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

3

4 Annika Agatz

5 Environment Department, University of York, Heslington, York, United Kingdom

6 Corresponding author : annika.agatz@york.ac.uk + 44-(0)-1904323118

7 ORCID [0000-0003-3228-8822](https://orcid.org/0000-0003-3228-8822)

8

9 Mario M. Schumann¹

10 Agricultural Entomology, Department of Crop Sciences, University of Göttingen, Göttingen,
11 Germany

12 Mario-Matthias.Schumann@kws.com

13

14 B. Wade French

15 North Central Agricultural Research Laboratory, Agricultural Research Service, United
16 States Department of Agriculture, Brookings, SD, USA

17 Wade.French@ars.usda.gov

18

19 Colin D. Brown

20 Environment Department, University of York, Heslington, York, United Kingdom

21 colin.brown@york.ac.uk

22

23 Stefan Vidal

24 Agricultural Entomology, Department of Crop Sciences, University of Göttingen, Göttingen,
25 Germany

26 svidal@gwdg.de

27

¹ Current address: Department of Phytopathology, KWS SAAT SE, Einbeck, Germany

28 1. Abstract

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

38 **RESULTS:** The order of compound concentrations needed to invoke a specific effect
39 intensity (EC₅₀ values) within the acute toxicity tests was chlorpyrifos > tefluthrin >
40 clothianidin. This order changed for the rhizotron experiments because application type, fate
41 and transport of the compounds in the soil profile and sub-lethal effects on larvae also
42 influence their effectiveness in controlling larval feeding on corn roots.

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

48

49 2. Key words

50 rootworm, corn, tefluthrin, clothianidin, chlorpyrifos, efficacy

51

52 **3. Introduction**

53 Efficacy assessment provides fundamental information for the placement of plant
54 protection products on the market¹. Efficacy describes the power of a product to produce an
55 effect that fulfils the claims made for it on the proposed label²; characterisation is particularly
56 challenging for products that control soil-based and root-damaging pests due to the complex
57 and dynamic interplay of the system components (compound/formulation, soil, roots, target
58 pest, and environmental conditions). Constant contact exposure of pest organisms via direct
59 dermal application³ or filter paper application^{4, 5} and subsequent assessment of mortality (to
60 derive dose-response relationships) are frequently conducted, but are least representative of
61 the system because behaviour of the pesticide in the soil and responses of target organisms to
62 these compounds are ignored. These types of efficacy trials are categorised as ‘laboratory or
63 growth chamber tests’ within the preliminary screening for the biological dossier according to
64 the Guidelines on Efficacy Evaluation for the Registration of Plant Protection Products⁶.
65 Field efficacy studies represent the other extreme of environmental realism, assessing
66 efficacy in standardised field experiments within a growing season. Standardisation of field
67 experiments often includes a rating system that is specific to the plant and target pest, such as
68 the measurement of reduced corn root injury from the corn rootworm compared to controls
69 via the node injury scale⁷. Such trials are categorised as ‘operational large scale trials’⁶. There
70 is uncertainty about the extent to which results from one field study (or a low number of
71 studies) are representative of the full range of possible environmental conditions (e.g.
72 different soils, different climates, different strains of pest) and farming practices.
73 Furthermore, the study design generally prevents identification of the driving mechanisms
74 behind observed efficacy. Between these two extremes of efficacy trials in terms of
75 environmental realism, there are several intermediate studies that are carried out in the
76 laboratory or greenhouse^{3, 8-10}. Examples of such studies are experiments where organisms

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

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

93

94 **4. Materials and methods**

95 Experiments were carried out at the Department of Crop Sciences, University of Göttingen
96 (Germany) with three technical grade pesticides obtained from Sigma-Aldrich, namely
97 clothianidin (CAS 210880-92-5), chlorpyrifos (CAS 2921-88-2) and tefluthrin (CAS 79538-
98 32-2). Pesticides used were chosen to be active against larvae of the western corn rootworm
99 whilst differing both in mode of action and physico-chemical properties. **Table 1** summarises
100 the characteristics of the three pesticides used.

