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The use of gene expression to unravel the single and mixture toxicity of abamectin and difenoconazole on survival and reproduction of the springtail *Folsomia candida*☆

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ABSTRACT

Pesticides risk assessments have traditionally focused on the effects on standard parameters, such as mortality, reproduction and development. However, one of the first signs of adverse effects that occur in organisms exposed to stress conditions is an alteration in their genomic expression, which is specific to the type of stress, sensitive to very low contaminant concentrations and responsive in a few hours. The aim of the present study was to evaluate the single and binary mixture toxicity of commercial products of abamectin (Kraft® 36 EC) and difenoconazole (Score® 250 EC) to *Folsomia candida*. Laboratory toxicity tests were conducted to access the effects of these pesticides on springtail survival, reproduction and gene expression. The reproduction assays gave EC_{50} and EC_{10} values, respectively, of 6.3 and 1.4 mg a.s./kg dry soil for abamectin; 1.0 and 0.12 mg a.s./kg dry soil for Kraft® 36 EC; and 54 and 23 mg a.s./kg dry soil for Score® 250 EC. Technical difenoconazole did not have any effect at the concentrations tested. No significant differences in gene expression were found between the abamectin concentrations tested (EC_{10} and EC_{50}) and the solvent control. Exposure to Kraft® 36 EC, however, significantly induced Cyp6 expression at the EC_{50} level, while VgR was significantly downregulated at both the EC_{10} and EC_{50}. Exposure to the simple pesticide mixture of Kraft® 36 EC + Score® 250 EC caused significant up regulation of ABC transporter, and significant down regulation of VgR relative to the controls. GABA receptor also showed significant down-regulation between the EC_{10} and EC_{50} mixture treatments. Results of the present study demonstrate that pesticide-induced gene expression effects precede and occur at lower concentrations than organism-level responses. Integrating "omic" endpoints in traditional bioassays may thus be a promising way forward in pesticide toxicity evaluations.

1. Introduction

Studies evaluating the environmental toxicity of pesticides have demonstrated that, besides mortality, they may exert several sub-lethal effects such as behavioral, immunological and endocrinal alterations (Brühl et al., 2013; Gill et al., 2012; Hayes et al., 2010). Despite the recognition of these effects, environmental risk assessments of pesticides have traditionally focused on the effects on a limited number of standard test parameters, such as mortality, reproduction and development (Biales et al., 2016; EFSA, 2013a). However, technological developments in the 21st century allowed substantial progress in identifying and quantifying toxic effects at the molecular level, including the evaluation of metabolites and other molecules (Paniagua-Michel and Olmos-Soto, 2016).

One of the first signs of adverse effects that occur in organisms exposed to stress conditions is an alteration in their genomic expression that reflects physiological shifts to adapt to new environmental conditions (Van Straalen and Roelofs, 2008). Genomic

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tools allow studying the function of each gene in organisms and how these interact and control phenotypic characteristics through the analysis of gene (transcriptome) and protein (proteome) expression (Mutch, 2005). In recent years, efforts have been made to evaluate the usefulness of incorporating genetic studies in risk assessment of chemical stressors such as pesticides and pharmaceuticals (Chen et al., 2014). Such studies have shown 'omics' technologies to be promising and complimentary to traditional laboratory toxicity tests since gene expression analysis is i) specific to the type of stress; ii) sensitive to very low contaminant concentrations; and iii) responsive in a few hours (Bahamonde et al., 2016).

The springtail *Folsomia candida* (Isotomidae) is a detritivore and therefore plays an important role in soil nutrient recycling and mineralization (Fountain and Hopkin, 2005). *F. candida* is sensitive to a wide range of soil pollutants. Toxicity tests with this species have been standardized and are often required in the terrestrial risk assessment of pesticides (EFSA, 2017; Environment Canada, 2014; ISO, 2014). This collembolan species has previously been used in studies evaluating the genomic effects of metals, drugs and altered environmental conditions such as pH and temperature (Chen et al., 2015; de Boer et al., 2010; Nota et al., 2011; Roelofs et al., 2013; Waagner et al., 2012; Yuan et al., 2014). However, studies on genomic responses of *F. candida* exposed to pesticides are underrepresented to date (Cardoso et al., 2017; Qiao et al., 2015).

The commercial production of strawberries in Brazil uses large amounts of pesticides such as the insecticide/acaricide Kraft® 36 EC (a.s. abamectin) and the fungicide Score® 250 EC (a.s. difenoconazole). Abamectin is an acaricide/insecticide that acts on vertebrate and invertebrate gamma-aminobutyric acid (GABA) receptors, as well as on glutamatergic receptors of invertebrate chloride channels (Novelli et al., 2016). After abamectin exposure, the elevated number of chloride ions after abamectin exposure leads to a hyperpolarization of the muscle and nerve cells, which ultimately interferes with the neuromuscular transmission and subsequent death through paralysis (Campbell, 1989). The fungicide difenoconazole is a triazole that is known to inhibit sterol biosynthesis, which is crucial for maintaining cell membrane integrity of fungi (Fishel, 2014; FRAC, 2018).

