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From laboratory to the field: Validating molecular markers of effect in *Folsomia candida* exposed to a fungicide-based formulation

Tiago Simões[a,b,c,*], Sara C. Novais[a,c], Tiago Natal-da-Luz[b], Mathieu Renaud[b], Sara Leston[a,b,d], Fernando Ramos[d], Jörg Römbke[e], Dick Roelofs[c], Nico M. van Straalen[c], José P. Sousa[b], Marco F.L. Lemos[a]

[a] MARE – Marine and Environmental Sciences Centre, ESTM, Polytechnic Institute of Leiria, Peniche, Portugal
[b] Centre for Functional Ecology, Department of Life Sciences, University of Coimbra, Portugal
[c] Department of Ecological Science, Vrije Universiteit, Amsterdam, the Netherlands
[d] REQUIMTE/LAQV, Faculty of Pharmacy, University of Coimbra, Portugal
[e] ECT Oekotoxikologie GmbH, Flörsheim, Germany

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ABSTRACT

Under controlled laboratory conditions, toxicity data tend to be less variable than in more realistic in-field studies and responses may thus differ from those in the natural environment, creating uncertainty. The validation of data under environmental conditions is therefore a major asset in environmental risk assessment of chemicals. The present study aimed to validate the mode of action of a commercial fungicide formulation in the soil invertebrate *F. candida*, under more realistic exposure scenarios (in-field bioassay), by targeting specific molecular biomarkers retrieved from laboratory experiments. Organisms were exposed in soil cores under minimally controlled field conditions for 4 days to a chlorothalonil fungicide dosage causing 75% reduction of reproduction in a previous laboratory experiment (127 mg a.i. kg^{-1}) and half this concentration (60 mg a.i. kg^{-1}). After exposure, organisms were retrieved and RNA was extracted from each pool of organisms. According to previous laboratorial omics results with the same formulation, ten genes were selected for gene expression analysis by qRT-PCR, corresponding to key genes of affected biological pathways including glutathione metabolism, oxidation-reduction, body morphogenesis, and reproduction. Six of these genes presented a dose-response trend with higher up- or down-regulation with increasing pesticide concentrations. Highly significant correlations between their expression patterns in laboratory and in-field experiments were observed. This work shows that effects of toxicants can be clearly demonstrated in more realistic conditions using validated biomarkers. Our work outlines a set of genes that can be used to assess the early effects of pesticides in a realistic agricultural scenario.

1. Introduction

In the international agreement between European Union (EU) member states, known as Treaty of Lisbon, it is stated in Article 191 that "the protection of the environment shall be based on the precautionary principle and on the principles that preventive action should be taken" (EU, 2007). Such a statement means that efforts must be made to establish new methodologies and develop predictive tools protective of environmental contamination. Predictive ecotoxicology aims to integrate the effects of stressors across different levels of biological organization, from molecular level to whole communities and ecosystems (van Gestel, 2012). This way, a generalized overview can be considered and regulated, based on biomarker endpoints or statistical models predicting real emerging scenarios, as a decision supporting system (De Coen et al., 2009). Most of the current ecotoxicological testing methods applied by regulatory agencies focus on apical endpoints with conservative standard laboratory toxicity tests, from which arbitrary application factors are applied and extrapolated to real ecosystems (Birnbaum et al., 2016; De Coen et al., 2009; Vighi and Villa, 2013). These traditional methodologies are still very valuable tools from a protective point of view but drag with them large margins of uncertainty to real scenarios of contamination. To circumvent this limitations, technological advances have now enabled an increased, faster, and more realistic understanding on the relationship between

* Corresponding author at: Edifício CETEMARES, Avenida do Porto de Pesca, 2520 – 630 Peniche, Portugal.
E-mail address: tiago.simoes@ipleiria.pt (T. Simões).

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contaminants and organisms (toxicity pathways), boosting also our ability to classify and characterize chemicals according to their mode of action (MoA), grouping them according to their specific toxicity and potential risk to the environment and human health. High-throughput technologies such as transcriptomics, proteomics, metabolomics or *in vitro* analysis allowed a dramatic increase of the data obtained from tissue and sub-cellular levels and link this data to higher levels of biological organization, resulting in faster, reliable and significantly more efficient approaches and methodologies to be considered in eco-toxicological studies. Allied to our improved ability to analyse, integrate, and model complex data, it is therefore possible to provide a more accurate scientific basis to predict early effects at a higher scale, which is of major concern in environmental risk assessment (Villeneuve and Garcia-Reyero, 2011). The European Commission (2012) has already recognized that advances in the identification and use of novel biomarkers for exposure are now possible and of major importance. Gene expression profiles have been a useful resource to use as biomarkers in human health for several years, but have also been gaining relevance in ecotoxicological studies (Chaousis et al., 2018). Chip-based technology is also an innovative pathway under development for risk assessments in the environment, using sensitive molecular biomarkers (Campana and Wlodkowic, 2018). Using biomarkers in risk assessment methods may therefore create the bridge between predicted and measured biological effects of contaminants in the environment.

