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Abstract

Pesticides taken up by plants from the soil or interstitial (pore) water can cascade to higher trophic levels, which are expected to be more affected due to cumulative bottom-up effects. Knowledge about the impact of indirect exposure to pesticides on non-target terrestrial trophic chains, however, is still lacking. Therefore, we examined the direct and indirect effects of three concentrations of the herbicide 2,6-dichlorobenzonitrile (DCBN) and an insecticide with a similar molecular structure (1,4-dichlorobenzene, DCB) on the fitness traits of a tritrophic system: the wheat plant *Triticum aestivum*, the aphid *Sitobion avenae* and its specialist parasitoid *Aphidius rhopalosiphi*. To mimic exposure via interstitial water the toxicants were added to the growth medium of the plant. Passive dosing between the medium and a silicon layer was used to achieve constant exposure of the poorly soluble pesticides. Wheat plants exposed to both pesticides grew smaller and had reduced biomasses. Negative effects on the reproductive rate, biomass and the number of aphids were only observable at the highest concentration of DCBN. Overall parasitism rate decreased when exposed to both pesticides and parasitoid attack rates decreased at lower concentrations of DCBN and at the highest DCB concentration. The parasitoid sex ratio was extremely male-biased in the presence of DCBN. Our results demonstrate that pesticides can alter the performance of higher trophic levels by sublethal effects, through a bypass of the second trophic level. In addition, the novel test system used was suitable for detecting such carryover effects on non-target organisms.

Keywords: *Aphidius rhopalosiphi*; 1,4-dichlorobenzene; 2,6-dichlorobenzonitrile; ecological interactions; indirect exposure; organic pollutants; passive dosing; non-target organisms; *Sitobion avenae*; sublethal; *Triticum aestivum*; trophic chain

Introduction

Pesticides are the largest group of manmade toxic organic compounds that have been introduced deliberately into the environment to exterminate pest organisms, mostly in agricultural settings (Holtze et al. 2008) but also in industry and for domestic use (Meharg et al. 2000). They may pose problems for non-target organisms, especially since they may unintentionally spread to various other environmental compartments like rain, soil and ground water (Björklund et al. 2011). Once persistent chemicals end up in the soil and the soil interstitial water (Meharg et al. 2000; Björklund et al. 2011), they can be taken up by plant roots and subsequently be translocated to other parts of the plants. There, they can pose a risk to the consumers of the plant (Barbour et al. 2005) and potentially to their natural enemies. Thus, chemicals taken up by plants may reach organisms in all trophic compartments of an ecosystem. However, most terrestrial toxicological studies mainly focus on the effects on single-species, applying the toxicants directly on the studied organism (van den Berg et al. 1998). To our knowledge only few recent studies included more than two trophic levels under controlled conditions (Guedes et al. 2016). Thus, the impact of toxic compounds on trophic chains in terrestrial systems has been neglected, while these interactions play a major role in ecosystem structure and functioning (Relyea and Hoverman 2006; Bezemer et al. 2013).

Multi-trophic terrestrial systems can be complex and generally involve several trophic levels interacting directly but also indirectly through the food chain via cascading effects. For instance, natural enemies, such as parasitoids or predators, can have cascading top-down effects on lower trophic levels by suppressing their phytophage host or prey and thereby enhancing the plant fitness (Gómez and Zamora 1994). On the other hand, phytophage populations are regulated by the quality and

quantity of the primary producers that depend on the abiotic environment (Schmitz 2008; Mooney and Singer 2012), which in return can affect higher trophic levels by a cascading bottom-up effect. Stress caused by disturbances of this abiotic environment, such as pesticide exposure, can cause considerable changes throughout all levels of a trophic chain (Elderd 2006; Hopcraft et al. 2009) and organisms in the upper trophic levels like parasitoids are expected to be particularly vulnerable due to cumulative bottom-up effects (Tscharntke and Brandl 2004; Hance et al. 2007).

Many parasitoids are hymenopteran insects that lay eggs inside specific phytophagous insects and their larvae feed inside or on the host, often killing them ultimately as a result of their development (Godfray 1994). Consequently, parasitoids depend on their hosts to reproduce and to survive during their larval stages. For this reason, abiotic stress on their host or its food source can indirectly affect both their oviposition behaviour and offspring development (Hance et al. 2007). Several studies suggest that the success of parasitism decreases with exposure to toxicants (i.e. Joseph et al., 2011). First, a decrease in both host quantity and quality due to pesticide exposure through the plants may alter parasitoid oviposition behaviour as the optimal foraging theory predicts that a parasitoid female selects the most profitable patches of hosts to lay her eggs in order to maximize her fitness (Godfray 1994; Wajnberg et al. 2008). Second, after oviposition, toxicant stress by pesticides may be directly lethal for the developing parasitoid offspring (Campbell and Duffey 1979; Gunasena et al. 1990) and third, similarly to defensive organic compounds produced by plants defense chemicals, they may also alter their development indirectly through a reduction of host performance, i.e. host quality (Sznajder and Harvey 2003; Harvey et al. 2005). Finally, sex ratio may change due to an alteration of sex allocation at the time of oviposition because of female choice (i.e. parasitic wasps can determine the

