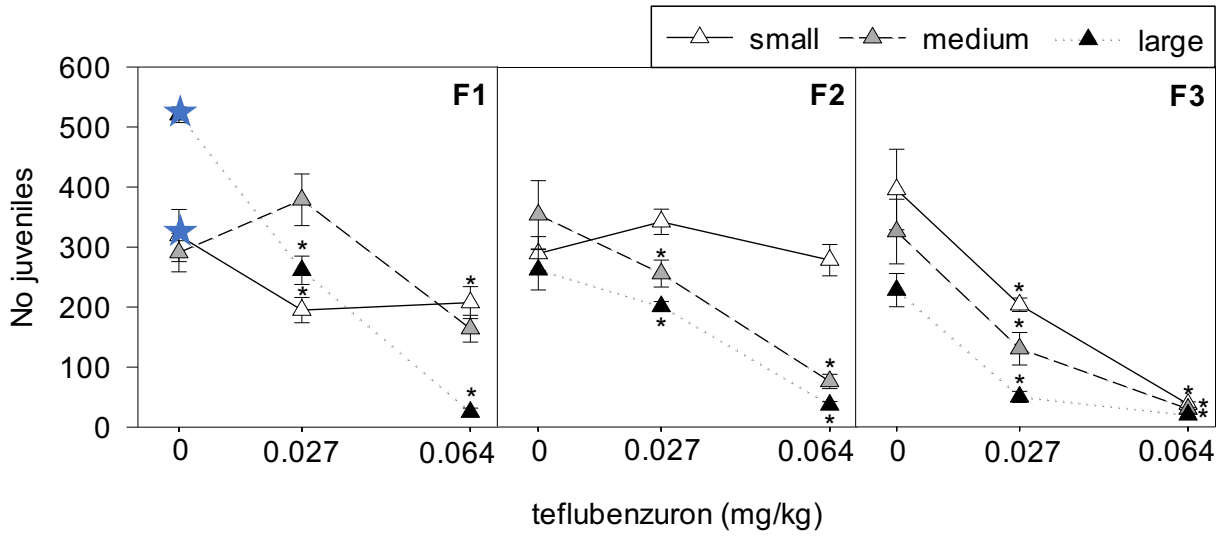

Figure 4: Results of *Folsomia candida* multigeneration test (endpoint: size as area) after exposure to teflubenzuron (0, 0.027, 0.064 mg/kg soil d.w., i.e., ca. 0, EC10, EC50) in LUFA 2.2 soil for 3 generations (F1, F2, F3). Values are expressed as average ± standard error. Small (S): S<0.05, Medium (M): 0.1>M>0.05 and Large (L): 0.8>L>0.1. $p<0.05$ (Dunnetts' post-Hoc test) *: between control and treatments within each size group. ★: solvent control data used: control (L=301±103; S: 202±63), solvent control (L=520±26; S: 319±44).