Current laboratory studies in regulatory ecotoxicology are not performed on the molecular level but acute or even chronic laboratory tests are required in the lowest tier. Molecular studies are not yet required in a regulatory document. Although lower-tier laboratory studies (molecular level) are essential to unravel modes of action of chemical compounds, higher-tier studies (*e.g.*, *in situ*) are crucial as a validation criterion in environmental risk assessment trials. This way the ecological relevance is increased, in order to better describe the actual consequences for ecosystems (Van Straalen, 2003). The main goal of the present study was to validate previous transcriptomics results obtained in the laboratory (Simões et al., 2018a) by exposing the collembolan *Folsomia candida* to a commercial fungicide formulation through an in-field bioassay, by targeting specific molecular biomarker genes. These genes were selected according to the previous biological effects observed under laboratory conditions. By binding to -thiol rich molecules, chlorothalonil is expected to affect normal protein metabolism, cellular respiration and oxidative stress defense, causing cytotoxicity and ultimately abnormal cell proliferation in the tissues of contact, leading to impairment in developmental features such as molting and reproduction. The set of genes for the present study were also selected according to the adverse outcome pathways (AOP) developed or under development for other chlorinated chemicals and fungicides, available in the international repository AOPWiki (https://aopwiki.org/) and compiled in Table A.1. The replicability of results was also one of the main goals, to evaluate the suitability of these markers, to assess the early effects of fungicide formulations in a real scenario of pesticide application. The tested species *F. candida* is a widespread arthropod that occurs in soils throughout the world and has been used as a standard test organism in past decades for estimating the effects of pesticides and environmental pollutants on non-target soil arthropods.

2. Materials and methods

2.1. Organisms, test soil and pesticide

The test species *Folsomia candida* (Isotomidae: Collembola) was obtained from cultures maintained at the Soil Ecology and Ecotoxicology Laboratory of the Centre for Functional Ecology, University of Coimbra (Portugal). The animals were cultured in vessels filled with a wet mixture of plaster of Paris and activated charcoal (11:1, w/w) in a climatic chamber at constant temperature of 20 ± 2 °C, and with a 16:8 h light:dark photoperiod and fed with baker's yeast (Vahiné, Mccormick, Italy). The eggs were isolated and culture media was renewed every 2–3 days until enough synchronized (10–12 days) organisms for the exposure were obtained.

The test soil was located in an agricultural area from the lower Mondego valley in Coimbra, Portugal (40°12′44.0″N; 8°27′02.4″W). Prior to soil collection, the vegetation was cut to about 50 mm of the top soil layer (organic) and 54 soil cores were collected from this field area to be defaunated. Each soil core consisted in a rounded PVC tube 50 mm wide and 150 mm long. Each core was forced 100 mm in the ground and then removed, maintaining soil structure intact inside the core. Soil cores were then defaunated in the laboratory using two consecutive freeze-thaw cycles (48 h at −20 °C followed by 48 h at 25 °C). After defaunation, cores were kept at 4 °C for no longer than one week before the start of experiments. The physicochemical and mineralogical characterization of this natural soil can be consulted in Simões et al. (2018b).

The fungicide formulation Bravo500® used in the present study was acquired from Syngenta, Portugal, with chlorothalonil as active ingredient (40%, a.i.).

2.2. Exposure conditions

All defaunated soil cores were placed back (buried 100 mm deep) in the exact same location where they were previously collected. Soil moisture content was maintained in all soil cores by spraying artificial rain, prepared according to Velthorst (1993), on a daily basis (30 mL).

Thirty-six cores were used for gene expression evaluation, consisting in twelve replicates from each treatment: 1) control (Ct); 2) a low concentration (1/2 EC50, 60 mg a.i kg^{-1}); 3) a high concentration (EC50, 127 mg a.i. kg^{-1}). The pesticide concentrations correspond to the estimated EC50 value for reproduction under laboratory conditions (Simões et al., 2018a) and sensibly half this value, respectively. Synchronized organisms were placed into the soil cores (220 organisms per core) and allowed to acclimate for 4 h. After this period, the cores were sprayed (using a commercial sprayer system) with the fungicide formulation to achieve the desired nominal concentrations of 60 and 127 mg a.i. kg^{-1} soil. The inner volume of soil inside the cores and soil density (1300 g L^{-1}) were used to calculate total soil weight inside each core. The fungicide formulation concentrations were diluted to a volume of 50 mL at the appropriate dose level using distilled water and added to each core. After dosing of soil cores, a plastic mesh tarp was placed above the experimental site to avoid excess exposure to sun resulting from the removal of vegetation. The organisms were exposed to those conditions for 4 days and collected at the end of that period. The disposition of the soil cores in the exposure site can be seen in Fig. 1.

The last 18 cores were used to follow compound migration during the exposure. These cores were handled in the same procedures as the other cores (6 cores for control, 6 cores sprayed with 60 mg kg^{-1} and 6 cores sprayed with 127 mg kg^{-1} of the fungicide), but no organisms were added. These cores were destructively sampled immediately after contamination, and after 2 and 4 days. Three soil cores were sampled per treatment in each sampling date. During destructive sampling, each of the eighteen cores was divided into three sections for compound migration analysis: 0–25 mm, 25–50 mm and 50–100 mm. Soil samples were kept at −20 °C until chemical analysis.

2.3. Pesticide analysis

Analytical determinations of the active ingredient chlorothalonil present in exposed soil samples followed the methodology described by Singh et al. (2002), with some adaptations. All solvents used were LC grade and were filtered through 0.45 µm Whatman nylon membrane filters (Whatman, Maidstone, USA) prior to degassing in ultrasonic bath. Ultrapure water was obtained daily from a Milli-Q water purification system (Millipore, Bedford, MA, USA). Nitrogen was generated

