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Assessment of acute toxicity tests and rhizotron experiments to characterise lethal and sub-lethal control of soil-based pests

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1. Abstract

BACKGROUND: Characterising lethal and sub-lethal control of soil-based pests with plant protection products is particularly challenging due to the complex and dynamic interplay of the system components. Here we present two types of studies: acute toxcity experiments (homogenous exposure of individuals in soil) and rhizotron experiments (heterogeneous exposure of individuals in soil) to investigate their ability of strengthening the understanding of driving mechanisms of effectivness of the plant protection product. Experiments were conducted with larvae of the western corn rootworm *Diabrotica virgifera virgifera* LeConte and three pesticide active ingredients (clothianidin (neonicotinoid), chlorpyrifos (organophosphate), and tefluthrin (pyrethroid).

RESULTS: The order of compound concentrations needed to invoke a specific effect intensity (EC_{50} values) within the acute toxicity tests was chlorpyrifos > tefluthrin > clothianidin. This order changed for the rhizotron experiments because application type, fate and transport of the compounds in the soil profile and sub-lethal effects on larvae also influence their effectiveness in controlling larval feeding on corn roots.

CONCLUSION: Beyond the pure measurement of efficacy through observing relative changes in plant injury to control plants, the tests generate mechanistic understanding for drivers of efficacy apart from acute toxicity. The experiments have the potential to enhance efficacy testing and product development and might be useful tools for assessing resistance development in the future.

2. Key words

rootworm, corn, tefluthrin, clothianidin, chlorpyrifos, efficacy

3. Introduction

Efficacy assessment provides fundamental information for the placement of plant protection products on the market[1]. Efficacy describes the power of a product to produce an effect that fulfils the claims made for it on the proposed label[2]; characterisation is particularly challenging for products that control soil-based and root-damaging pests due to the complex and dynamic interplay of the system components (compound/formulation, soil, roots, target pest, and environmental conditions). Constant contact exposure of pest organisms via direct dermal application[3] or filter paper application[4, 5] and subsequent assessment of mortality (to derive dose-response relationships) are frequently conducted, but are least representative of the system because behaviour of the pesticide in the soil and responses of target organisms to these compounds are ignored. These types of efficacy trials are categorised as 'laboratory or growth chamber tests' within the preliminary screening for the biological dossier according to the Guidelines on Efficacy Evaluation for the Registration of Plant Protection Products[6]. Field efficacy studies represent the other extreme of environmental realism, assessing efficacy in standardised field experiments within a growing season. Standardisation of field experiments often includes a rating system that is specific to the plant and target pest, such as the measurement of reduced corn root injury from the corn rootworm compared to controls via the node injury scale[7]. Such trials are categorised as 'operational large scale trials'[6]. There is uncertainty about the extent to which results from one field study (or a low number of studies) are representative of the full range of possible environmental conditions (e.g. different soils, different climates, different strains of pest) and farming practices. Furthermore, the study design generally prevents identification of the driving mechanisms behind observed efficacy. Between these two extremes of efficacy trials in terms of environmental realism, there are several intermediate studies that are carried out in the laboratory or greenhouse[3, 8-10]. Examples of such studies are experiments where organisms

are exposed in soil where the compound is homogeneously present and mortality of individuals is reported, plant pot studies in greenhouses, and an exposure in soil where proposed field application of the compound is simulated in rhizotrons.

Here we compare two sets of experiments from the above mentioned intermediate category of efficacy trials conducted with three pesticide active ingredients (clothianidin, chlorpyrifos, and tefluthrin). We assessed the ability of the tests to deliver more detailed insights into compound-specific modes of action that could benefit the biological dossier for evaluation of plant protection products. Experiments were conducted with larvae of the western corn rootworm (*Diabrotica virgifera virgifera* LeConte) as a model species. The first set of experiments comprised three acute toxicity tests in growth cabinets where larval survival and changes in larval behaviour were investigated over time at different exposure concentrations for a period of five days. The second set of experiments were set up as rhizotron studies conducted in a greenhouse over 3-4 weeks where the spatial appearance of larvae and their behaviour was recorded over time after exposure to concentrations of the active ingredients that were comparable to those in the field (i.e. specific compound placement at sowing and at field-relevant rates).

4. Materials and methods

Experiments were carried out at the Department of Crop Sciences, University of Göttingen (Germany) with three technical grade pesticides obtained from Sigma-Aldrich, namely clothianidin (CAS 210880-92-5), chlorpyrifos (CAS 2921-88-2) and tefluthrin (CAS 79538-32-2). Pesticides used were chosen to be active against larvae of the western corn rootworm whilst differing both in mode of action and physico-chemical properties. Table 1 summarises the characteristics of the three pesticides used.

Non-diapausing eggs[11] were obtained from the USDA-ARS North Central Agricultural Research Laboratory (Brookings, South Dakota, USA) and stored under dark and cold (7°C) conditions. Prior to laboratory experiments, eggs were prepared for hatching (incubated for 12 days at 25°C and 65% relative humidity in an incubator cabinet (Mytron GmbH, Heiligenstadt, Germany)) and reared on untreated maize roots (cultivar: Ronaldinio, KWS SAAT SE, Einbeck, Germany) from plants at growth stage BBCH 11[12] in peat soil (Fruhstorfer Erde, Typ P, Hawita Gruppe GmbH, Vechta, Germany). Larvae were extracted from the culture containers via an adapted Kempson chamber[13]. Further details on the methods for egg hatching and larval rearing are described by Brandl et al. (2016)[10].

Acute toxicity tests investigated the time- and concentration-dependence of acute toxicity, ultimately identifying the effect concentration for 50% of the tested individuals (EC_{50}) after different exposure durations and under constant environmental conditions. Rhizotron experiments investigated how effects varied over time when pesticides were used similarly to the field situation (seed or band application at sowing), but still under controlled environmental conditions. Both sets of experiments were conducted with late 2nd larval instars for practicality. First instar larvae were considered to be too small to achieve an acceptable recovery rate of larvae from the soil (acute toxicity test) and observational success (rhizotron experiments).