3.4 Cellular and biochemical markers

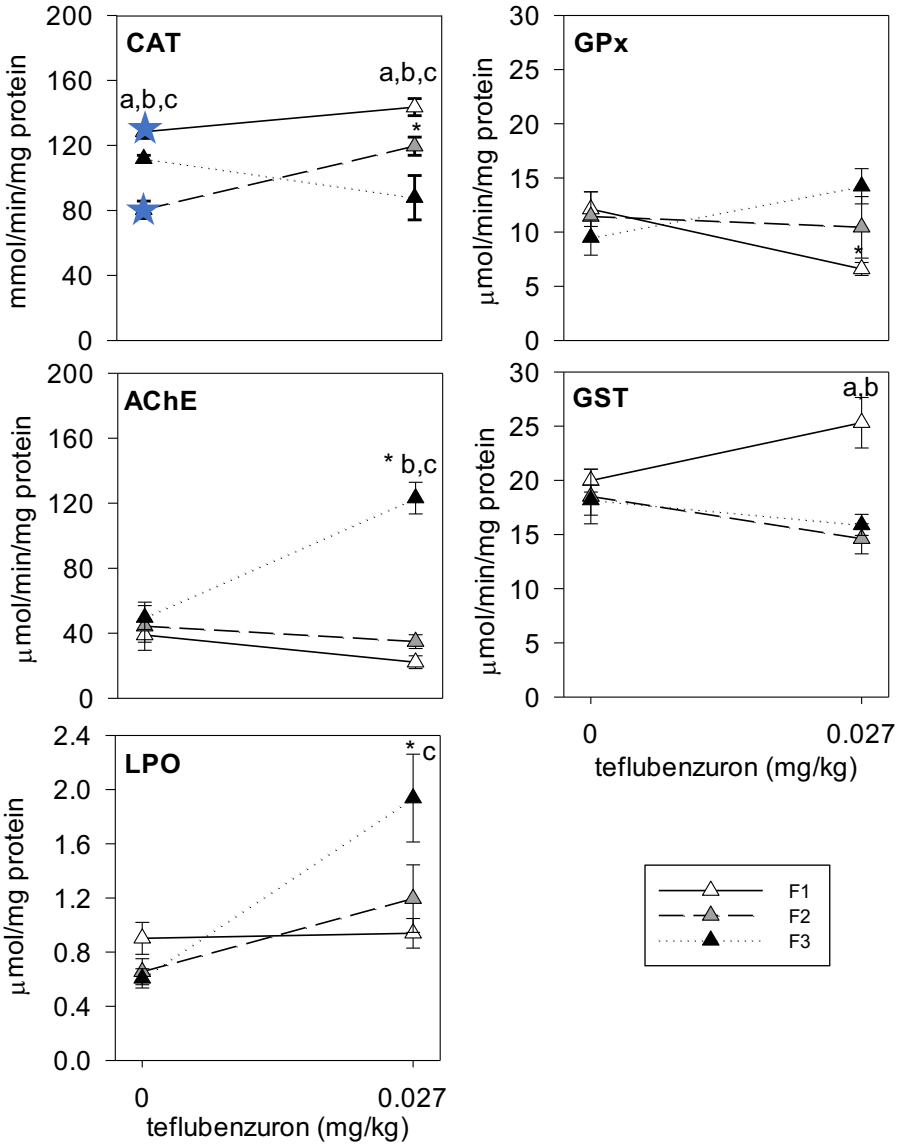

Figure 5: Results of *Folsomia candida* multigeneration test (endpoint: cellular and biochemical markers) after exposure to teflubenzuron (0, 0.027 mg/kg soil d.w.) in LUFA 2.2 soil for 3 generations (F1, F2, and F3, i.e. 28, 56, and 84 days after experiment start). Values are expressed as average ± standard error. AChE: acetylcholinesterase; GPx: glutathione peroxidase; CAT: catalase; GST: glutathione-S-transferase; LPO: lipid peroxidation. *p<0.05 (t-test): between control and TFB; a, b, c (p<0.05, Dunnetts' post-Hoc test): between generations: a: between F1-F2, b: between F1-F3 and c: between F2-F3. ★: solvent control data used: F1-control (105±20), solvent control (128±9); F2-control (121±4), solvent

control (80±4).

Increase ($p<0.05$) of CAT activity occurred in F2 (and not in F1 and F3) (Fig. 5). GPx activity decreased in F1 and AChE increased in F3. GST increased in F1, but this was reverted in the next generations. LPO showed that damage did occur in F3 organisms.

4. DISCUSSION

F. candida was not able to avoid TFB contaminated soil. Similar effects (lack of avoidance) have been observed with other contaminants, e.g. ivermectin (Guimarães et al., 2019) or dimethoate (Pereira et al., 2013). The reasons for inability to avoid these compounds could be because the organisms' olfactory or other sensory receptors are not responsive to TFB or related with the inhibition of the neurotransmission, e.g. the cholinergic or GABAergic synapses (Bicho et al., 2015), although we cannot confirm either in the present study. The major concern associated with this study is related to the fact that both survival and reproduction are affected for concentrations where no avoidance occurs. In fact, toxic effects on reproduction are quite severe with an EC50 of 0.06 mg TFB/kg soil d.w.. This is comparable with values reported in the literature for F1 (Campiche et al., 2006). The PEC in soil is 0.06 mg/kg soil d.w. and the Toxicity Exposure Ratio is 0.1 (Campiche et al., 2006), which highlights the ecological relevance of the study and the consequent environmental impact.

The observed stimulating reproduction effect of acetone was previously reported (Guimarães et al., 2019; Römbke et al., 2010) and the use of acetone as solvent could be responsible for increased variability in the results obtained in *F. candida* tests.

Results from the multigenerational exposure showed that TFB toxicity increased in F3. Other authors have shown the potential transfer of TFB effects between generations, e.g. Campiche et al. (2007) reported an effect of TFB on the reproduction of the F1 generation when exposing F0 during 10 days (no exposure of the F1). These results seem to indicate that effects of TFB are transferred

between generations (in this case the effects increased). This is not the case for all chemicals, e.g. in a multigenerational exposure to ivermectin (Guimarães et al., 2019), imidacloprid or thiacloprid (van Gestel et al., 2017) no increase of effects was observed in reproduction.