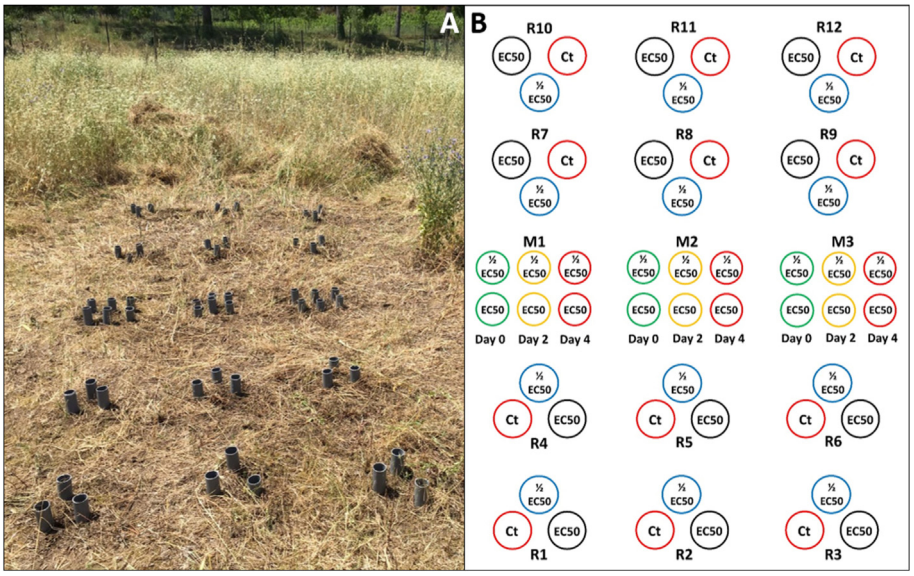

Fig. 1. Field disposition of the soil cores, sprayed with the chlorothalonil-based formulation in two different nominal concentrations (60 and 127 mg a.i. kg^{-1} soil): A – real disposition; B – schematic representation. Replicates R1 to R12 were used to follow the targeted gene expression effects on *Folsomia candida*. Replicates M1 to M3 were used to evaluate chlorothalonil vertical migration in soil. Ct = Control (0 mg a.i. kg^{-1} soil); EC50 = concentration causing 50% reduction in reproduction under laboratory exposures (127 mg a.i. kg^{-1}); 1/2 EC50 = half the concentration causing 50% reduction in reproduction under laboratory exposures (60 mg a.i. kg^{-1}).

in-house with a nitrogen generator from Peak Scientific Instruments Ltd. (Chicago, IL, USA).

From each sample, 5 g of soil were weighed into polypropylene centrifuge tubes, to which 4.5 mL of acetonitrile and 0.5 mL of ultrapure water were added. Samples were then vortex mixed and placed in an orbital shaker for 1 h. After this time, tubes were centrifuged at 5000*g*, for 5 min, the supernatant filtered and injected to the HPLC system.

HPLC–UV determination was conducted with a Gilson modular system (Gilson, Middleton, WI, USA) equipped with a pump (Gilson 321) and an automatic injector (Gilson 234) coupled to an UV/Vis detector (Gilson 155). The chromatographic column used for separation was an ACE C18 column (Advanced Chromatography Technologies Ltd., Aberdeen, Scotland) and a NewGuard C18 pre-column (PerkinElmer, Norwalk, USA) equilibrated at 25 °C. Scanning a solution of chlorothalonil allowed the selection for the most accurate wavelength for elution monitoring. Maximum absorption was achieved at 325 nm. Chlorothalonil was analyzed in isocratic mode with a mobile phase constituted by 0.1% formic acid in water (70%) and acetonitrile (30%). Twenty µL of sample was injected, flowing a rate of 1.2 mL/min for a total run time of 17 min. Limits of detection (LOD) and quantification (LOQ) were established at 0.17 and 0.5 mg kg^{-1}, respectively.

2.4. Sample collection and RNA isolation

Each core with the exposed organisms was retrieved from the site and placed in a tray with water. The organisms were floated to the surface, collected to RNase free microtubes and immediately placed in dry ice to be transported to the laboratory. Samples were kept at −80 °C until further analysis. Total RNA from each sample was extracted with the TRIZOL® extraction method (Invitrogen, Belgium), following the manufacturer's instructions. Organisms were homogenized mechanically, after adding 0.5 mL of TRIZOL® reagent. After the first RNA precipitation, wash and resuspension steps, total RNA was submitted to a DNase treatment using the RQ1 RNase-Free DNase kit from Promega (Madison, USA). RNA integrity was validated in a 1% gel electrophoresis for every sample before proceeding with the qPCR assays. Total RNA content and purity along with gDNA contamination were determined with Nanodrop 2000 (Thermo Scientific, USA) and Qubit® 2.0 Fluorometer (ThermoFisher Scientific, USA), respectively. Taking into consideration RNA yield, integrity and quality (Table A.2), the best six samples from each treatment (plus control) were then selected for a second DNase purification treatment and further gene expression evaluations.

2.5. cDNA synthesis and gene expression analysis

Total RNA (200 ng) was reversely transcribed into first-strand cDNA using the iScript™ cDNA Synthesis kit from Bio-Rad, containing a modified Moloney murine leukemia virus (MMLV) reverse transcriptase and both oligo (dT) and random hexamer primers in its reaction mixture. Synthesis was performed according to the manufacturer's instructions in a total volume of 20 µL for each sample.

The fungal non-systemic mode of action (MoA) of chlorothalonil is by binding to glutathione, inhibiting glutathione-dependent enzymes (Cox, 1997). Several other reports correlate chlorothalonil with other -thiol rich molecules activities (McMahon et al., 2011; Zhang et al., 2016). For the present study, targeted genes were based on a combined criterion between the fungicide's MoA, detoxification mechanisms of *F. candida*, and the verified results from a previous laboratorial exposure, where gene expression and reproduction of *F. candida* were evaluated upon exposure to the fungicide (Simões et al., 2018a). This previous laboratorial data was obtained from whole *F. candida* transcriptome sequencing, using Illumina HiSeq 2000 platform (Illumina, USA). Differential gene expression was evaluated using Limma and edgeR packages in R (version 3.1.3), following a GLM (Generalized Linear Model) regression, and data was deposited in NCBI SRA under accession SRP152014 (Bioproject PRJNA477642). Only differentially expressed genes identified during the laboratory exposure were considered. From these, twelve specific genes were used in the present study (Table 1). For the amplification reactions, the iTAQ™ Universal SYBR® Green Supermix was used. Each reaction (final volume of 20 µL) consisted of: 2 µL of DNA template, 2 µL of forward primer, 2 µL of reverse primer, 4 µL of nuclease-free water, and 10 µL of the reaction mix. The thermal cycling protocol comprised an activation step of 30 s at 95 °C and 40 cycles of a combined denaturation (5 s at 95 °C) and annealing (30 s at 60 °C) step. Melt curves were generated by measuring fluorescence after each temperature increase of 0.5 °C for 5 s over a range from 65 to 95 °C to verify the presence of the desired amplicon. Each assay was 3× technically replicated and amplification was performed on a CFX Connect™ Real-Time PCR System (Bio-Rad). Primer sequences were designed using Oligo Explorer software (version 1.1.2, Gene Link™), according to the annotated transcriptome of *F. candida* (Faddeeva et al., 2015) and are presented in Table 1.