Recently, Oliveira et al. (2018) demonstrated that among three test species (i.e. the potworm *Enchytraeus crypticus*, the collembolan *F. candida*, and the mite *Hypoaspis aculeifer*), *F. candida* was the most sensitive to both abamectin and difenoconazole. These authors further noted that environmentally relevant concentrations of both pesticides are likely to significantly affect this collembolan species (Oliveira et al., 2018). In addition, abamectin and difenoconazole are intensively used throughout the year in Brazilian strawberry crops so they are likely to occur simultaneously (Novelli et al., 2012; Sanches et al., 2017). Recent studies evaluating the binary mixture toxicity of these pesticides demonstrated both antagonistic and synergistic mixture effects to the aquatic microcrustacean *Macrothrix flabelligera* (Moreira et al., 2017) and the fish *Danio rerio* (Sanches et al., 2017, 2018). These mixture interactions were attributed to the mode of action of these pesticides, although both studies also concluded that the exact underlying mixture toxicity mechanisms may only be elucidated by conducting studies at the molecular level (Moreira et al., 2017; Sanches et al., 2017).

The aim of the present study was to evaluate the single and binary mixture toxicity of commercial products of abamectin (Kraft® 36 EC) and difenoconazole (Score® 250 EC) to *F. candida*. To this end, laboratory toxicity tests were conducted to access the effects of these pesticides on springtail survival, reproduction and gene expression. In addition, effects on gene expression of the active substances were also evaluated through the polymerase chain reaction (PCR) technique. Several candidate genes were selected to identify if the exposure to these compounds affects gene expression through biological processes previously suggested to be involved in the stress response to these compounds.

2. Materials and methods

2.1. Experimental overview

The work was divided in two steps. The first step consisted of toxicity tests to analyze the effects of the pesticides studied, individually and as mixtures, on the survival and reproduction of *F. candida*. In the second step, *F. candida* was exposed to the pesticides individually and as mixtures at concentrations corresponding with the EC_{10} and EC_{50} derived from the reproduction tests in step one. Following exposure, the expression of selected genes was quantified using qPCR and compared between treatments and concentrations.

2.2. Test organisms

The laboratory bioassays were conducted in the laboratory of the Department of Ecological Science at the Vrije Universiteit Amsterdam, the Netherlands. *F. candida* (Berlin strain, VU Amsterdam) was obtained from an in-house culture that has been kept in the laboratory for many years. Organisms were maintained and cultured in PET containers (volume: 0.75L) with a plaster of Paris water-saturated base, containing 10% charcoal, in a climate room at $16 \pm 1\,^{\circ}C$, 75% relative humidity (RH) and a 12/12 h light-dark regime. The animals were fed *ad libitum* thrice a week with dried baker's yeast (Instant yeast from Algist Bruggeman N.V, Ghent, Belgium). Since *F. candida* reproduces parthenogenetically, all individuals have identical genotypes (de Boer et al., 2013).

2.3. Survival and reproduction tests

The first step was to determine the 28-d EC_{10} and EC_{50} for all single compounds (active substances and commercial products) using the standardized ISO protocol 11267 (ISO, 2014). Following this standard protocol, ten juvenile (10–12 day old) animals were exposed in 100 ml test jars to 30 g moist spiked LUFA 2.2 soil per replicate (soil pH_{CaCl2}: 5.5; total organic carbon content: 2.1%; water-holding capacity: 46.5%; LUFA-Speyer, Speyer, Germany). Five replicates were used for each treatment. All tests were incubated in a climate room at 20 °C, 75% RH and a 16 h/8 h light/dark cycle. During the tests, moisture content and food availability were checked regularly and adjusted if needed. After a 28-d exposure period, the number of surviving adults and new-born juveniles were assessed by extracting the animals through flotation with water, after which digital photographs were made and animals were counted using the Cell^{D} software (Olympus Europe, Hamburg, Germany, www.olympus-europa.com).

Stock solutions for the active substances abamectin (PESTANAL®, Sigma- Aldrich - CAS Number: 71751-41-2; purity 98,6%) and difenoconazole (PESTANAL®, Sigma-Aldrich - CAS Number: 119446-68-3; purity 99,7%) were prepared with acetone. A small portion of dry LUFA 2.2 soil (5 g/replicate) was spiked with 5 mL acetone stock solution in a glass jar (100 ml). The lid was closed and the jar incubated overnight to allow equilibration, after which it was opened to allow evaporation of the acetone. After the acetone was evaporated, the spiked soil was mixed with untreated Lufa 2.2 soil to achieve the desired test concentration. Mixing was done with a spoon, and for each treatment the same mixing time (approx. 5 min) and same mixing procedure was applied. After mixing, the soil was moistened to 50% of the water holding capacity using distilled water and mixed again. For the

Table 1
Primers used in the quantitative PCR assay to assess the effects of different pesticides on the gene expression of *Folsomia candida* exposed in Lufa 2.2 soil.