101 Non-diapausing eggs¹¹ were obtained from the USDA-ARS North Central Agricultural
102 Research Laboratory (Brookings, South Dakota, USA) and stored under dark and cold (7°C)
103 conditions. Prior to laboratory experiments, eggs were prepared for hatching (incubated for
104 12 days at 25°C and 65% relative humidity in an incubator cabinet (Mytron GmbH,
105 Heiligenstadt, Germany)) and reared on untreated maize roots (cultivar: Ronaldinio, KWS
106 SAAT SE, Einbeck, Germany) from plants at growth stage BBCH 11¹² in peat soil
107 (Fruhstorfer Erde, Typ P, Hawita Gruppe GmbH, Vechta, Germany). Larvae were extracted
108 from the culture containers via an adapted Kempson chamber¹³. Further details on the
109 methods for egg hatching and larval rearing are described by Brandl et al. (2016)¹⁰.

110 Acute toxicity tests investigated the time- and concentration-dependence of acute toxicity,
111 ultimately identifying the effect concentration for 50% of the tested individuals (EC₅₀) after
112 different exposure durations and under constant environmental conditions. Rhizotron
113 experiments investigated how effects varied over time when pesticides were used similarly to
114 the field situation (seed or band application at sowing), but still under controlled
115 environmental conditions. Both sets of experiments were conducted with late 2nd larval
116 instars for practicality. First instar larvae were considered to be too small to achieve an
117 acceptable recovery rate of larvae from the soil (acute toxicity test) and observational success
118 (rhizotron experiments).

119

120 **4.1. Acute toxicity tests**

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

126 Ten larvae (2nd instar) were placed onto 200 mL soil containing 0, 0.05, 0.15, 0.44, 1.33 or
127 4 mg active substance per litre soil volume (ppm) of tefluthrin, clothianidin, or chlorpyrifos,
128 respectively. Each experiment began with three replicates for each assessment point (24, 72
129 and 120 h) and concentration. Independent replicates were needed for each assessment day
130 because manual recovery of larvae from the soil disrupted the sample. Sample preparation
131 involved mixing 200 mL soil (air dried; sieved to < 2 mm; sand 24.3%, silt 56.7%, clay
132 19.0%, organic carbon content 1.7%) with 25 mL solution (tap water containing 0.8%
133 acetone for the control treatment or 0.20, 0.59, 1.78, 5.33 and 16.0 mL of a 50 mg/L stock
134 solution that contained 2% acetone topped up with tap water to a volume of 25 mL). Samples
135 were left standing at room temperature for three to five hours to allow the acetone to
136 evaporate prior to placing six pre-germinated maize seeds (Ronaldinio; 15MAO1128; KWS
137 SAAT SE), grown to the category 07 on the Zadoks Scale by placing them for two days at 25
138 +/- 1°C on wet towels in the dark at humidity >65%, 1-2 cm beneath the soil surface and
139 adding ten rootworm larvae at the soil surface. Subsequently, beakers were stored in a
140 controlled climate chamber until larval recovery. Environmental conditions were constant
141 temperature of 20 +/- 1°C, constant relative humidity of 65%, and a light-dark cycle of 16:8
142 h.