All primer sets were tested for their efficiency and specificity. Real-time PCR amplification efficiencies (E) were calculated from the given

Table 1
Primer sequences and efficiencies of the selected genes from *F. candida*, evaluated by qPCR. Analysis was performed after exposing the organisms under minimally controlled field conditions to the formulated fungicide.

Gene abbreviation	Functional description	Accession number	Primer sequences (5′ → 3′)	Efficiency	r squared
Rps18	40S ribosomal protein S18	GAMN01000369	FW: TCCCTGACTGGTTCCTCAAC RV: GTGGGCTCGGATCTTCTTCA	97.0	0.998
Mth2	G-protein coupled receptor	GAMN01017015	FW: AACTAAGGATGACACCCAGC RV: CAGAGCCAGAAATAACCCAC	98.4	0.999
Hsp23	Heat shock protein 23	GAMN01004140	FW: GCTCCGAAGAAGGTGGTAG RV: TATCCCCAGTTTCTCCGTC	99.5	0.999
Hspβ1	Heat shock protein beta-1	GAMN01000935	FW: CGACGATCTCCTTGGGGTTT RV: GCAGCACTCATGTCACGTTC	100.0	0.978
GST	Glutathione S-transferase	GAMN01000417	FW: CCACTCCGTCATGAACCCAA RV: CTGTCCTCCCTAAAGCGTGG	99.1	0.994
RDH12	Retinol dehydrogenase 12	GAMN01003999	FW: CGACTCTGCCAGCTTTCTCA RV: GAAGAAGTCCTCGGCGTGAA	97.0	0.983
Ctns	Cystinosin	GAMN01037779	FW:ACTGAGGGGCAAATTGGGAG RV: TCTCACATGGCCCCTGTTTC	99.6	0.977
Cyp4C1	Cytochrome P450 4C1	GAMN01025638	FW: GTGGTTGCTAGGCGAAGGAT RV: CCAGGAATCCGTCGAAGCTT	99.7	0.957
VMO1	Vitelline membrane outer layer protein 1	GAMN01016071	FW: AGAAATGGAGCTGTGCCCTC RV: TGTTCCGCTGTTTACTCGCT	97.9	0.987
PDI-2	Disulfide-isomerase 2	GAMN01002621	FW: TTCTACTTTGTGCCCTCGCC RV: GGCAGATGTCAGAGTGTCCC	91.7	0.987
CTSL	Cathepsin L1	GAMN01028105	FW: AAGTATCCCTGCTGACCCCA RV: TGGTGGTTGGATATGGTGCC	95.3	0.997
celD	Endoglucanase E-4	GAMN01014347	FW:AGGACTACGTGGCAAATGAG RV: ACGTGCGACAGTAGATGTTG	90.2	0.998

slope of the standard curve according to equation: $E = (10^{(-1/\text{slope})} - 1) \times 100$. Simultaneously, a melting curve analysis was conducted to assess the specificity of each primer to produce a single, specific amplification product. Possible gDNA contaminations and primer dimers formation were also verified by using –RT controls (replicates without reverse transcriptase) and non-template control replicates (NTC), respectively.

To evaluate the effects of the fungicide in the exposed *F. candida*, relative expressions of the 10 selected target genes described in Table 1, were assessed in this study. Expression values were normalized with two housekeeping genes (HK). A ribosomal protein S18 (*Rps18*) and G-protein coupled receptor (*Mth2*) were tested and used as HK genes, based on previous results from Simões et al. (2018a). *Rps18* and *Mth2* revealed an amplification variability of less than one cycle between non-treated and treated samples ($\Delta C_T = 0.90$ and 0.34, respectively). Gene expression calculations were performed according to the methodology proposed by Hellemans et al. (2007). This is an adaptation from the Pfaffl (2001) methodology, using the equation: Relative fold change (RFC) = $(E_{GOI})^{\Delta Ct\ GOI}$/GeoMean$[(E_{REF})^{\Delta Ct\ REF}]$, where E is the efficiency of the primer for each gene of interest (GOI) and for the housekeeping gene (REF). The geometric mean (GeoMean) between HK genes relative quantities was used, accordingly. Results were considered as RFC to control and were $\log_2$ normalized.

2.6. Data analysis and statistics

To assess differences of chlorothalonil migration in soil, Student *t*-test analysis or one-way analysis of variance (ANOVA) followed by Tukey *post hoc*, were performed. For the statistical differences on the survival of *F. candida* after the in-field exposure bioassay, multiple comparisons between treatments were performed using Kruskall-Wallis, followed by *post hoc* Games-Howell tests. For the differential gene expression analysis, ANOVA was performed, followed by Dunnett's test. Correlation tests between laboratory and in-field gene expression datasets were performed using Pearson's correlation coefficient. Data normality was checked (through Kolmogorov-Smirnov test) before evaluating the correlations.