Gene	Oligos (5' – 3')	Efficiency (%)
SDHA	Forward: ACACTTTCCAGCAATGCAGGAG Reverse: TTTTCAGCCTCAAATCGGCA	98.6
YWAZ	Forward: TCGCCCTCAACTTTTCCG TT Reverse: TGCTATCGCTTCATCGAATGCT	93.2
ABC (Fc_ABC-rt-3)	Forward: GTGTGAAATCTGGCGAAAAGGT Reverse: TTGAGCAGCAGAAGGCACTAATC	90.6
Cyp6 (Fcc01780)	Forward: GCGTTAAAAGCGAGGCAAGA Reverse: GCGATATCCACGTTCGAATTGT	89.3
GABA (Fc_05090)	Forward: CAATGGCTACTCAAACGTCGG Reverse: GTTAGGCACACACCAGTCCACA	89.4
VgR (Fc_10990)	Forward: TGACAATGGTAACGCAGACGTC Reverse: AGGTTAAGCATACCCCAGTCCAC	85.6

commercial formulations of abamectin (Kraft® 36 EC; 36 g abamectin/L; Cheminova, Brazil) and difenoconazole (Score® 250 EC; 250 g difenoconazole/L; Syngenta Crop Protection Ltda, Brazil), stock solutions were prepared in distilled water. After spiking, the soils were thoroughly mixed with a spoon as described above and transferred to the test containers after which test animals were introduced. For the mixture, two stock solutions were made for each of the compounds, Kraft® and Score®, both were added to the soil and manually mixed with the help of a spoon, for complete soil homogenization.

Based on preliminary testing, the selected test concentrations were 0.33, 1.0, 3.3, 10, and 33 mg active substance (a.s.)/kg dry soil for all the test compounds. One additional concentration was added in the test evaluating abamectin (100 mg a.s./kg), while for the tests with difenoconazole and Score® 250 EC two more concentrations were evaluated (100 and 333 mg a.s./kg). Since the pure compounds were dissolved in acetone and the commercial formulations in distilled water, two controls were included: one with acetone ("solvent control") and one without ("control").

2.4. Gene expression

The effects of single and mixture exposure to the pesticides on gene expression were evaluated in a 2-d test conducted as described by e.g. de Boer et al. (2010), Nota et al. (2011) and Roelofs et al. (2012). The following treatments were evaluated at both the EC_{10} and EC_{50} levels for effects on reproduction, determined as described above: i) control; ii) solvent control; iii) abamectin; iv) Kraft® 36 EC; v) Kraft® 36 EC and Score® 250 EC. Single exposures to difenoconazole and Score® 250 EC were not evaluated since it was not possible to derive proper EC_{10} and EC_{50} values because only slight adverse phenotypic effects were observed at the highest concentration tested.

Animals were age-synchronized prior to exposure according to Fountain and Hopkin (2005). Twenty-four days old *F. candida* were used for the tests with five replicates, each containing 60 collembolans, per treatment. After a 2-d exposure period, the animals were extracted from the soil by flotation and left to dry on a plaster of Paris base. Subsequently, animals were snap frozen in liquid nitrogen for RNA extraction.

Selected genes were analyzed by applying Q-PCR in accordance with the methods described in de Boer et al. (2009a,b). The selected genes for study were: i) the cytochrome P450 Cyp6 subfamily, which is involved in the biotransformation of xenobiotic organic chemicals; ii) the γ-aminobutyric acid (GABA) receptor, which is the major inhibitory neurotransmitter in invertebrates and a potential target of abamectin; iii) the vitellogenin receptor (VgR), responsible for the uptake and transport of vitellogenin (Vg), the major energy source of developing embryos; and iv) ATP-binding cassette transporter (ABC), which transports a wide variety of substrates across extra- and intracellular membranes. These genes were chosen since they were indicative for adverse effects of abamectin in invertebrates (Riga et al., 2014; Wolstenholme, 2010). To the best of our knowledge, no molecular toxicity studies have been conducted with difenoconazole-exposed animals, precluding the selection of relevant candidate genes to be used in this study (Mu et al., 2016; Liang et al., 2015b).

After cDNA synthesis with reverse transcriptase, quantitative PCR (two technical replicates per gene expression assay) was performed with primers (Table 1) of a set of genes of interest on a CFX Connect Real Time PCR Detection System (BIO-RAD, USA) using standard cycling conditions: 10 min DNA polymerase activation and DNA denaturation at 95 °C, followed by 40 cycles of 15 s at 95 °C and 1 min 60 °C.

Table 2
Nominal and measured concentrations of abamectin and difenoconazole in Lufa 2.2 soil at the beginning and the end of the reproduction toxicity tests and gene expression tests with *Folsomia candida*. All values are the mean of two measurements, except for the ones for the mixture exposures of the formulations Kraft® 36 EC and Score® 250 EC, which were not replicated. Also shown is the half-life (DT_{50}) estimated from the loss of the compound over the 28-d experimental period.