4.1. Acute toxicity tests

Larvae were exposed to pesticide-active substances mixed into soil (silt loam collected from a field in Göttingen (DE) (51°33'09.3"N 9°53'55.9")) to determine time- and concentration-dependent effects of the pesticides. The number of surviving and immobile larvae was assessed and changes in larval appearance and/or behaviour were recorded following exposure for 24, 72 or 120 h.

Ten larvae (2nd instar) were placed onto 200 mL soil containing 0, 0.05, 0.15, 0.44, 1.33 or 4 mg active substance per litre soil volume (ppm) of tefluthrin, clothianidin, or chlorpyrifos, respectively. Each experiment began with three replicates for each assessment point (24, 72 and 120 h) and concentration. Independent replicates were needed for each assessment day because manual recovery of larvae from the soil disrupted the sample. Sample preparation involved mixing 200 mL soil (air dried; sieved to < 2 mm; sand 24.3%, silt 56.7%, clay 19.0%, organic carbon content 1.7%) with 25 mL solution (tap water containing 0.8% acetone for the control treatment or 0.20, 0.59, 1.78, 5.33 and 16.0 mL of a 50 mg/L stock solution that contained 2% acetone topped up with tap water to a volume of 25 mL). Samples were left standing at room temperature for three to five hours to allow the acetone to evaporate prior to placing six pre-germinated maize seeds (Ronaldinio; 15MAO1128; KWS SAAT SE), grown to the category 07 on the Zadoks Scale by placing them for two days at 25 +/- 1°C on wet towels in the dark at humidity >65%, 1-2 cm beneath the soil surface and adding ten rootworm larvae at the soil surface. Subsequently, beakers were stored in a controlled climate chamber until larval recovery. Environmental conditions were constant temperature of 20 +/- 1°C, constant relative humidity of 65%, and a light-dark cycle of 16:8 h.

Organisms were manually removed from soil at the respective sampling interval (24, 72 or 120 h) by sifting the entire sample with tweezers until all organisms were recovered. Organisms were placed on a Petri dish and categorised as either dead, immobile, knocked down, or mobile. Individuals with directed movement across the Petri dish were categorised as mobile, whilst those that were moving without a sign of directed movement were categorised as knocked down. Immobile and dead individuals were distinguished by touching both ends of the larvae with a brush; those showing an avoidance reaction but without the

ability for whole body movement (i.e. twisting the head or abdomen) were categorised as immobile.

Larvae were assessed for their body size (measured as head capsule width) immediately before placement onto the soil and reassessed for head capsule width again after their recovery from the soil following 120 h of exposure. Head capsule width was measured as a proxy for moulting during the experimental phase (because it is not possible to recover the remains of the exoskeleton from the soil sample) and to assess any size dependency of mortal effects. A method previously developed for aquatic invertebrates was used, where organisms were transferred to a Petri dish and a picture of the Petri dish was taken using an ordinary flatbed scanner. Pictures were subsequently manually analysed with purposely developed and freely available software[14].

4.2. Rhizotron experiment

The rhizotrons consisted of a thin soil layer (same soil as used for the acute toxicity tests) between glass sheets (30 cm width * 60 cm height * 0.6 cm depth). Plastic sticks (0.6 cm thick) separated the glass sheets at both sides and perforated adhesive tape formed the base of the rhizotrons. The glass sheets were marked with a first- and second-order grid that divided the entire soil profile into 50 equally sized squares of 6x6 cm (first-order grid) and the area of intense root growth into 36 equally sized squares of 3x3 cm (second-order grid). Figure 1 illustrates the set-up of the rhizotrons and the experiment.

For larval insertion, straws with a diameter of 2 cm were squeezed between the glass sheets on both sides of the rhizotrons prior to filling with soil. Rhizotrons were then filled with soil until only the top 6 cm were soil free. Subsequently, the soil in the rhizotrons was wetted to field capacity by letting water drain through the soil profile and allowing excess water to leach out of the rhizotrons. Then a maize seed (cultivar: Ronaldinio, KWS SAAT SE,

Einbeck, Germany) was placed in the centre of the soil surface and covered with soil until the top 3 cm of the rhizotrons was soil free, and pesticide was applied dissolved in tap water containing 2% acetone. Clothianidin was applied in a single spot directed onto the seed to treat the seed with 0.6 mg active substance which represents the mass equivalent of a seed treatment with Poncho®1250 (Bayer CropScience). Tefluthrin and chlorpyrifos were applied in a 25-cm band onto the soil just above seeding depth (with 125 µL stock solution per cm band width) to simulate a band application with Force® 3G (Syngenta) for the tefluthrin treatment and Lorsban® 15G, Saurus® 15G (Dow AgroSciences) or Nufos® 15G (Cheminova Inc.) for the chlorpyrifos treatment. Band application rates were derived from application rates in the field[15] so that the total load of the compound per soil volume available for each seed equalled the total load per soil volume and seed from the field application rate. For this calculation, only the soil volume of the upper 30 cm in the field was considered, because it is known that eggs and larvae of the rootworm mainly occur at this depth in the field[16]. In total 0.338 mg tefluthrin or 3.38 mg chlorpyrifos was applied in the rhizotron. Application in the rhizotron was calculated as follows. In the field, there were 6.2 seeds per row meter and the soil volume per row meter was 228 L (accounting for row spacing of 76 cm (i.e. 30 inches) and a soil depth of 30 cm). Thus each seed in the field had 36.77 L of soil dedicated to them. In the rhizotron the soil volume available was 1.08 L. Thus, a factor of 33.43 (i.e. 36.77/1.08) was applied to the field application rate to derive the soil mass equivalent application per seed. Lorsban® 15G (chlorpyrifos content 15%) for example was applied as 8 oz /1000 ft of row[16]. This equals 111.6 mg active ingredient per m row. Dividing this by the soil equivalent factor gives an application rate of 3.38 mg/seed.