143 Organisms were manually removed from soil at the respective sampling interval (24, 72 or
144 120 h) by sifting the entire sample with tweezers until all organisms were recovered.
145 Organisms were placed on a Petri dish and categorised as either dead, immobile, knocked
146 down, or mobile. Individuals with directed movement across the Petri dish were categorised
147 as mobile, whilst those that were moving without a sign of directed movement were
148 categorised as knocked down. Immobile and dead individuals were distinguished by touching
149 both ends of the larvae with a brush; those showing an avoidance reaction but without the

150 ability for whole body movement (i.e. twisting the head or abdomen) were categorised as
151 immobile.
152 Larvae were assessed for their body size (measured as head capsule width) immediately
153 before placement onto the soil and reassessed for head capsule width again after their
154 recovery from the soil following 120 h of exposure. Head capsule width was measured as a
155 proxy for moulting during the experimental phase (because it is not possible to recover the
156 remains of the exoskeleton from the soil sample) and to assess any size dependency of mortal
157 effects. A method previously developed for aquatic invertebrates was used, where organisms
158 were transferred to a Petri dish and a picture of the Petri dish was taken using an ordinary
159 flatbed scanner. Pictures were subsequently manually analysed with purposely developed and
160 freely available software¹⁴.

161

162 **4.2. Rhizotron experiment**

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

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

175 Einbeck, Germany) was placed in the centre of the soil surface and covered with soil until the
176 top 3 cm of the rhizotrons was soil free, and pesticide was applied dissolved in tap water
177 containing 2% acetone. Clothianidin was applied in a single spot directed onto the seed to
178 treat the seed with 0.6 mg active substance which represents the mass equivalent of a seed
179 treatment with Poncho[®] 1250 (Bayer CropScience). Tefluthrin and chlorpyrifos were applied
180 in a 25-cm band onto the soil just above seeding depth (with 125 µL stock solution per cm
181 band width) to simulate a band application with Force[®] 3G (Syngenta) for the tefluthrin
182 treatment and Lorsban[®] 15G, Saurus[®] 15G (Dow AgroSciences) or Nufos[®] 15G (Cheminova
183 Inc.) for the chlorpyrifos treatment. Band application rates were derived from application
184 rates in the field¹⁵ so that the total load of the compound per soil volume available for each
185 seed equalled the total load per soil volume and seed from the field application rate. For this
186 calculation, only the soil volume of the upper 30 cm in the field was considered, because it is
187 known that eggs and larvae of the rootworm mainly occur at this depth in the field¹⁶. In total
188 0.338 mg tefluthrin or 3.38 mg chlorpyrifos was applied in the rhizotron. Application in the
189 rhizotron was calculated as follows. In the field, there were 6.2 seeds per row meter and the
190 soil volume per row meter was 228 L (accounting for row spacing of 76 cm (i.e. 30 inches)
191 and a soil depth of 30 cm). Thus each seed in the field had 36.77 L of soil dedicated to them.
192 In the rhizotron the soil volume available was 1.08 L. Thus, a factor of 33.43 (i.e. 36.77/1.08)
193 was applied to the field application rate to derive the soil mass equivalent application per
194 seed. Lorsban[®] 15G (chlorpyrifos content 15%) for example was applied as 8 oz /1000 ft of
195 row¹⁶. This equals 111.6 mg active ingredient per m row. Dividing this by the soil equivalent
196 factor gives an application rate of 3.38 mg/seed.

197 Following application of any pesticide, the rest of the rhizotron was filled with soil; thus
198 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 literature¹⁵.

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 10-11¹²) 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 $25 \pm 1^\circ\text{C}$, 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.

249

250 **5. Results and discussion**

251 **5.1. Acute toxicity tests**

252 Across all three experiments (each with one active ingredient), all six pre-germinated
253 maize seeds emerged within 5 days in treatments above 0.44 mg/L whilst only 1-5 seedlings
254 emerged from the soil in the concentrations 0.05 and 0.15 mg/L and only 1-3 seedlings
255 emerged across all control beakers. This indicates that root damage beyond tolerable levels
256 for plant growth occurred due larvae introduced into the system and that extent of damage
257 was dependent on pesticide concentration. Effects on larvae observed as mortality or
258 immobility varied not only with concentration but also with duration of exposure. **Table 2**
259 summarises the EC₅₀ values and their 95% confidence intervals after 24, 72 and 120 h of
260 exposure. The efficacious dose based on the median EC₅₀ values decreased in the order
261 chlorpyrifos > tefluthrin > clothianidin (organophosphate > pyrethroid > neonicotinoid).
262 Taking into account the 95% confidence interval however, only clothianidin is significantly
263 different from chlorpyrifos and tefluthrin after 24 and 48h of exposure whilst chlorpyrifos is
264 significantly different from the other two compounds tested after 120h of exposure.