3. Results

Weather conditions during the exposure period consisted in temperatures ranging from 16 ± 0.8 °C and 31.3 ± 2.6 °C, with no precipitation events registered. After the exposure period, organisms were collected in the entire core, individuals in each soil core were counted and percentage survival was estimated. On average, 77.9, 79.5 and 55.4 organisms were collected in control, 1/2 EC50 and EC50 treatments, respectively, corresponding to recovery percentages of 35%, 36% and 25% from the initial 220 organisms added in every replicate from the different treatments (control and contaminated). The observed mortality was significant in all treatments (Games-Howell, $p < 0.001$) but not statistically different between them ($F_{2,33} = 1.363$, $p = 0.270$).

3.1. Vertical migration of chlorothalonil

To evaluate the downward migration of chlorothalonil (applied as a commercial formulation), residues of the fungicide were determined at different depths in the soil columns and at different time-points, as mentioned in Section 2.2. The vertical migration of chlorothalonil over 4 days (sprayed as a formulated product in two different concentrations) is presented in Fig. 2.

The chlorothalonil migration was distinct between applied chemical concentrations, with a faster migration when a higher concentration (EC50; 127 mg a.i. kg^{-1}) was applied, after spraying the pesticide formulations (Day 0). This can be inferred by the difference in average concentrations observed between the top layers 0–25 and 25–50 mm after spraying the lower formulation concentration ($t = 3.4$, df = 4, $p = 0.03$). The same result was not observed for the higher concentration tested, presenting no differences in the top layers ($t = 1.04$, df = 4, $p = 0.37$). Moreover, two days after exposure, the samples with the high concentration presented a more homogeneous distribution along the soil column (0 to 100 mm), while the samples with the low concentration (1/2 EC50; 60 mg a.i. kg^{-1}) presented higher concentrations in the top and middle layers (0 to 50 mm) comparatively to the bottom layer ($F_{2,6} = 7.59$, $p = 0.02$). This is evidence of a slower vertical migration of chlorothalonil when the lower concentration was applied. However, at the end of the experiment (after 4 days), high amounts of chlorothalonil were also found in the bottom layer

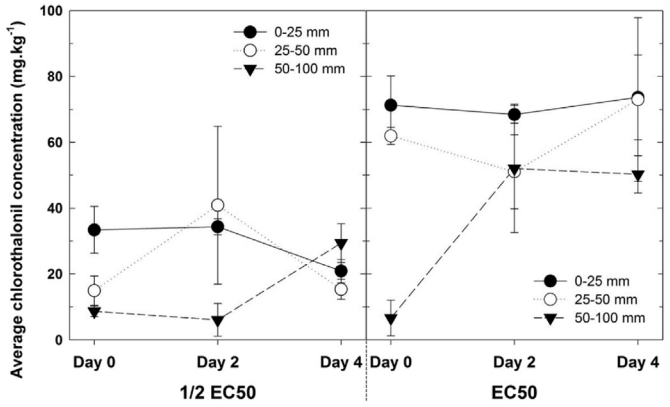

Fig. 2. Vertical migration of chlorothalonil in soil after 0, 2, and 4 days of application, using two sprayed formulated concentrations (1/2 EC50–60 and EC50–127 mg a.i. kg^{-1}). Results are presented as average (3 biological replicates) residual concentrations in each layer, plus standard deviation.

(50–100 mm) for both concentrations tested (no significant differences observed between layers), demonstrating a uniform distribution of the contaminant along the soil column. The fungicide migrated along the entire depth of the soil cores, for both tested concentrations, during the exposure period.

3.2. Gene expression

In the present study, transcript abundance was evaluated by qPCR analysis. All products showed a single peak in the melting curve analysis. The calculated amplification efficiency for primers ranged from 90.2% to 100%, $r^2 \geq 0.957$ (Table 1).

Relative fold change expression results for the 10 selected targets can be seen in Fig. 3, where results for untreated (control) and contaminated samples (1/2 EC50 and EC50) are displayed. For a comparative purpose, also average expression results obtained for these specific genes under previous laboratorial exposure conditions (after 4 days exposed to the EC50 concentration, 127 mg kg^{-1}), are presented in Fig. 3.

Expression values were in agreement for all the tested genes (up- or down-regulated) between laboratory and in-field bioassay exposures, although the expression fold changes were in general less pronounced in the in-field experiment. From the selected and tested genes, six were differentially expressed in the in-field bioassay experiment. *Ctns*, R*DH*, and *GST* were significantly up-regulated for the two tested concentrations of the formulated fungicide. The same number of genes were significantly down-regulated (*VMO1*, *PDI-2*, and *CTSL*), although CTSL was differentially expressed only for the higher concentration tested. From the detoxification and stress-related genes, glutathione S-transferase (*GST*) was the only differentially expressed gene and for both concentrations. Cystinosin (*Ctns*) presented the highest average level of over-expression, followed by retinol dehydrogenase gene (*RDH12*). The reproductive related gene, *VMO1*, was the most down-regulated gene, followed by a protein disulfide isomerase (*PDI-2*) and cathepsin L1 (*CTSL*). Despite the trend of increased average expression levels with contamination, heat shock stress related genes (*HSPb1*, *HSP23*) and cytochrome *Cyp4C1* did not reveal differential expressions upon exposure of *F. candida* to any of the tested concentrations of the formulation. It is important to mention that all the six differentially expressed genes increased their average expression patterns, positively or negatively, when fungicide concentration was increased, in a clear dose-response pattern. Overall, gene expression levels in the two in-field treatments (considering all the genes tested) proved to be highly correlated ($r_p = 0.84$, $p < 0.001$, $r^2 = 0.71$). All data passed normality test (Kolmogorov-Smirnov, $p > 0.05$) and therefore normal distributions were assumed.

For comparison and validation purposes, the relations between datasets from the different environments (laboratory and field) were also tested here (Fig. 4), correlating expression levels from 2 and 4 days exposure in laboratory with expression levels from the in-field bioassay (4 days exposure), using the same concentration used in the lab (127 mg kg^{-1}, EC50) and half this concentration (60 mg kg^{-1}, 1/2 EC50).