Following application of any pesticide, the rest of the rhizotron was filled with soil; thus sowing depth was 6 cm. The top soil was carefully wetted by adding small amounts of water

199 sequentially and letting it distribute through the dry soil (indicated with a change in colour).
200 This procedure was repeated until the top soil was consistent in colour (no dry soil remained).
201 Filled rhizotrons contained a soil volume of 1.08 L with an average soil mass of 1.20 kg. The
202 sides of the rhizotrons were covered with a black cloth to prevent light interfering with root
203 growth and larval behaviour. Rhizotrons were then placed in trays filled with tap water to 5-
204 cm height, and kept in a greenhouse for three weeks (average temperature 25°C and relative
205 humidity of 65%); water demand for plant growth was satisfied through maintenance of the
206 5-cm water layer in the trays. In total 24 rhizotrons were prepared with six replicates for the
207 control and six for each of the three pesticide treatments. Test solutions of each of the three
208 pesticides were prepared from the same technical grade active substances, whilst application
209 type and application rates were derived from field efficacy studies reported in the literature[15].
210 Seed germination and subsequent plant growth (growth stage BBCH 12-13 according to
211 Lancashire et al.[12]) was similar in 22 out of the 24 rhizotrons prepared. Plants in the other two
212 rhizotrons showed delayed germination and thus produced a significantly smaller root system
213 and less plant growth (growth stage BBCH 10-11[12]) within three weeks. The experiment
214 continued with only the 22 rhizotrons that showed similar plant and root growth, reducing the
215 number of replicates for the control and the clothianidin treatment to five. Following plant
216 growth in the greenhouse for 18 days, rhizotrons were moved to a controlled climate room
217 (constant temperature of 25 ± 1°C, constant relative humidity of 65%, and a light-dark cycle
218 of 16:8 h) to acclimatise the rhizotrons to the same environmental conditions under which the
219 corn rootworm larvae were cultured. After three days of acclimatisation, 20 late 2nd instar
220 larvae were inserted at the edge of each side of each rhizotron 12 cm beneath the soil surface
221 by dropping them through the prepared straws.
222 A snap shot of larval appearance and larval behaviour was taken on a daily basis for four
223 consecutive days starting one day after larval placement into the rhizotrons. Rhizotrons were

224 moved to a dark room to avoid stressing the individuals with extended exposure to light, the
225 black cloth was removed and all grids were examined on each side of the rhizotron with a
226 flashlight. The number of larvae in each grid was recorded and physiological status of each
227 larva was reported under the following categories 'moving', 'resting', 'feeding', 'pupated',
228 'knocked down' or 'dead'. An illustration of the rhizotron experiment containing an
229 explanation of the physiological status is given in Figure 1.
230

231 ## 4.3.Data analysis

232 Dead and immobile larvae observed in the acute toxicity test were summed and data
233 (fraction of dead and immobile individuals over number of individuals introduced to the
234 system i.e. 20)) were analysed using SigmaPlot (version 13.0, Systat Software, San Jose, CA)
235 to derive dose response curves (by fitting a Sigmoid, 3 parameter function to the raw data) to
236 calculate the EC_{50} values after 24, 72 and 120 hours of exposure. We use the term EC_{50} rather
237 than LC_{50} because we merged the data on dead and immobile larvae; immobile larvae cannot
238 reach the food source and thus do not contribute to root damage within the assessment period.
239 Data on width of the head capsule were used to calculate a percentage increase over 120 h for
240 each of the three replicate beakers of the control and the three smallest concentrations tested.
241 These values were tested with a one-way ANOVA in SigmaPlot (version 13.0, Systat
242 Software, San Jose, CA).
243 Results from the rhizotron experiments were also analysed in SigmaPlot (version 13.0,
244 Systat Software, San Jose, CA) performing either a two-way ANOVA (observation time and
245 compound as variables) or a three-way ANOVA (observation time, compound and soil depth
246 as variables). All ANOVAs used the Shapiro-Wilk test to test for normality and the Brown-
247 Forsythe test to test for equal variance. The pairwise multiple comparison procedures were
248 conducted using the Holm-Sidak method with an overall significance level of 0.05.

5. Results and discussion

5.1. Acute toxicity tests

Across all three experiments (each with one active ingredient), all six pre-germinated maize seeds emerged within 5 days in treatments above 0.44 mg/L whilst only 1-5 seedlings emerged from the soil in the concentrations 0.05 and 0.15 mg/L and only 1-3 seedlings emerged across all control beakers. This indicates that root damage beyond tolerable levels for plant growth occurred due larvae introduced into the system and that extent of damage was dependent on pesticide concentration. Effects on larvae observed as mortality or immobility varied not only with concentration but also with duration of exposure. Table 2 summarises the EC_{50} values and their 95% confidence intervals after 24, 72 and 120 h of exposure. The efficacious dose based on the median EC_{50} values decreased in the order chlorpyrifos > tefluthrin > clothianidin (organophosphate > pyrethroid > neonicotinoid). Taking into account the 95% confidence interval however, only clothianidin is significantly different from chlorpyrifos and tefluthrin after 24 and 48h of exposure whilst chlorpyrifos is significantly different from the other two compounds tested after 120h of exposure.