265 How far EC₅₀ values represent actual 50% effect doses for larvae of laboratory cultures
266 and field populations of the western corn rootworm cannot be determined. It is possible that
267 the values differ between strains more than they do between compounds tested. Magalhães
268 and co-authors (2007)⁴ obtained EC₅₀ values for clothianidin testing neonates from 19 field
269 collected and laboratory cultured western corn rootworm populations exposed on filter paper
270 for 24 h and found a maximal difference between the EC₅₀ values of a factor of 20⁴. Direct
271 comparison of EC₅₀ values obtained here with EC₅₀, LC₅₀ values (lethal concentration for
272 50% of tested organisms) and LD₅₀ values (lethal dose for 50% of tested organisms) from the
273 literature for any of the compounds is not possible because exposure types (soil application in

274 the current study, direct dermal application³, and filter paper application^{4, 5} were different and
275 studies were also conducted with different strains and differently sized / aged larvae ranging
276 from neonates to late 3rd instars. Average LD₅₀ from direct dermal application for 3rd instar
277 larva for example ranged for chlorpyrifos between 3.16 and 11.3 ng /mg larva and between
278 2.55 and 78.3 ng/mg larva for tefluthrin after exposing larva for 24h³. LC₅₀ values for
279 clothianidin derived using neonates of different strains exposed for 24h on filter paper ranged
280 between 1.5 and 21.9 ng/cm² filter paper. Though the actual values we obtained here for the
281 EC₅₀ after 24 h of exposure are one to two orders of magnitude lower than those found in the
282 literature, we cannot state whether the test generated similar results or not. The differences in
283 exposure methods and units of the results do not allow direct comparison.

284 In addition to causing immobility and mortality, each compound showed at least one
285 different characteristic effect on the larvae during the acute toxicity test. These effects were
286 observed consistently but were not thoroughly quantified due to time constraints during the
287 experiment. Tefluthrin made the larvae move in a specific and uncontrolled pattern in all
288 exposure concentrations at and above 1.78 mg/L from the first observation (exposure duration
289 24 h) onwards. A demonstration of this behaviour and comparison with that of larvae
290 recovered from the control has been recorded (see video “**Twist and Curl**” in comparison to
291 the video “**Control**” accessible [here](#)). Larvae exposed to clothianidin that were categorised as
292 dead or immobile looked different to those that were dead or immobile following exposure to
293 the other pesticides. An abnormally enlarged abdomen, swollen to a diameter approximately
294 three times the normal width (**Figure 2**), was observed from the first observation onwards.

295 Organisms exposed to chlorpyrifos did not show an immediate (24 h after exposure)
296 observable impact in contrast to the other compounds, but comparison of their
297 growth/moulting (measured as increase in head capsule width) after five days of exposure
298 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 emergence¹⁷. 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

324 the rhizotrons treated with tefluthrin, followed by chlorpyrifos and clothianidin and the
325 overall lowest number of organisms were retrieved from the control rhizotrons. The temporal
326 decline in organisms retrieved was only significant for the control and the clothianidin
327 treatment; the significance for the latter treatment derives from the exceptionally high
328 retrieval at the first observation period in comparison to the other treatments. The low overall
329 retrieval observed as well as its temporal decline is a typical source of error for this kind of
330 experiment^{10,18}. Observations are constrained by the thickness of the soil layer, and the
331 resulting restriction in observation of all larvae introduced into the systems. Especially 2nd
332 instar larvae were reported to burrow extensively into roots, making it difficult to observe
333 larval behaviour and thus reducing the chance to find larvae following introduction into the
334 rhizotron¹⁸. The overall retrieval was likely lowest in the control treatment because of the
335 higher feeding activity of unexposed larvae ($p < 0.001$) that was observed compared to those in
336 the pesticide treatments (Figure 4).