All datasets from the different environments have shown to be significantly and positively correlated. The most significant correlation was observed between 2 days exposure in the lab and field higher concentration (*Lab_2 days EC50 vs Field EC50*; Fig. 4A). However, when considering the same time and concentration of exposure between the different environments (*Lab_4 days EC50 vs Field EC50*), also highly significant correlations were observed (Fig. 4B). Datasets from two days of exposure in the lab and field lower concentration were also significantly correlated (*Lab_2 days vs Field 1/2 EC50*; Fig. 4C) and the lower correlation, although still significant, was registered between the datasets from 4 days exposure in the lab and field lower concentration (*Lab_4 days EC50 vs Field 1/2 EC50*; Fig. 4D). Overall correlations indicate that biological responses of *F. candida* to the fungicide were highly conserved for the selected genes, despite the potential variability resulting from different environments, time-points and pesticide concentrations.

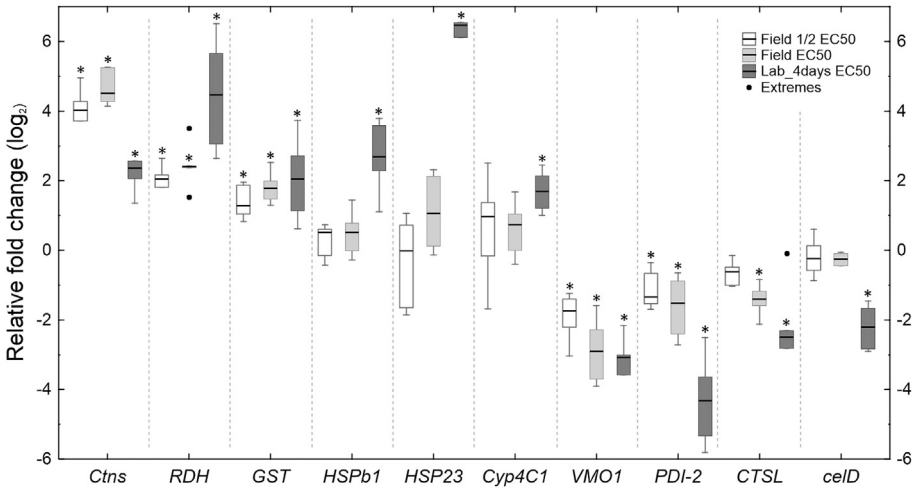

Fig. 3. Relative fold change (log_2) in expression of targeted *Folsomia candida* genes, after exposure to two sprayed concentrations of a chlorothalonil-based formulation (1/2EC50 and EC50) during four days in an in-field bioassay. For comparative purpose, average laboratorial fold changes (Lab_4 days EC50) for each gene were included. (*) differentially expressed genes compared to the respective laboratorial or in-field control.

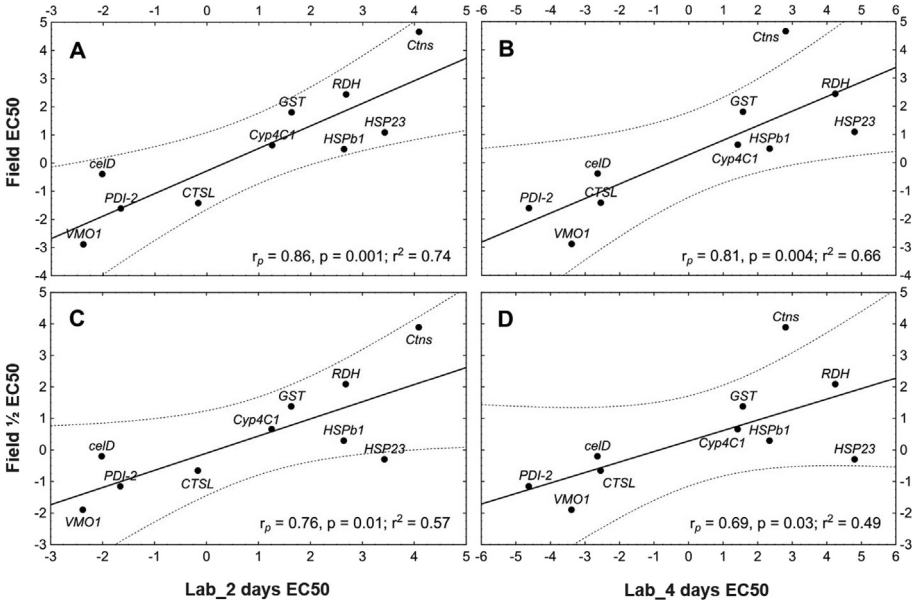

Fig. 4. Correlation and regression analysis between gene datasets from laboratory and in-field bioassay exposures, using average expression levels (fold change, log_2) from each of the 10 selected genes, represented by *x* and *y* axis. 1/2 EC50, (60 mg a.i. kg^{-1}); EC50, (127 mg a.i. kg^{-1}). Confidence intervals (95%) presented by trace lines.

4. Discussion

The in-field bioassay exposing *F. candida* to the fungicide formulation for four days was based on transcriptomics and proteomics results obtained in a previous experiment, where organisms were exposed to similar concentrations of the formulation, under laboratorial conditions (Simões et al., 2018a). The main objective in the present work was therefore to validate the applicability of a set of genes, criteriously selected and assumed to be linked to the mode of action of the fungicide, to be used as ecotoxicological markers under more realistic field exposure scenarios for chlorothalonil-based formulations.

As realism increases in chemical exposures, much more variability is expected, comparatively to laboratorial environment, where conditions are much more controlled and stable (Rivas et al., 2016; Römbke et al., 2006; Scott et al., 2009). This variability results from several factors that cannot be controlled in the field. Thus, despite the minimally controlled conditions (physical confinement and moisture), environmental factors could have originated the low recovery rates observed at the end of the exposure. Therefore, variables such as the daily temperature ranges or predation from higher invertebrates must be indicated here as possibilities, since arachnids, mites and annelids were found in the exposure site and inside soil cores (data not shown).