Experiment	Pesticide treatment	Nominal concentration (mg a.s./kg)	Measured concentration (mg a.s./kg)	Initial concentration (% nominal)	Concentration on day 28 (mg a.s./kg)	DT_{50} (d)
Survival/ reproduction test	Abamectin	3.3	1.65	50	0.92	35
	Kraft® 36 EC	1	1.1	110	0.43	21
test	Difenoconazole	100	118	118	–	–
	Score® 250 EC	100	82	82	78	420
	Score® 250 EC	33	32	95	25	117
	Abamectin	1.4	1.2	75	–	–
	Abamectin	6.3	5.1	80	–	–
Gene expression	Abamectin	1.4	1.1	75	–	–
	Abamectin	6.3	5.1	80	–	–
	Kraft® 36 EC	0.12	0.25	200	–	–
	Kraft® 36 EC + Score® 250 EC	1.00 + 22.5	1.1 + 25.4	110 + 113	–	–
	Kraft® 36 EC + Score® 250 EC	0.12 + 22.5	0.12 + 21.7	100 + 96	–	–

2.5. Chemical analyses of the test compounds

Chemical analyses and quantification of the compounds in the spiked soil were performed by the company Groen Agro Control (Delfgauw, the Netherlands) using liquid chromatography tandem-mass spectrometry (LC-MS/MS), with detection limits of 0.01 mg/kg for all compounds. Soil samples were analyzed at the start and at the end of the reproduction test. Compound concentrations were also quantified at the start of the exposure of the gene expression test.

2.6. Statistical analyses

The Trimmed Spearman-Karber method was used to calculated LC_{50} values (Hamilton et al., 1977). EC_{10} and EC_{50} values were estimated using a three-parameter logistic model, and NOECs were derived by ANOVA followed by Dunnett's test, differences were considered significant when $p < 0.05$. Before NOEC determination, the reproduction data were checked for normality (with Kolmogorov-Smirnov test) and homogeneity of variances (with Levene test). The EC_{10}, EC_{50} and NOEC calculations were conducted in IBM SPSS Statistics 22 for Windows.

The cycle threshold values resulting from the gene expression assay were retrieved using the CFX manager software (Biorad) by applying a baseline subtraction and a manual threshold setting at 0.005. Relative expression values were calculated by normalization of target genes with two reference genes, SDHA and YWAZ (de Boer et al., 2009a,b) expression were in the CFX Manager software based on the mathematical method developed by Muller et al. (2002) which corrects for primer pair efficiency. The resulting mean normalized expression (MNE) values were used to perform statistical analysis using the software package SPSS23 (IBM). A one-way ANOVA followed by Dunnett's test was used to evaluate differences between control and compounds tested. Statistical significance was accepted at the $p < 0.05$ level.

3. Results and discussion

3.1. Test performance and pesticide degradation

In the survival/reproduction test, the initial concentrations were between 78 and 120% of intended concentrations for all treatments except for abamectin (50%; Table 2). In the gene expression test, the initial abamectin concentrations were also at the lower end (80%) when compared with the other treatments (96–200%; Table 2).

Abamectin concentrations after 28 days showed a considerable decline, showing this compound is degraded with an estimated DT_{50} (half-life or degradation time 50%) of 20–35 days (Table 2). Difenoconazole appeared much more persistent with the major part still being present after 28 days, and an estimated half-life of 117–420 days (Table 2). Aerobic microbial degradation has been indicated as one of the primary mechanisms responsible for the dissipation of abamectin in soils (Dionisio and Rath, 2016). Subsequently, DT_{50} values will depend on microbial activity and other soil parameters (e.g. Amorim et al., 2005; Jänsch et al., 2005). Laboratory degradation studies have indicated DT_{50} values for abamectin ranging from 11 to 50 days (EU, 2006a), with a typical value of 29 days (Lewis et al., 2016). These values are hence in line with the dissipation rate noted in the present study.

Difenoconazole was more persistent in the present study than abamectin, and DT_{50} values recorded were in agreement with the values of 53–709 days from laboratory studies (EFSA, 2011), and a typical value of 130 days reported in the literature (Lewis et al., 2016). Triazole pesticides have indeed been reported to have low biodegradability (Bromilow et al., 1999; Wang et al., 2011), which has been attributed to the ability of these compounds to adsorb to organic matter present in the soil and therefore become less available for degradation by microorganisms (Svobodová et al., 2018; Yu et al., 2006).