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

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

350 The spatial data on where individuals were found show a tendency for tefluthrin and
351 clothianidin to provoke the larvae to move further down in the soil profile in comparison to
352 larvae in the chlorpyrifos exposure and the control (Figure 5). This only becomes apparent
353 when comparing observations from the first and last observation period. Vertical distribution
354 of the larvae was similar across all treatments and control 24h after placing them in the
355 rhizotrons (Figure 5 top). Most larvae remained in the horizontal plane where they were
356 introduced to the system (13-18 cm depth) and an almost equal number of larvae moved up or
357 down. Within 96 h, however, larvae that were not affected moved extensively within the soil
358 profile as demonstrated by an overall significant interaction between time and depth (p
359 < 0.001). The direction of the movement was treatment-specific (Figure 5 bottom) and
360 significant for the comparison of interaction between depth and compound ($p < 0.001$).

361 The statistically-significant increased downwards movement following treatment with
362 tefluthrin and clothianidin was probably caused by the presence of the compounds in the
363 upper soil layers and the known repellent effect of both compounds. Michaelides et al.
364 demonstrated a repellent effect of tefluthrin at sub-lethal concentrations for the northern corn
365 rootworm (*Diabrotica barberi* Smith & Lawrence)¹⁹. Movement away from thiamethoxam (a
366 precursor for clothianidin) was observed for neonate western corn rootworm larvae exposed
367 on filter paper⁵. Repellency was not only apparent from spatial location data in the vertical
368 plane, but also in the horizontal plane (Figure 6). Whilst for all other treatments and the
369 control a movement of larvae from the edges of the rhizotron to the centre was observed over
370 time, this pattern of movement was much less pronounced in the rhizotrons treated with
371 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 translaminal and root
383 systemic activity of clothianidin²⁰ 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 material²¹. 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

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

411 Acute toxicity experiments in soil increase environmental realism in comparison to direct
412 dermal application³ and filter paper application^{4, 5} because the former incorporate pesticide
413 sorption and biodegradation, and thus differences in bioavailability between compounds;
414 these differences will be soil-specific as soil type influences both sorption and
415 biodegradation. At the same time, uniform mixing of pesticide into soil removes the effect of
416 spatial distribution that will influence efficacy under field conditions, thus providing a
417 measure of potential efficacy that is comparable with that of simpler tests. Taking
418 measurements at three or more time points generates suitable data to parameterise a
419 toxicokinetic toxicodynamic (TKTD) model such as the general unified threshold model of
420 survival (GUTS²⁴) which theoretically allows the prediction of acute toxic effects from any
421 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 management²⁵. 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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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

546 **Tables**547 **Table 1:** Properties of the pesticides²⁰.

	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 X 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

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

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

553

554 **Table 3:** Average and standard deviation of increase in head capsule width (%) over a period
 555 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

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559 **Table 4:** Results of the statistical analysis for data presented in Figure 4. Shown are the P-

560 values from three-way ANOVA (Shapiro-Wilk test to test for normality, Brown-Forsythe test

561 to test for equal variance, and Holm-Sidak method for pairwise multiple comparison) with an