How far EC_{50} values represent actual 50% effect doses for larvae of laboratory cultures and field populations of the western corn rootworm cannot be determined. It is possible that the values differ between strains more than they do between compounds tested. Magalhães and co-authors (2007)[4] obtained EC_{50} values for clothianidin testing neonates from 19 field collected and laboratory cultured western corn rootworm populations exposed on filter paper for 24 h and found a maximal difference between the EC_{50} values of a factor of 20[4]. Direct comparison of EC_{50} values obtained here with EC_{50}, LC_{50} values (lethal concentration for 50% of tested organisms) and LD_{50} values (lethal dose for 50% of tested organisms) from the literature for any of the compounds is not possible because exposure types (soil application in

the current study, direct dermal application[3], and filter paper application[4, 5] were different and studies were also conducted with different strains and differently sized / aged larvae ranging from neonates to late 3rd instars. Average LD_{50} from direct dermal application for 3rd instar larva for example ranged for chlorpyrifos between 3.16 and 11.3 ng /mg larva and between 2.55 and 78.3 ng/mg larva for tefluthrin after exposing lrava for 24h[3]. LC_{50} values for clothianidin derived using neonates of different strains exposed for 24h on filter paper ranged between 1.5 and 21.9 ng/cm^2 filter paper. Though the actual values we obtained here for the EC_{50} after 24 h of exposure are one to two orders of magnitude lower than those found in the literature, we cannot state whether the test generated similar results or not. The differences in exposure methods and units of the results do not allow direct comparison.

In addition to causing immobility and mortality, each compound showed at least one different characteristic effect on the larvae during the acute toxicity test. These effects were observed consistently but were not thoroughly quantified due to time constraints during the experiment. Tefluthrin made the larvae move in a specific and uncontrolled pattern in all exposure concentrations at and above 1.78 mg/L from the first observation (exposure duration 24 h) onwards. A demonstration of this behaviour and comparison with that of larvae recovered from the control has been recorded (see video "Twist and Curl" in comparison to the video "Control" accessible here). Larvae exposed to clothianidin that were categorised as dead or immobile looked different to those that were dead or immobile following exposure to the other pesticides. An abnormally enlarged abdomen, swollen to a diameter approximately three times the normal width (Figure 2), was observed from the first observation onwards.

Organisms exposed to chlorpyrifos did not show an immediate (24 h after exposure) observable impact in contrast to the other compounds, but comparison of their growth/moulting (measured as increase in head capsule width) after five days of exposure revealed that survivors grew/moulted 54% less than larvae from the control treatment (11.2%

299 increase in comparison to 24.3% with marginal significance, p = 0.063) (Table 3); no
300 difference in head capsule width was measured prior to exposure (p = 0.58). An indication
301 that the non-significant reduction in growth observed here might be an actual effect is the
302 previous observation of delayed emergence of adult beetles from soil following chlorpyrifos
303 application. Sutter et al.[17] studied the field efficacy of several compounds in corn and
304 observed a delayed emergence of rootworm in plots treated with chlorpyrifos. Reduced
305 growth, as observed here, can lead to prolonged development of larvae and thus delayed
306 emergence[17]. Whether or not the observed change in the overall head capsule width really is
307 an indication of reduced growth cannot be verified with the experimental data alone because
308 changes in overall head capsule width can arise from size selective mortal effects that might
309 have led to a bias of the measured data. Whether clothianidin and tefluthrin might also trigger
310 growth inhibition could not be assessed accurately due to limitations of the method used. The
311 measurement method only works when the organisms (or in this case the head capsules) are
312 close to or touching the surface of the scanner. Excessive movement of organisms (twisting
313 and curling triggered by tefluthrin exposure) and elevation of the head capsule from the
314 surface of the Petri dish (due to an enlarged abdomen triggered by clothianidin exposure)
315 limited the production of pictures of sufficient quality for analysis. A more accurate method
316 for the assessment of growth and thus growth inhibition should be included into the acute
317 toxicity experiment in the future. Most likely weighing the organisms before and after
318 exposure would yield more accurate and statistically powerful measures that are not affected
319 by other physiological alterations of the organisms.
320

321 ## 5.2. Rhizotron experiment

322 Figure 3 summarises the relative retrieval of individuals placed into the rhizotrons over
323 time. Retrieval in each treatment decreased over time, whilst retrieval was greatest overall in

the rhizotrons treated with tefluthrin, followed by chlorpyrifos and clothianidin and the overall lowest number of organisms were retrieved from the control rhizotrons. The temporal decline in organisms retrieved was only significant for the control and the clothianidin treatment; the significance for the latter treatment derives from the exceptionally high retrieval at the first observation period in comparison to the other treatments. The low overall retrieval observed as well as its temporal decline is a typical source of error for this kind of experiment[10,18]. Observations are constrained by the thickness of the soil layer, and the resulting restriction in observation of all larvae introduced into the systems. Especially 2nd instar larvae were reported to burrow extensively into roots, making it difficult to observe larval behaviour and thus reducing the chance to find larvae following introduction into the rhizotron[18]. The overall retrieval was likely lowest in the control treatment because of the higher feeding activity of unexposed larvae ($p<0.001$) that was observed compared to those in the pesticide treatments (Figure 4).

Significant ($p<0.001$) effects of the pesticides are apparent when looking at the number of individuals found to be dead or severely affected (characterised as 'knocked down'). Despite the treatments being different in terms of the application zone (seed or band application) and the amount of active ingredient applied per litre of soil (clothianidin 0.6 mg; tefluthrin 0.313 mg; chlorpyrifos 3.13 mg), the relative number of individuals dead or knocked down increased in all pesticide treatments over time and reached between 40 and 48% within 96 h (tefluthrin 42.2 ± 12.6; clothianidin 47.9 ± 28.9; chlorpyrifos 40.0 ± 21.2). Thus the total compound load used in the rhizotron experiment resulted in average pesticide concentrations in soil that were 4 to 11 times higher than the EC_{50} values observed in the acute toxicity experiments without reaching higher mortality. In addition to these effects that were observed in all treatments tested, there was a specific effect of clothianidin that caused an increased

number of larvae to pupate ($p \leq 0.015$), in turn leading to a smaller number of moving organisms (Figure 4).

The spatial data on where individuals were found show a tendency for tefluthrin and clothianidin to provoke the larvae to move further down in the soil profile in comparison to larvae in the chlorpyrifos exposure and the control (Figure 5). This only becomes apparent when comparing observations from the first and last observation period. Vertical distribution of the larvae was similar across all treatments and control 24h after placing them in the rhizotrons (Figure 5 top). Most larvae remained in the horizontal plane where they were introduced to the system (13-18 cm depth) and an almost equal number of larvae moved up or down. Within 96 h, however, larvae that were not affected moved extensively within the soil profile as demonstrated by an overall significant interaction between time and depth ($p < 0.001$). The direction of the movement was treatment-specific (Figure 5 bottom) and significant for the comparison of interaction between depth and compound ($p < 0.001$).