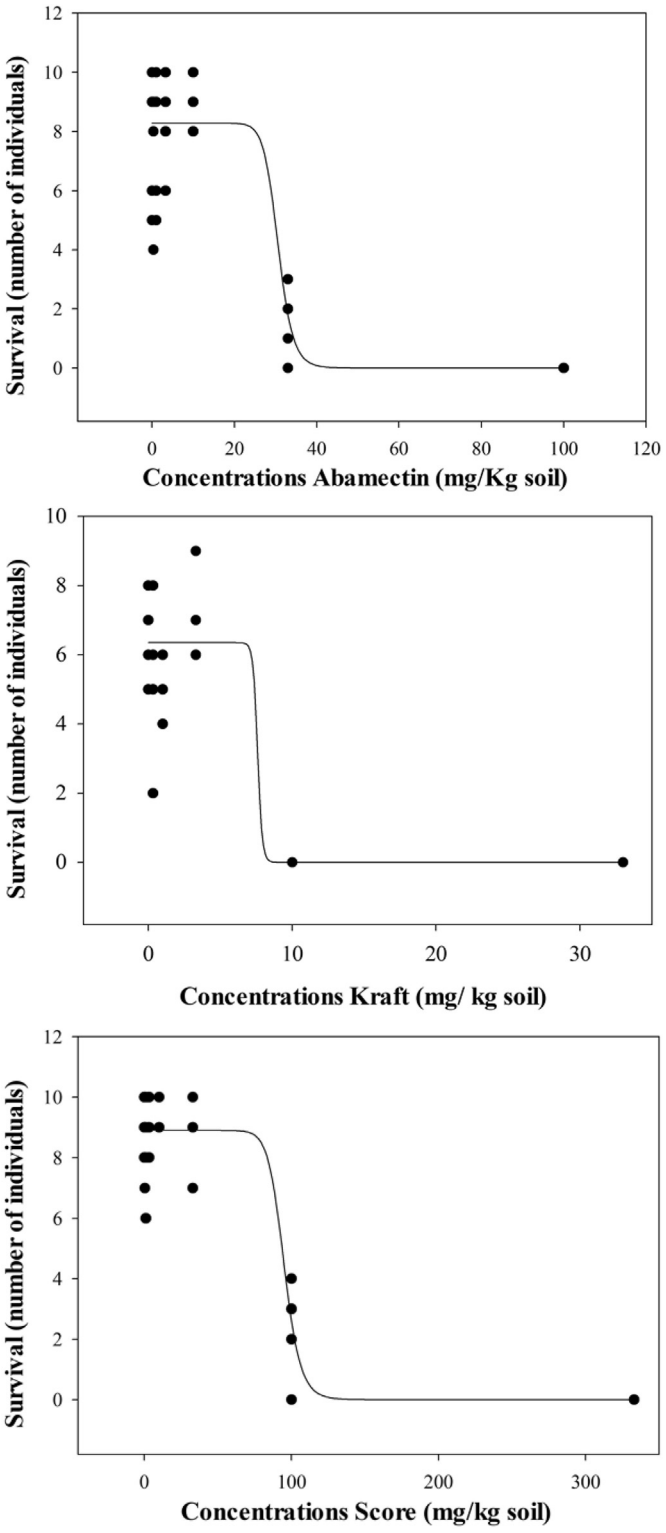

Fig. 1. Dose-response relationships for the effect of all compounds tested on the survival of *Folsomia candida* after 28 days exposure in Lufa 2.2 soil. A- Abamectin. B- Kraft® 36 EC. C- Score® 250 EC. The points represent the measured survival data, the lines show the fit of a logistic dose-response model to the data.

3.2. Survival and reproduction

The controls showed mean adult survival of 80 ± 20% for abamectin, 70 ± 10% for Kraft® 36 EC, and 90 ± 10% for difenoconazole and Score® 250 EC. So, control survival was only slightly below the validity criterion of 80% for Kraft® 36 EC. The mean numbers of juveniles in the controls were 489 ± 167, 306 ± 125, 602 ± 86, 852 ± 118 for the tests with abamectin, Kraft®, difenoconazole and Score®, respectively, so all well above the validity criteria of 100 juveniles per replicate (ISO, 2014). For Kraft® 36 EC variation in control reproduction was 41%, so slightly above the validity criterion of 30% set by ISO (2014), but for all other tests this validity criterion was met.

The dose-response curves and derived toxicity values indicate that the commercial formulations of abamectin and difenoconazole were more toxic to *F. candida* than their active substances (Figs. 1 and 2, Table 3). The presence of ingredients other than the active substances (antioxidants, solvents, adjuvants, defoamers, etc.) in commercial formulations has often been suggested to increase the toxicity of active substances (Arena et al., 2017; Oakes and Pollak, 2000; Rajini et al., 2016; Solomon and Thompson, 2003). In line with this, a possible greater toxicity of Kraft® 36 EC as compared with technical abamectin to aquatic and terrestrial organisms has previously been discussed (Novelli et al., 2012; Nunes et al., 2016).