562 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

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566 **Figures**

567 **Figure 1**



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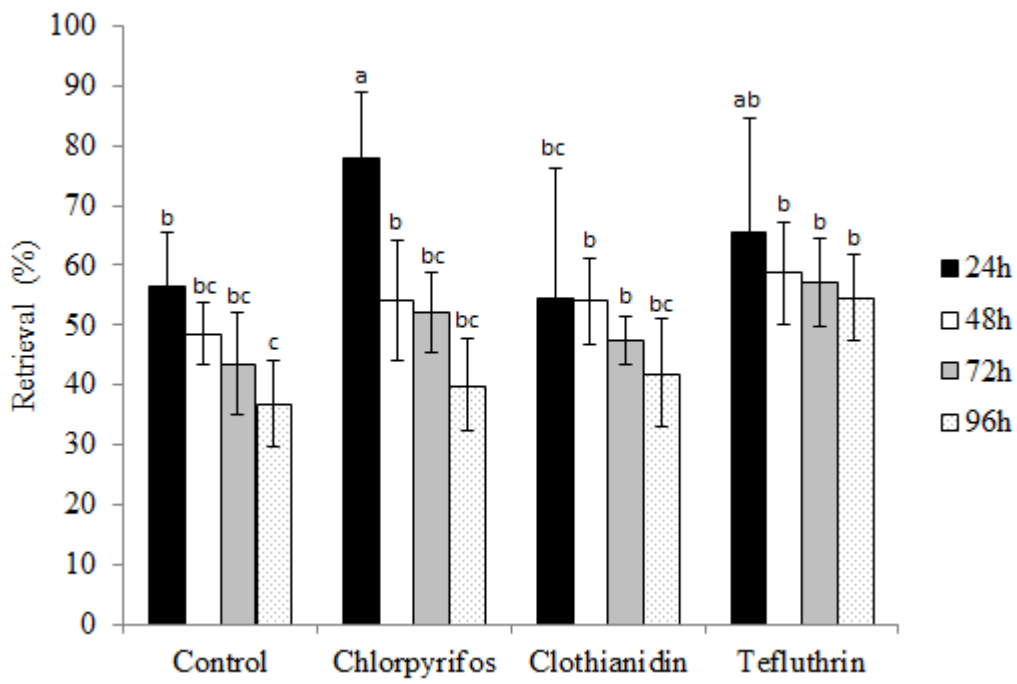
570 **Figure 2**



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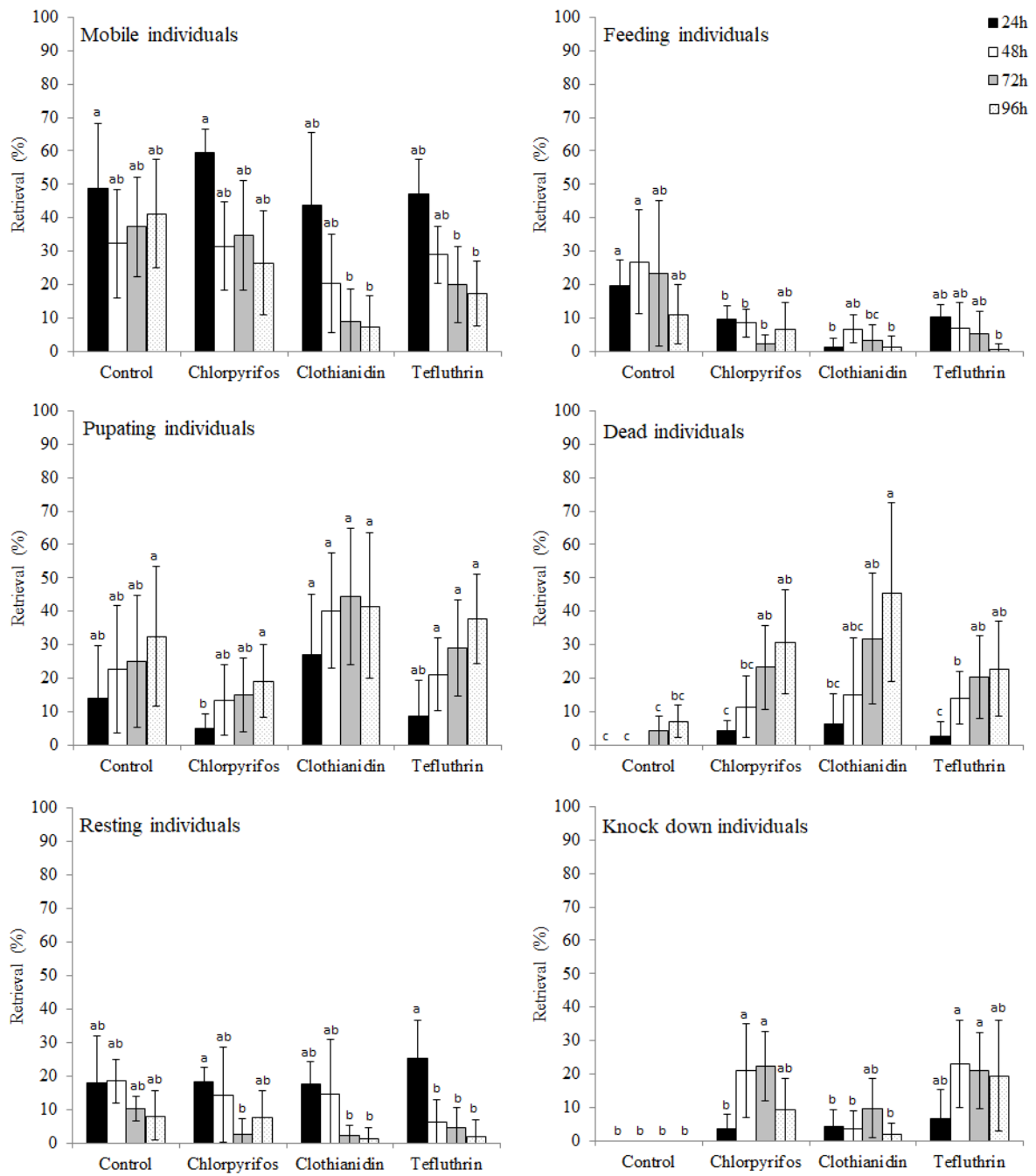
573 **Figure 3**