The statistically-significant increased downwards movement following treatment with tefluthrin and clothianidin was probably caused by the presence of the compounds in the upper soil layers and the known repellent effect of both compounds. Michaelides et al. demonstrated a repellent effect of tefluthrin at sub-lethal concentrations for the northern corn rootworm (*Diabrotica barberi* Smith & Lawrence)[19]. Movement away from thiamethoxam (a precursor for clothianidin) was observed for neonate western corn rootworm larvae exposed on filter paper[5]. Repellency was not only apparent from spatial location data in the vertical plane, but also in the horizontal plane (Figure 6). Whilst for all other treatments and the control a movement of larvae from the edges of the rhizotron to the centre was observed over time, this pattern of movement was much less pronounced in the rhizotrons treated with tefluthrin. The clothianidin treatment most likely did not provoke reduced horizontal

372 movement of larvae as it was not distributed widely in the horizontal plane following local
373 application onto the seed.
374 All larvae in soil within 12 cm below the compound application zone (indicated with the
375 dashed line in Figure 5) were severely affected (i.e. not able to move or dead). It is not
376 possible to verify whether clothianidin really moved downwards because compound
377 distribution in space and time was not measured. The physico-chemical properties of this
378 compound combined with the absence of water infiltrating from the top of the rhizotrons
379 would suggest that the compound would be transported upwards rather than downwards due
380 to upwards movement of water for soil evaporation. Water extraction from the soil profile
381 from roots that grew below the application zone, however, could have contributed to partial
382 downwards movement of the compound. Furthermore, the known translaminar and root
383 systemic activity of clothianidin[20] likely allowed the compound to be distributed within the
384 root system and affect larvae feeding on roots. Alford and Krupke (2017) found that
385 clothianidin was present in root tissue of corn up to 34 days post planting of seed-treated
386 seedlings and that up to 1.34 and 0.26% of the applied compound was recovered from tissue
387 and root material[21]. For the other treatments, there were still some active larvae found within
388 this area (zone of most dense root appearance), an indication that either the compound did not
389 move deep enough or did not have the concentration to cause almost instant mortality or
390 repellency three weeks after application.
391

392 ## 5.3. General discussion

393 Efficacy in terms of root damaging pests is defined here as any impact of the compound
394 on the organism that reduces the pest pressure on the root (i.e., reduced feeding activity).
395 Theoretically, this can occur from a range of effects where mortality is the only effect that is
396 not potentially reversible. Sub-lethal impacts that directly or indirectly reduce feeding on

roots may be reversible but effectively make the same contribution to efficacy in terms of reducing root feeding whilst apparent, and ultimately can cause mortality through starvation. One example is the loss of foraging and/or feeding ability deriving from inability to either sense the roots or reach roots due to immobility or loss of controlled movement. Another example is the avoidance of regions treated with the pesticide, as observed here and found by Woodson et al.[22] in choice experiments on soil with different organophosphates (terbufos, chlorethoxyfos, fonofos). To some extent all of these mechanisms of efficacy were at least qualitatively observed in the experiments presented in this study. They may explain previous field observations of seed and in-furrow applications with plant protection products containing imidacloprid, fipronil, thiamethoxam, tefluthrin, chlorpyrifos and diazinon that were shown to reduce root damage without causing an overall reduction in *Diabrotica* population size (measured as number of emerging beetles)[23]. Though not quantified directly within the acute toxicity experiment, reduced feeding reduction was measured indirectly through rate of emergence of corn seedlings.

Acute toxicity experiments in soil increase environmental realism in comparison to direct dermal application[3] and filter paper application[4, 5] because the former incorporate pesticide sorption and biodegradation, and thus differences in bioavailability between compounds; these differences will be soil-specific as soil type influences both sorption and biodegradation. At the same time, uniform mixing of pesticide into soil removes the effect of spatial distribution that will influence efficacy under field conditions, thus providing a measure of potential efficacy that is comparable with that of simpler tests. Taking measurements at three or more time points generates suitable data to parameterise a toxicokinetic toxicodynamic (TKTD) model such as the general unified threshold model of survival (GUTS[24]) which theoretically allows the prediction of acute toxic effects from any exposure pattern (duration and concentration).

422 The total compound load used in the rhizotron experiment resulted in average pesticide
423 concentrations in soil that were 4 to 11 times higher than the $EC_{50(120h)}$ values observed in the
424 acute toxicity experiments. Nevertheless, the lethal effect (death or knock down) observed
425 within four days was very similar across the compounds tested. This illustrates the
426 importance of considering the temporal, spatial and potential systemic distribution of a
427 compound in the soil profile. The rhizotron experiments gave insights into potential sub-
428 lethal impacts (i.e. avoidance and accelerated pupation) that may contribute to the efficacy in
429 terms of reducing root damage without causing direct mortal effects. This in turn might be
430 useful to gain insights into possibilities of resistance development as knowledge of insect
431 behaviour and biology has been identified as pivotal for resistance management[25]. Though
432 not done in our experiment, a direct measure of root damage in comparison to the control can
433 be included within the study design by assessing the plant and/or root biomass at the
434 assessment point.
435 Rhizotron experiments require more time overall due to the need for preparation of plants
436 but the actual assessment does not take longer than the acute toxicity experiments and has the
437 advantage of being able to assess impacts of a compound/treatment in relation to field
438 application types. Understanding the interplay of organism distribution in the soil profile
439 under different application types could be enhanced if soil samples at the end of the
440 experiment were analysed to provide the spatial distribution of both organisms and
441 compound. An advantage of rhizotrons is that the experiments are controllable in terms of
442 environmental conditions and less effort in comparison to field trials, thus allowing in-depth
443 comparison between compounds/products. However, the rhizotron experiments conducted
444 here do not represent the real behaviour of the compound moving through the soil profile as
445 absence of precipitation (rainfall and irrigation) and constant water availability from the
446 bottom of the rhizotron do not mimic field conditions; this could be addressed by undertaking

447 the experiments within a rainfall simulator. Furthermore, the effect intensities observed here
448 are likely to differ from those in the field because experiments were conducted with 2nd instar
449 larvae to achieve an acceptable recovery rate of larvae from the soil (acute toxicity test) and
450 observational success (rhizotron experiments). In the field, however, neonate larvae that are
451 substantially smaller (and thus likely to be more susceptible) are typically exposed to the
452 compounds because pesticide application is carried out at sowing, and sowing normally
453 precedes egg hatch.