The toxicity of abamectin (both active substance and commercial product) was greater than that of difenoconazole (Figs. 1 and 2, Table 3). In line with this, previous studies also noted a greater sensitivity of both aquatic (Sanches et al., 2017) and terrestrial (Oliveira et al., 2018) organisms to abamectin as compared to difenoconazole. This may be explained by their respective mode of action since collembolans have been demonstrated to be more sensitive to insecticides than fungicides (Daam et al., 2011 and references therein).

The only study available to us evaluating the toxicity of difenoconazole to *F. candida* noted a 28-d NOEC (reproduction) and a 28-d LC_{50} of 500 and > 1000 mg/kg, respectively for technical difenoconazole (EU, 2006b). These values are in line with the results of the present study (c.f. Figs. 1 and 2, Table 3). For abamectin, however, reported toxicity values vary substantially among studies: available 21 to 28-d EC_{50} values for *F. candida* reproduction are 0.06 (Oliveira et al., 2018), 0.68 (Diao et al., 2007) and 13 mg a.s./kg (Kolar et al., 2008). It is indeed known that toxicity values may vary greatly depending on experimental conditions such as soil composition and chemical form (a.s. versus commercial product) (Amorim et al., 2005; Jänsch et al., 2005; Nunes et al., 2016). For example, the quantity and quality of the organic matter in the test soil in known to play a significant role in organic chemical sorption to soil, especially when compounds have a high Koc value such as abamectin (Huang et al., 2003; Nunes et al., 2016; Song et al., 2002). An earthworm ring test conducted in 18 laboratories showed a spread in LC_{50} values up to a factor of 5, even though the same experimental conditions were used in all laboratories (Moser et al., 2009). Oliveira et al. (2018) and Diao et al. (2007) used a natural soil, whereas Kolar et al. (2008) used the same soil (LUFA 2.2) as used in the present study to evaluate the toxicity of technical abamectin. This may hence at least partly explain why the EC_{50} value reported in the latter study corresponds best to that derived in the present study (6.3 mg/kg; Fig. 2, Table 3).

3.3. Gene expression

Among the four genes considered, there was no significant difference ($p > 0.05$) in gene expression between the abamectin concentrations tested (EC_{10} and EC_{50}) and the solvent control (Fig. 3). Exposure to Kraft® 36 EC, however, significantly induced Cyp6 expression at the EC_{50} level, while VgR was significantly downregulated at both the EC_{10} and EC_{50} levels, with a more severe downregulation in the EC_{50} treatment. This suggests a dose-dependent relationship for these two gene expression profiles. In addition, these results are in line with the reproduction data,

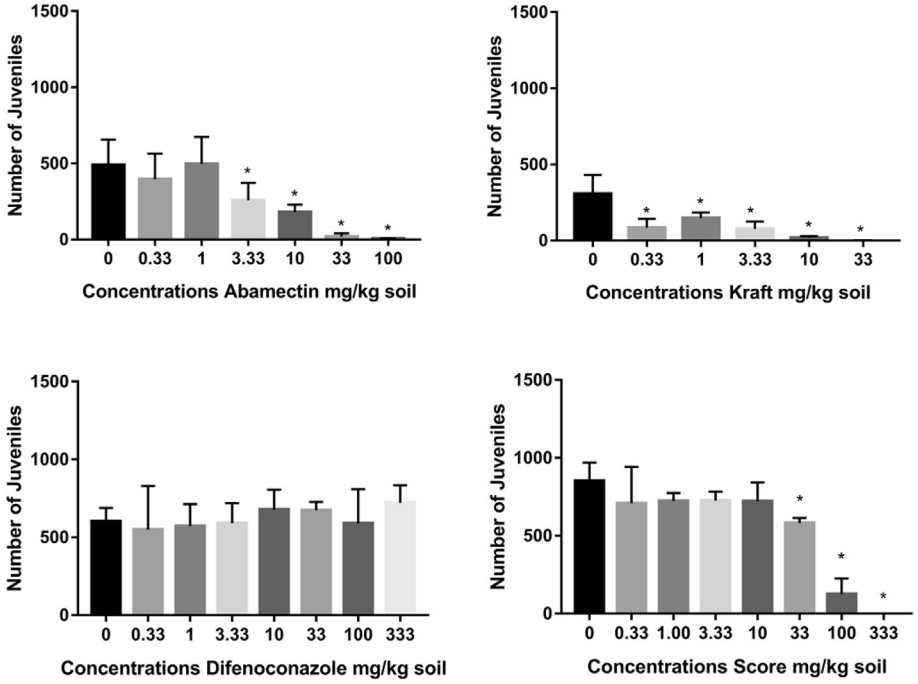

Fig. 2. Effect of the selected pesticides on the reproduction (mean number of juveniles ± standard deviation; n = 5) of *Folsomia candida* after 28 days exposure in Lufa 2.2. soil: Abamectin (A); Kraft® 36 EC (B); difenoconazole (C) and Score® 250 EC (D). Exposure levels significantly differing from the control according to an ANOVA followed by Dunnett's test (c-control) are indicated with an * ($p < 0.05$).