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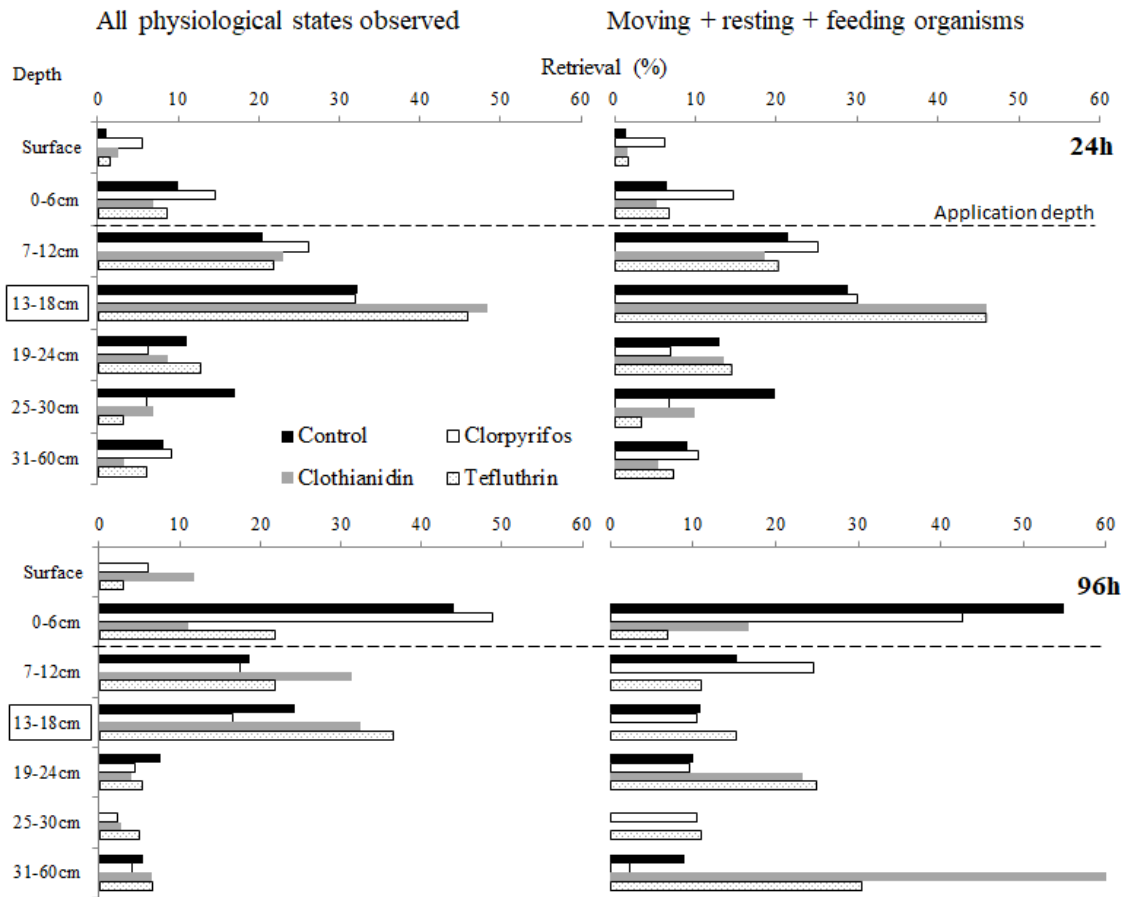
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576 **Figure 4**