454 There are several aspects of the experiments reported here that hinder the extrapolation of
455 experimental effect intensities as a measure for efficacy in the field. Nonetheless, they are
456 time- and cost-effective compared to field trials, and have potential to enhance efficacy
457 testing and product development by generating mechanistic understanding of processes
458 determining field efficacy. The additional focus on sub-lethal impacts that are potentially
459 reversible could be particularly important in studying the potential for development of
460 resistance in pest populations.

461

462 ## Acknowledgement

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465 products in this publication is solely for the purpose of providing specific information and
466 does not imply recommendation or endorsement by the US Department of Agriculture.
467 USDA is an equal opportunity provider and employer.

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545

Tables

Table 1: Properties of the pesticides[20].

	Tefluthrin	Clothianidin	Chlorpyrifos
Substance group	Pyrethroid	Neonicotinoid	Organophosphate
Mode of action	Sodium channel modulator	Acetylcholine receptor (nAChR) agonist	Acetylcholinesterase (AChE) inhibitor
	Contact and respiratory action with some repellent effects	Translaminar and root systemic activity	Non-systemic with contact and stomach action.
Molecular mass [g/mol]	418.73	249.7	350.89
Vapor pressure (mPa)	8.4	2.8×10^{-8}	1.43
Half-life in soil at 20°C [d]	37	545	76
Soil organic carbon partition coefficient [L/kg]	112900	123	8151
Water solubility [mg/L]	0.016	340	1.5

Table 2: Average effect concentrations [mg/L] (mortality and immobility) observed in the acute toxicity tests after 24, 72, and 120 h of exposure. Brackets show the 95% confidence intervals.

	Tefluthrin	Clothianidin	Chlorpyrifos
$\mathbf{EC_{50(24h)}}$	0.697 (0.557 – 0.956)	0.242 (0.189 – 0.50)	0.753 (0.604 – 0.906)
$\mathbf{EC_{50(72h)}}$	0.241 (0.211 – 0.292)	0.092 (0.080 – 0.108)	0.365 (0.211 – 0.621)
$\mathbf{EC_{50(120h)}}$	0.092 (0.053 – 0.195)	0.093 (0.079 – 0.105)	0.327 (0.241 – 0.474)

Table 3: Average and standard deviation of increase in head capsule width (%) over a period of 120 h in the acute toxicity test with chlorpyrifos.

Concentration (mg/L)	Average of growth (%) within 120 h	SD
0.00	24.31	10.81
0.05	30.47	2.97
0.15	11.15	8.58
0.44	11.51	10.19

Table 4: Results of the statistical analysis for data presented in Figure 4. Shown are the P-values from three-way ANOVA (Shapiro-Wilk test to test for normality, Brown-Forsythe test to test for equal variance, and Holm-Sidak method for pairwise multiple comparison) with an overall significance level of 0.05.

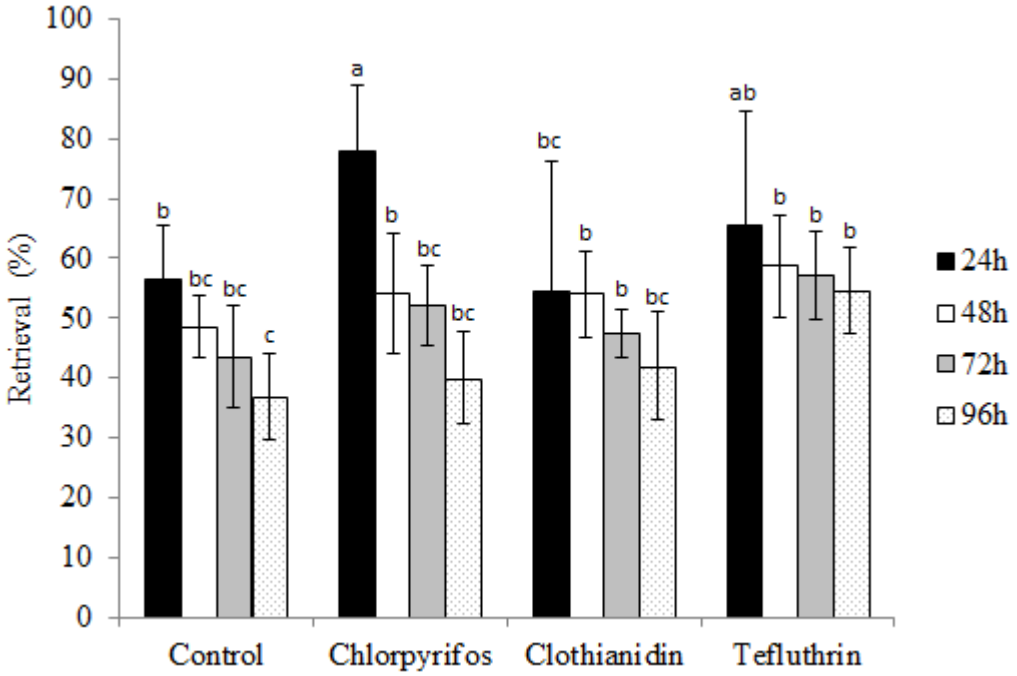
Overall	**Total abundance**	**Mobile**	**Feeding**	**Puppa**	**Dead**	**Resting**	**Knocked down**
Treatment	<0.001	<0.001	<0.001	<0.001	<0.001	0.289	<0.001
Time	<0.001	<0.001	0.032	<0.001	<0.001	<0.001	0.003
Treatment x Time	0.123	0.363	0.372	0.969	0.229	0.267	0.119
Treatments compared							
Tefluthrin vs. Control	<0.001	0.028	<0.001	0.884	0.007	0.444	<0.001
Chlorpyrifos vs. Control	0.015	0.663	<0.001	0.054	<0.001	0.704	<0.001
Clothianidin vs. Control	0.346	<0.001	<0.001	0.015	<0.001	0.388	0.173
Tefluthrin vs. Clothianidin	0.015	0.105	0.516	0.012	0.03	0.803	<0.001
Chlorpyrifos vs. Clothianidin	0.12	<0.001	0.379	<0.001	0.105	0.847	0.005
Tefluthrin vs. Clorpyrifos	0.559	0.05	0.652	0.042	0.477	0.85	0.187