In a real environment, the activity of contaminants may be affected, as it may be the case for migration in the soil column or degradation rates, due to environmental factors such as eg. puddling that affect the soil properties and consequently contaminant activities (Kah and Brown, 2006). Collembolan species such as *F. candida* can be found in the litter or top soil layer, feeding on the surface of organic or mineral materials and the water film (Kaneda and Kaneko, 2002). However, these arthropods can migrate vertically in soil (Aldaya et al., 2006) and therefore are able to avoid surface stressful conditions. Considering the above-mentioned possibilities, a compound migration assessment was carried out to exclude the possibility of no contact between the pesticide and the test organisms due to avoidance behavior, considering that the effective damage of the pollutant in *F. candida* was intended as primary objective. Since chlorothalonil is a fungicide with reported low solubility (Sw = 0.85 mg L^{-1} at 25 °C) and high organic-carbon normalized sorption coefficient (Koc) of 1600–14,000 (Bending et al., 2007), low vertical mobility rates are expected in the soil column, as reported by Van Scoy and Tjeerdema (2014). Results, however, indicated that the active ingredient in the formulation migrated to the lower level of the soil column. While chlorothalonil concentrations were not quantified in the organisms themselves, results are demonstrative that the organisms were in contact with the pesticide along the soil core. The announced co-formulant in the formulation, propylene glycol (propane-1,2-diol), is miscible with a wide range of solvents (including water) and could therefore drag the free chlorothalonil downward. Chlorothalonil is a very low to moderately persistent fungicide in soil, with reported half-life (DT50) in the range from a few days to 2 months (EFSA, European Food Safety Authority, 2018). Singh et al. (2002) reported that the half-life of chlorothalonil in a natural soil was 8.6 days, applied at a concentration of 10 mg a.i. kg^{-1} dry soil. Since the concentrations tested in the present study were 6 to 12 times higher, significant degradation (DT50) would be less likely to occur within the time window applied in this study (4 days).

4.1. Gene expression

From the set of genes tested in the present study, the majority revealed significant expression responses to the pesticide.

Four of the selected genes, namely *GST*, *Ctns*, *CTSL*, and *PDI-2*, were initially selected as they are potentially implicated in the mechanisms of toxic action of the chemical, chlorothalonil. *GST* catalyzes the conjugation of glutathione with xenobiotic substrates during detoxification metabolism. Since glutathione is also targeted by chlorothalonil, an up-regulation of glutathione synthesis and glutathione dependent enzymes such as *GST* was anticipated and these results were confirmed here. Similar expression results for *GST* were reported in the literature for other non-target organisms (Elskus, 2012). The most up-regulated gene, *Ctns*, codes for a cystinosin protein that functions as an active transporter of cystine out of the lysosome. Cystines are oxidized dimers from two cysteine molecules. Any deficiency in cystinosin activity may lead to kidney disorders, as described with COS cells by Kalatzis et al. (2001) or with rodents (Wilkinson and Killeen, 1996). Considering the reported affinity of chlorothalonil to these simple -thiol molecules, *Ctns* up-regulation was, much likely related to the fungicide. Chlorothalonil could affect normal cysteine metabolism, especially in the detoxification and excretion systems in the gut of *F. candida*, as previously discussed in Simões et al. (2018a). In opposition, *CTSL* gene (Cathepsin L1) was down-regulated. The protein encoded by this gene is a lysosomal cysteine protease, playing a major role in intracellular protein catabolism. The active site of cathepsin L is composed of a reactive cysteine (Siklos et al., 2015). The interaction mechanism between the enzyme and chlorothalonil is not yet well explored, but the presence of

the fungicide significantly inhibited the normal expression of the coding gene when a higher chlorothalonil concentration was used.

Disulfide isomerase proteins (PDIs) are tangled in the progression and maturation of secretory proteins in the lumen of the endoplasmic reticulum (ER) (Ellgaard et al., 2018). PDIs catalyze the formation, reduction and rearrangement of disulfide bonds between cysteine monomers in proteins of eukaryotes, also acting as molecular chaperones in assisting polypeptide folding under stress conditions (Peng et al., 2017). Peng et al. (2017), also working with a polychlorinated compound (trichlorophenol), demonstrated that the molecule binds to and inactivates PDI. With such arguments, it would be expected for *PDI-2* coding gene to be over-expressed upon fungicide exposure to compensate for PDI inactivation by the fungicide. However, since the opposite was consistently observed here, the probable cause of this down-regulation might be questioned. In a study conducted by Winter et al. (2007), using the soil nematode worm *Caenorhabditis elegans*, the authors analyzed the PDI family of enzymes and they found that unlike the other disulfide isomerases, PDI-2 has an essential independent catalytic activity related to normal morphological (collagenous cuticle) and post-embryonic developments. In a more recent study, other authors described a PDI-2 enzyme to strongly interact with a nuclear embryo transcription factor (MEE8) with a role in regulating genes in maternal tissue necessary for normal embryo development (Cho et al., 2011). It is therefore possible that this particular PDI is also more related to developmental and reproductive features in *F. candida*, and less to protein folding (often associated to stress) and trafficking. This could explain the constant down-regulation of this gene, indicating a developmental impairment and a reproductive suppression upon fungicide exposure, which was previously demonstrated for *F. candida* under the same concentrations (Leitão et al., 2014; Simões et al., 2018a) and other non-target species (Du Gas et al., 2017; Yu et al., 2013). In this study, the reproductive related *VMO1* gene was also intentionally selected as target, since reproduction is of upmost ecological interest and normally addressed in classical ecotoxicological studies. VMO1 protein is found in the outside layer of the egg, in the vitelline membrane, acting as a barrier between the egg yolk and the outside environment. As expected, it was consistently suppressed in all tested conditions.