The sharp dose-response curve (survival, reproduction) could be related with the mode of action of TFB, i.e. the chitin biosynthesis inhibition can compromise the organisms' development (growth, which involves molting) and maturity, e.g. unability to reach the adult stage (Tunaz and Uygun, 2004). Because a minimum size is required for *F. candida* to be able to reproduce (Crommentuijn et al., 1993) the observed reduction in size and survival rates could be associated with reductions in the metabolic activity (Crouau and Moia, 2006; Smit and Van Gestel, 1997).

The inclusion of the endpoint organism size (in particular when given as size classes) for juveniles, shows the added value in the ecotoxicological interpretation. The quantified reduction of the number of larger sized animals shows the effect of the compound (growth inhibition) and also the potential consequences thereafter. Smaller and weaker organisms are more susceptible to stress and predation, which can result in an increased risk for springtails.

TFB exposure activated some of the oxidative stress mechanisms, e.g. GST in F1, CAT in F2, although these activations were not able to prevent lipid peroxidation in F3. It seems that exposure to TFB did not cause damage in the lipid membrane in F1 and F2. Instead it must have been elsewhere in the organism, e.g. reactive oxygen species generated by destruction of the cuticle.

This study showed that, for this particular substance, toxicity increased in the third generation. This was not predictable based on the standard one generation test. The effects of long-term exposure, especially for persistent contaminants with long-term release should be assessed. The risk assessment framework should be improved to include long-term testing requirements, especially for persistent substances and long-term contamination scenarios.

5. CONCLUSIONS

Although no avoidance behaviour was recorded, effects of TFB on survival and reproduction were high, which increases environmental concern and the associated risk of TFB. Multigenerational exposure to TFB increased toxicity to *F. candida* in F3 in terms of survival and reproduction. This could be related to the mode of action of TFB, which does not seem to sufficiently activate oxidative stress mechanisms of response (general pathways studied here). Further, TFB caused a reduction in the organisms' size which has potential consequences and may increase the risk to species. Together, all these endpoints render TFB added risks for the environment.

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Chapter V: Final remarks

Soil organisms are continuously being exposed to a number of substances released to the environment due to anthropogenic activities. Despite the significant amount of studies focusing on the effects of these pollutants to soil fauna, there are still methods that could be improved and/or included in the current guidelines for risk assessment.

The results showed in this thesis demonstrated that the evaluation of different life stages other than the ones currently used in the guidelines are beneficial to understand the impact of unfavourable conditions to soil organisms. For instance, in Chapter I, cadmium decreased reproduction of *F. candida* after the exposure of adults. However, exposure of eggs showed no effect in the hatching success, survival and reproduction.

Chapter II demonstrated that the conceptual model Concentration addition (CA) was able to predict effects of mixtures to *F. candida* reproduction rates, however strongly underestimated impact on avoidance behaviour. Although more tests should be performed, the CA model may be use for regulatory risk assessments regarding reproduction.

Tests using synchronized age individuals with a fixed exposed time may under or overestimate risks, with respective consequences to the environment and economic activities. It is therefore important to develop and integrate different test methods with the ones already recommended to improve reliability of the Environmental Risk Assessment (ERA). Current guidelines (OECD and ISO) recommend testing the juveniles (10-12 days old) of the ecotoxicological model species *Folsomia candida* for 28 days to assess effects on survival and reproduction. Although these tests are cost effective and easy to implement, they may lead to interpretations that diverge from of the exposure of organisms in more realistic scenarios, e.g. after prolonged exposure.

Chapter III showed that the impact of ivermectin on survival and reproduction of three generations of *F. candida* was similar. However, a reduction in organism's size was detected within generations. Because reproduction depends on the size of the respective organism, this may indicate an additional risk for next

generations. After multigenerational exposure of *F. candida* to the pesticide teflubenzuron, effects on size were also observed in Chapter IV, with the associated possible future consequences referred already in Chapter III. In addition, survival and reproduction were also reduced with increasing time of exposure. These results showed the importance to test more than one generation of exposed organisms to better understand effects and mechanisms involved after prolonged exposure of soil organisms to pollutants. Additionally, the multi-endpoint approach used in this thesis in Chapters III and IV, which included measurement of organism's size and analysis of cellular and biochemical markers in combination with survival, reproduction and avoidance, indicates the usefulness for inclusion of a more integrative approach using more parameters in combination with the endpoints already recommended.