Figures

Figure 1

Figure 2

573 **Figure 3**



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576 **Figure 4**

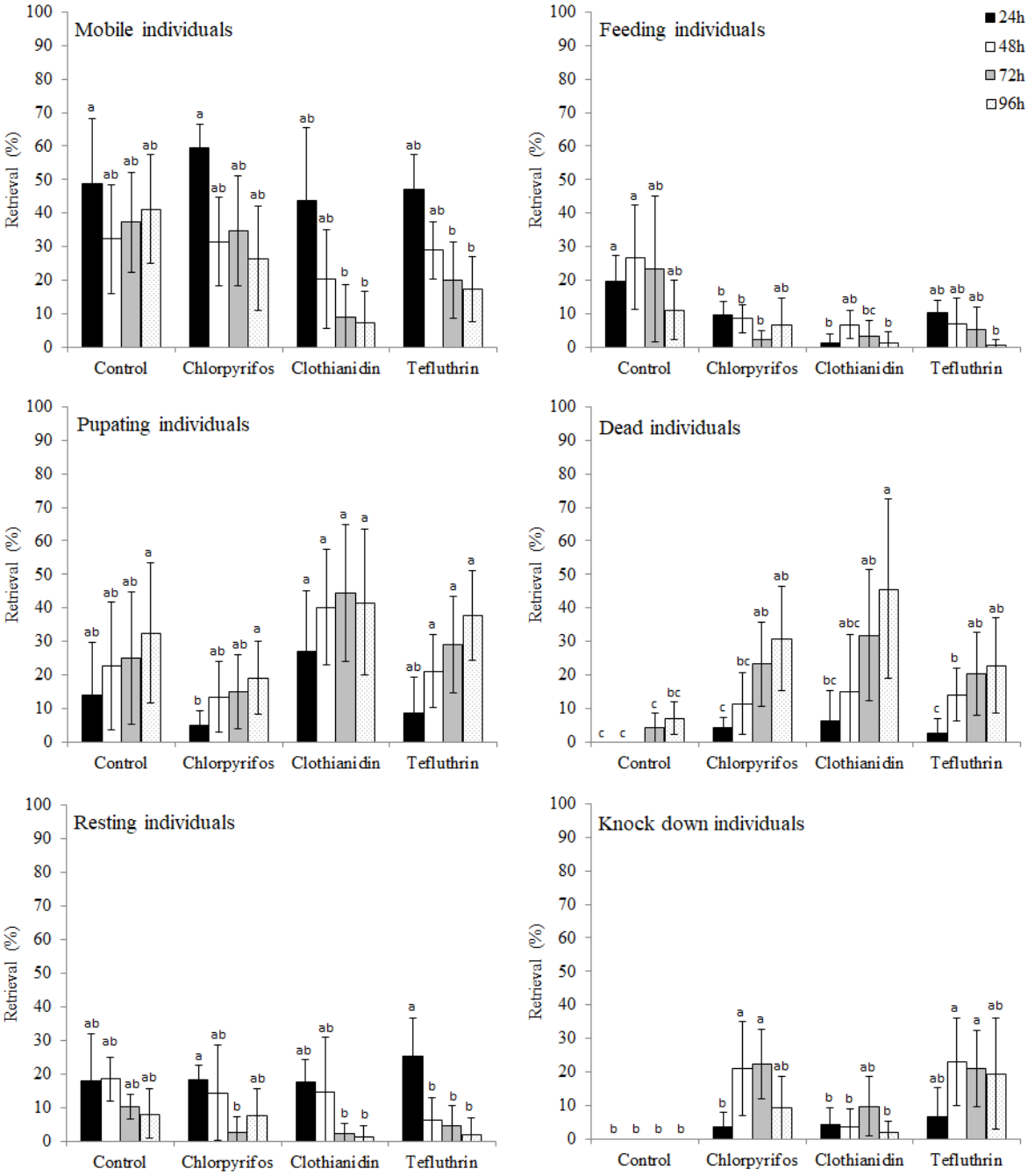

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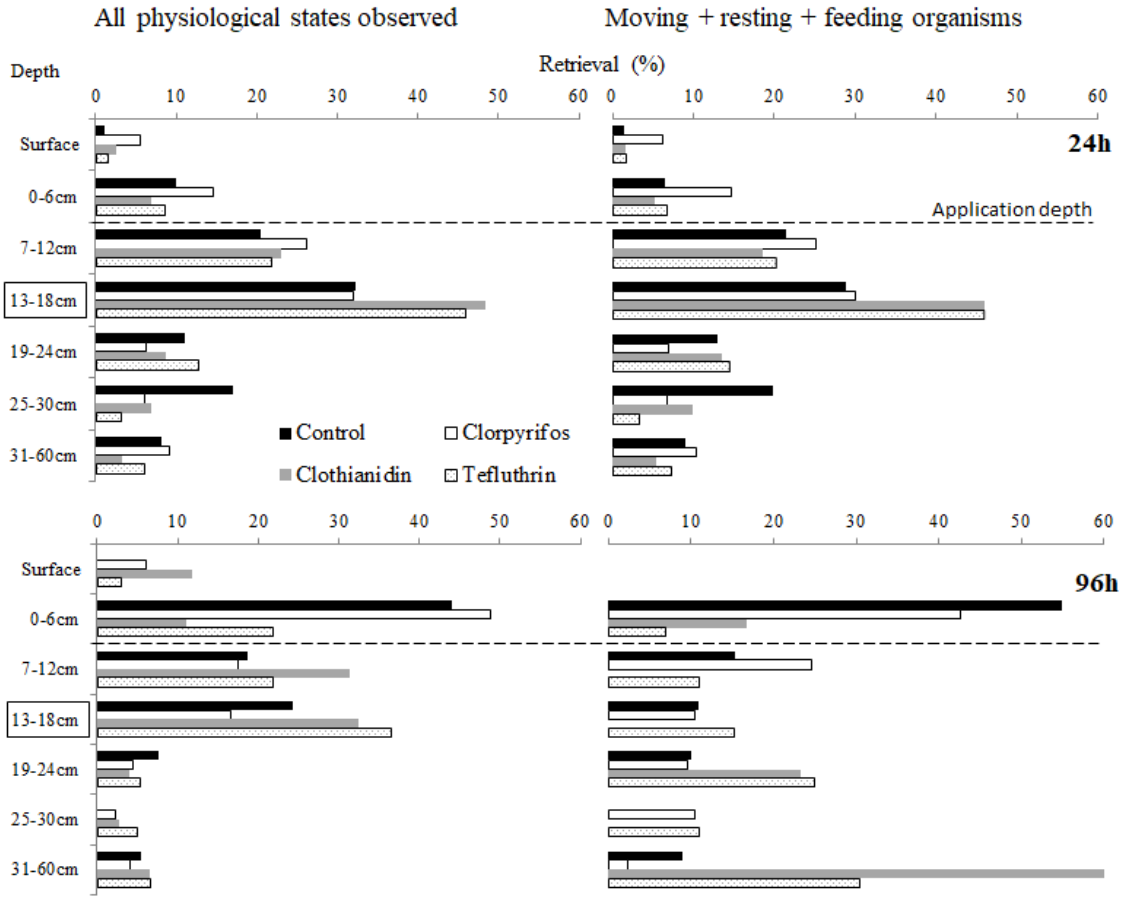
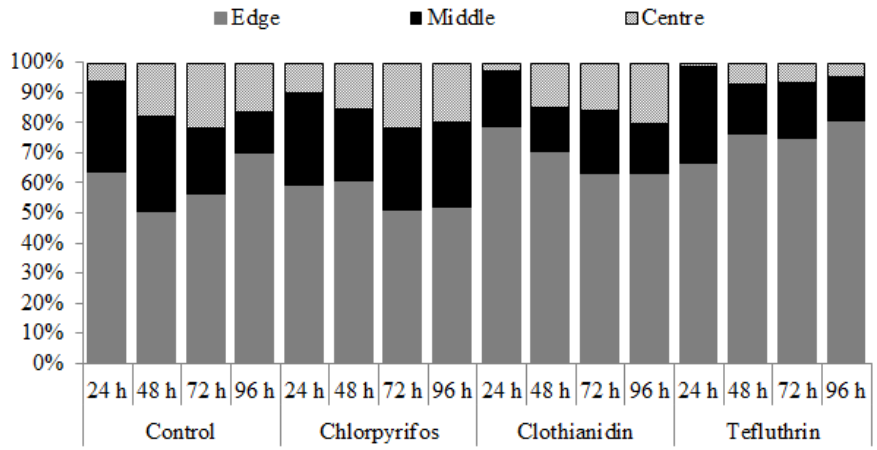
Figure 5


All physiological states observed
Moving + resting + feeding organisms
Depth
Retrieval (%)
0
10
20
30
40
50
60
Surface
0-6cm
7-12cm
13-18cm
19-24cm
25-30cm
31-60cm
24h
Application depth
Control
Clorpyrifos
Clothianidin
Tefluthrin
96h

582 **Figure 6**



583

584 ## Figure legends

585 **Figure 1:** Illustration of the rhizotron experiment.

586 **Figure 2:** 2nd instar larvae of the western corn rootworm extracted from the control soil (left)
587 or the soil treated with clothianidin (right) from the acute toxicity test.

588 **Figure 3:** Percent retrieval of organisms (average and standard deviation) as a function of
589 treatment and time. Columns with the same letter across the control and treatments and over
590 time are not significantly different from each other (three-way ANOVA; Holm-Sidak
591 method; $p>0.05$).

592 **Figure 4:** Percent retrieval of organisms (average and standard deviation) with different
593 physiological states as a function of treatment and time. Columns with the same letter across
594 the control and treatments and over time are not significantly different from each other (three-
595 way ANOVA; Holm-Sidak method; $p>0.05$).

596 **Figure 5:** Percent retrieval of organisms (average and standard) over depth of the soil layer
597 one and four days after exposure as a function of treatment. Larvae were introduced at 13-18
598 cm depth and pesticide placement was at 6 cm [---].

599 **Figure 6:** Relative distribution in the horizontal plane of recovered individuals as a function
600 of treatment and time. **Edge**: first 6 cm from both sides of the rhizotron; **Middle**: next 6 cm
601 towards the centre of the rhizotron; **Centre**: 3 cm towards each side of the seed.

Graphical abstract

Assessment of acute toxicity tests and rhizotron experiments to characterise lethal and sub-lethal control of soil-based pests

Annika Agatz*, Mario M. Schumann, B. Wade French, Colin D. Brown, Stefan Vidal

Assessing the efficacy of products to combat soil based pests is challenging. We present laboratory and greenhouse experiments and investigate their ability to assess the driving mechanism of efficacy prior to conducting field trials.