RDH12 presented an invariable significant up-regulation in all laboratorial time-points (2 to 10 days) after exposure to the same fungicide concentration as the higher concentration tested in this study (*Field EC50*). Based on these laboratory results it was selected for the in-field bioassay as a stress marker. This gene codes for a retinol dehydrogenase, responsible for the retinoid metabolism. Retinoids such as vitamin A are involved in a wide range of physiological processes such as oxidative stress response and immune function (Chen et al., 2015). In fact, these compounds have become widely used biomarkers of exposure to environmental pollutants and there are several reported studies indicating that the oxidative stress caused by chemical pollutants induces retinol metabolism (as in the present study) and storage to prevent oxidative damage (Novák et al., 2008). The other stress related genes evaluated here (*Hspβ1*, *Hsp23* and *Cyp4C1*) proved to be weak markers for *F. candida* exposure to chlorothalonil in the field, since their expression levels were not significantly different from control treatment (Fig. 3). Similar results were reported for heat shock proteins, using temperature as a stressor to *F. candida* (de Boer et al., 2010), although the authors conducted their tests under lab conditions and discuss the adaptability of *F. candida* to different temperature conditions. One should mention that the organisms were transposed from laboratory conditions to the exposure site and were not from a “natural field” population. By including natural conditions, like in the present study, it is probable that control organisms were also stressed due to other environmental factors and confinement in soil cores. Therefore, there was no significant difference between control and exposed organisms.

The overall effects of the tested concentrations of the fungicide formulation in expressions of targeted genes were positive and highly correlated, with the majority of the tested genes presenting differential expression patterns. It is also worth mentioning that these genes demonstrated a very evident dose-response pattern, when considering the different fungicide concentrations tested here. This was particularly clear for *Ctns*, *RDH*, *GST*, *VMO1*, *PDI-2*, and *CTSL* genes.

4.2. Laboratory and in-field comparisons

The expression patterns of the selected genes, between laboratory and in-field bioassay exposures, also showed positive, significant correlations and this was consistently observed between all selected treatments (Fig. 4). Interestingly, the best correlation was found between the results from the 2 days' exposure in laboratory and *Field EC50* (after 4 days), and not between expressions after 4 days for both treatments. These results lead one to believe that under more controlled conditions, the interaction of the subjects with the fungicide may be more direct and forward, compared to an in-field scenario, where a possible initial avoidance may be achieved by the organisms. In the outdoor experiment, the contamination was made by spraying, while in laboratory the soil was spiked with the fungicide, allowing a faster scattering and probably a more efficient organism-chlorothalonil interaction. Other possible explanations may be related to specific metabolic functions related to some of the selected genes, possibly triggered with some delay under field conditions (comparatively to laboratory), due to the higher constraints presented in this environment. Such events could also explain the general less pronounced fold changes in expression found for the in-field experiment (Fig. 4). There are numerous reports in the literature exploring biomarkers of effect to pollutants, under laboratory, or controlled field scenarios (*in situ* exposures). However, results from these studies are often assumed to represent different environments without a comparative validation, considering other possible influencing factors. There are few ambitious studies from the past few years attempting to validate specific biological markers in different environments, revealing however limited correlating evidence (Baillon et al., 2016; Kleinhenz et al., 2016; McClain et al., 2003; Quirós et al., 2007). This is more evident when addressing sensitive endpoints at the sub-cellular level such as gene expression (Song et al., 2018), probably hampered by background noise caused by the large variability of results expected from an exposure under less controlled conditions, with many more influencing factors (as already discussed), both biotic and abiotic (Li et al., 2010; Mikó et al., 2015).

In soil ecotoxicology, there are also few standardized tests to be performed in the field (van Gestel, 2012) and laboratory exposures are not commonly validated *in situ* as well, which would logically present higher ecological relevance. According to Kumpiene et al. (2019) this could very well be due to conservative institutional structures, contributing to the slow transition from laboratory to higher tier demonstrations. Regarding soil studies, there are prospective reports in the literature exploring gene makers and reference genes to be used under more realistic exposure conditions. However, most of these studies are performed under controlled conditions in laboratory and from these, only a fraction mention a naturally contaminated soil matrix (Brulle et al., 2011; Poynton et al., 2008; Roelofs et al., 2012). The comparative information between laboratorial and higher tier (semi-field, in-field bioassays) validation of specific gene markers for anthropogenic soil exposure is missing in the literature. This goal was attempted here and provided very compelling results for future natural exposure assessments. From a regulatory point of view, the cross-validation of results through different methodologies is of major importance (Martyniuk, 2018). The strong correlations observed here resulting from different methodological approaches also highlight the significance of the present work. Despite considering additional abiotic factors (*e.g.* different soils) or biotic interactions would benefit the study, the results confirm the close relation between the tested genes and chlorothalonil, making them optimal candidates as early warning indicators in field risk assessment schemes for chlorothalonil-based exposures.

5. Conclusions

A set of differentially expressed genes, *Ctns*, *RDH*, *GST*, *VMO1*, *PDI-2*, and *CTSL*, were demonstrated as optimal candidates to be used as early warning indicators in the field for chlorothalonil-based exposures. These results indicate that laboratory testing to determine sensitive genes was validated under more realistic exposure scenarios. Thus, these laboratory approaches have the potential for a more widespread field use. With the emergence of cost-efficient and sensitive analytical techniques such as omics or chip-based technologies in the field of ecotoxicology, this work presents a scientific basis to potentially develop an array to be used under natural chlorothalonil (and possibly related chlorinated chemicals) exposure scenarios.

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Declarations of interest

None.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envint.2019.03.073.

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