Table 3
Values of EC_{10}, EC_{50} (reproduction) and LC_{50} (adult survival) with their 95% confidence intervals (in parenthesis) obtained for the test compounds (abamectin, Kraft® 36 EC, difenoconazole and Score® 250 EC) in the 28-d *Folsomia candida* toxicity tests. All values are in mg a.s./kg dry soil. NP = not possible to calculate 95% confidence intervals.

Compounds	EC_{10}	EC_{50}	LC_{50}
Abamectin	1.4 (−0.88–3.7)	6.3 (1.8–11)	23 (20–27)
Kraft® 36 EC	0.12 (−0.15–4.0)	1.0 (0.17–1.8)	5.4 (NP)
Difenoconazole	>333	>333	>333
Score® 250 EC	23 (12–34)	54 (40–67)	81 (69–93)

because the studied genes were more affected by the formulated products than by the active substance of each pesticide.

When gene expression was evaluated for the simple pesticide mixture (Kraft® 36 EC + Score® 250 EC), we observed several statistically significant differences ($p < 0.05$) when compared to the controls. The ABC transporter was significantly up-regulated, whereas VgR was significantly down-regulated. GABA also showed significant down-regulation, but only at EC_{50} treatments when compared to control and EC_{10} gene expression levels (Fig. 3).

It is well known that organic compounds cause up-regulation of CYP genes since these genes are involved in the biotransformation and detoxification of xenobiotic compounds through hydrolyzing the carbon ring structure (Liang et al., 2015a). This was confirmed by de Boer et al. (2013), who also observed up-regulation of CYP genes when exposing *F. candida* to phenanthrene. A specific cytochrome P450 gene, Cyp392A16, catalyzes the hydroxylation of abamectin, which results in a metabolite that is substantially less toxic than abamectin (Riga et al., 2014). However, Sun et al. (2014) noted that 12 CYP genes exhibited different expression patterns in the Asian gypsy moth *Lymantria dispar* after exposure to three insecticides, some of which were up-regulated and others down-regulated. This hence suggests substrate specificity of this gene family.

In the present study, we observed activation of ABC in the Kraft® 36 EC and Score® 250 EC mixture treatment (Fig. 3). This was also noted by Epis et al. (2014) in the mosquito *Anopheles stephensi* at different timepoints (48 h–30 min) of exposure to the insecticide permethrin. Based on their findings, they concluded that ABC transporters act in different phases of the detoxification process (Epis et al., 2014). More particular, the ABC transporter family has been shown to transport xenobiotic compounds and their resulting metabolites and to act in concert with P450 enzymes to detoxify these compounds both in vertebrates and invertebrates (Dermauw and Van Leeuwen, 2014).

GABA-gated chloride channels are receptors responding to the neurotransmitter GABA. Arthropod GABA receptors are known targets of abamectin leading to neurotoxicity through inhibition of the neurotransmission (Lunt, 1991), and anesthetic effects (Bicho et al., 2015). Interestingly, no effects on GABA at the transcriptional level were recorded after single exposure to abamectin or Kraft® 36 EC (Fig. 3). This may suggest that detoxification of the compound through the biotransformation pathway is more likely to play a role in the physiological response of *F. candida* to abamectin toxicity than synthesizing additional receptor proteins to compensate for receptors occupied by abamectin. A similar conclusion was reached by Ilias et al. (2015) who noted up-