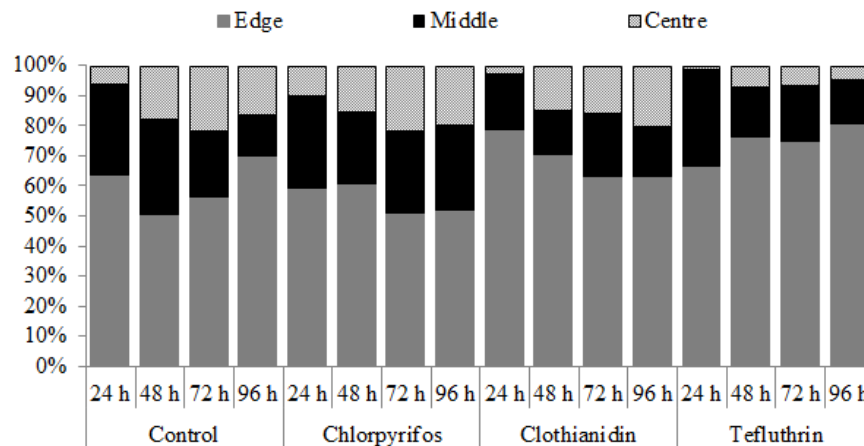
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579 **Figure 5**



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582 **Figure 6**

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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 [---].

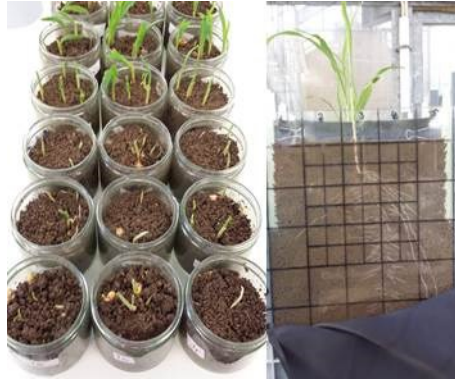
599 **Figure 6:** Relative distribution in the horizontal plane of recovered individuals as a function
600 of treatment and time. **Edge:** first 6 cm from both sides of the rhizotron; **Middle:** next 6 cm
601 towards the centre of the rhizotron; **Centre:** 3 cm towards each side of the seed.

602 **Graphical abstract**603 **Assessment of acute toxicity tests and rhizotron experiments to characterise lethal and**
604 **sub-lethal control of soil-based pests**

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

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