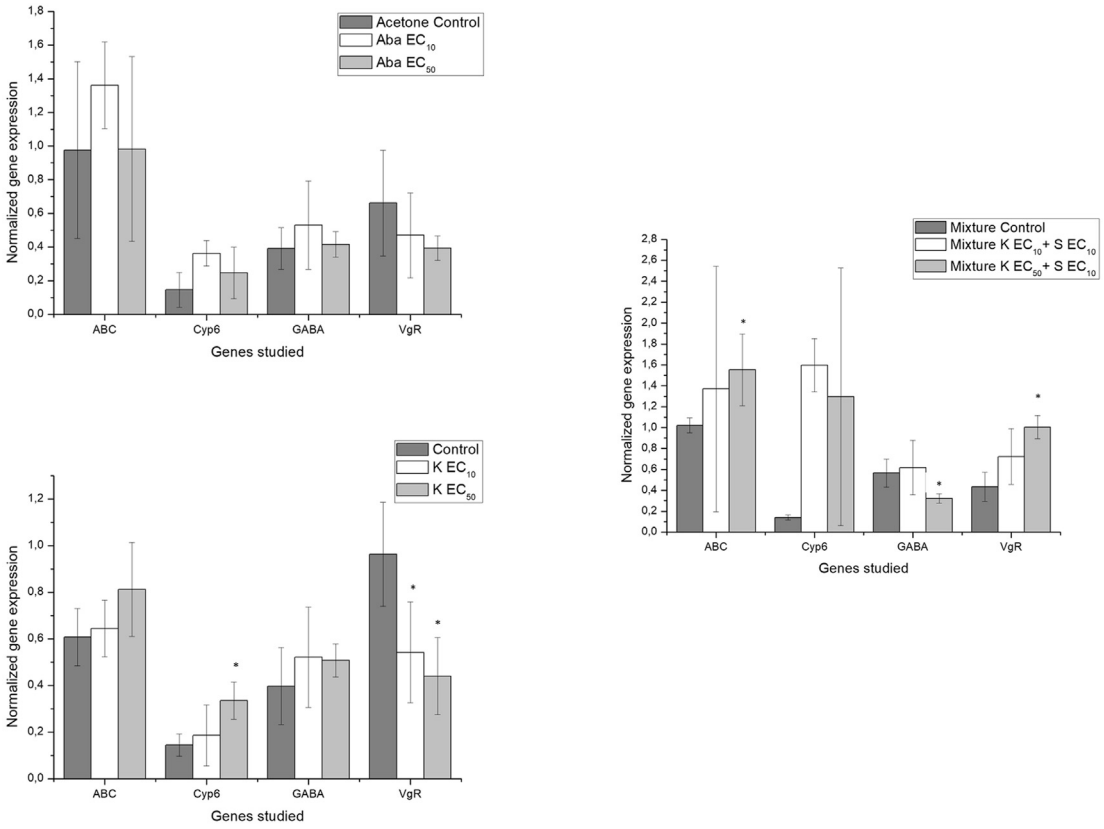

Fig. 3. Normalized gene expression of ABC transporter, Cyp6, gamma-aminobutyric acid − GABA, and vitellogenin receptor (VgR) genes in *Folsomia candida* exposed for 2 days to different pesticides at concentrations corresponding to the EC_{10} and EC_{50} for effects on reproduction (mean ± standard deviation, n = 5). Expression values of two stable reference genes (SDHA and YWAZ) were used to normalize for input cDNA. Exposures included abamectin (A), Kraft® 36 EC (B) and Kraft® 36 EC + Score® 250 EC mixtures (C). Exposure levels significantly differing from the control according to an ANOVA followed by Dunnett's test (c-control) are indicated with an * ($p < 0.05$).

regulation of P450 unigenes in the whitefly *Bemisia tabaci* but no response in nAChR (nicotinic acetylcholine receptors) despite the fact that the test compound imidacloprid is known to bind to insect nicotinic acetylcholine receptors (nAChR). Also, unexpectedly, GABA was downregulated in the mixture treatment. This response is difficult to explain in the context of mixture toxicity. We hypothesized that GABA would also not be affected in the mixtures, since it was not differentially expressed in the Kraft® 36 EC abamectin treatments and Score® 250 EC is not known to target this receptor. The insecticides that are known to interfere with GABA receptors are pyrethroids and phenylpyrazole (Davies et al., 2007; Raymond-Delpech et al., 2005). In any case, it exemplifies the notion that effects of mixtures are very hard to predict solely based on information of the single compounds, which most probably is the result of biological complexity and redundancy in response pathways.

The VgR aids in the uptake of vitellogenin by the oocytes and is essential for egg maturation during vitellogenesis (Upadhyay et al., 2016). It has therefore often been studied in the oocyte membranes of invertebrates, especially insects (Raikhel and Dhadialla, 1992). Lafontaine et al. (2016), for example, observed an activation of the Vg and VgR gene expression in the freshwater prawn *Macrobrachium rosenbergii* after exposure to the organochlorine insecticide chlordecone. In the present study, a significantly increased expression of VgR was noted at the EC_{50} level of the Kraft® 36 EC + Score® 250 EC mixture. Interestingly, the gene expression after single exposure to Score® 250 EC was not significantly affected and single Kraft® 36 EC exposure at the EC_{50} level showed a downregulation (Fig. 3).

Studies with information about toxicokinetics and toxicodynamics of pesticides are still limited, which hinders research on the gene expression of these compounds in target and non-target organisms. Ideally, molecular effects should be investigated in a more explorative way, such as whole-transcriptome RNA sequencing analysis. However, the present study made use of some previous biochemical information in order to guide candidate gene selection. Obviously, this is only a first step in elucidating the molecular mechanisms underlying these stress responses, which can aid in indicating the effects of these compounds on organisms, even when studied at low concentrations. Eventually this should allow protective measures to preserve ecosystem structure and functioning.

Toxicokinetic-toxicodynamic models have increasingly been used in toxicity evaluations of single compounds (EFSA, 2013b). However, studies of this type, and studies at the molecular level (such as the present study), are also imperative to allow increasing our mechanistic understanding of chemical mixture toxicity (Jager et al., 2014). Ultimately, this will also aid in developing practical criteria for the selection of chemical mixtures that are likely to exert synergistic interactions at environmentally realistic concentrations and hence require further (legislative) attention (Altenburger et al., 2013; Coors et al., 2013; Silva et al., 2015).

Conflicts of interest

The authors declare that they have no conflict of interest.

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