sex of their offspring by fertilizing the egg or not, and end to lay males in small hosts and females in bigger ones) and/or because of differential survival of the two genders within the hosts (Idris and Grafius 1993).

The aim of this study was to quantify the direct and indirect effects of two lipophilic pesticides, the herbicide 2,6-dichlorobenzonitrile (DCBN) and the insecticide 1,4-dichlorobenzene (DCB), on a tritrophic terrestrial food chain via exposure through the plant growth medium to mimic exposure via interstitial (pore) water. To mimic this exposure route and to maintain a constant concentration of the lipophilic pesticides, we applied a recently developed passive dosing method using silicone poly-dimethylsiloxane (PDMS) (Mayer and Holmstrup 2008; Smith et al. 2010). The trophic chain to test this consisted of the winter wheat *Triticum aestivum* Linnaeus, the aphid *Sitobion avenae* Fabricius (Carter et al. 1980) and its specialist parasitoid *Aphidius rhopalosiphi* de Stefani-Perez (Krespi et al. 1994). The two pesticides used were selected for their spread and persistence within various ecosystems (Wang et al. 1995; Newhart 2007; Björklund et al. 2011). They have both been used on a large scale and residues can still be found in the soil and interstitial water (Wang et al. 1995; Björklund et al. 2011).

Overall, we expected that this experimental set-up would allow us to demonstrate the effects two pesticides, with similar molecular structure but different modes of action, can have on a tritrophic system exposed through interstitial water. We hypothesized that the higher trophic level will be more affected by pesticides than the lower trophic levels due to cumulative bottom-up effects. This could result in a change of *A. rhopalosiphi* oviposition behaviour when encountering pesticide-exposed hosts, if it perceived them as being less profitable (Le Lann et al. 2008; van Baaren et al. 2009). Alternatively, parasitism could occur, but the development of the

parasitoid may be altered through exposure to pesticide residues stored in its host fat reserves (Joseph et al. 2011).

Materials and methods

Rearing

The aphid *S. avenae* and the solitary endoparasitoid *A. rhopalosiphi* populations originated from the Walloon Agricultural Research Centre and were collected in organic cereal crops near Gembloux in Belgium (50° 34'0"N, 04° 41'0"E) in spring 1994 (JP Jansen, Personal communication). The aphids used for this study derived from one parthenogenetic female (C Le Lann, unpublished data) and were reared on organic winter wheat, *T. aestivum*, provided by "La Ferme des Petits Chapelais" of Chavagne in France (48° 04'18"N, 1° 45'52"W). The parasitoids were reared on a mixed-age culture of *S. avenae* and were supplemented each year with field-collected parasitoid females to maintain genetic diversity in the cultures. Both cultures were kept in Plexiglas cages (33 x 33 x 33 cm) in climate rooms at 20 ± 1 °C, 75 ± 10 % relative humidity and a 16:8h light:dark regime.

For the parasitoids used in the experiment, mummies (which are nymph stages of parasitoids, inside dried exoskeletons of dead aphids) were collected from the culture and placed individually in gelatine capsules (L = 2 cm, Ø = 0.7 cm) until emergence. Newly emerged females were mated before experiments by placing one female together with 3-4 males of the same age in a Plexiglas cylinder (L = 15 cm, Ø = 1.5 cm) for one day, while provided with some droplets of *Acacia* honey (Florimel, Belgium) and moist cotton wool.

Pesticide characteristics and concentration determination

DCBN, also known as dichlobenil or dichlorobenil, was used as a broad-spectrum meristem-effective herbicide and can still be found in environmental compartments like soil and ground water (Björklund et al. 2011). DCB, also known as para-dichlorobenzene or p-dichlorobenzene, is used as a moth repellent and as toilet block deodorant (Meharg et al. 2000). DCB is also a persistent pollutant in agroecosystems as it was formerly used for eradicating a variety of crop pests (Newhart 2007). DCB was found in sewage sludge applied on agricultural land (Wang et al., 1995) and is a degradation product of Lindane, an insecticide also formerly widely used (Lal et al. 2010).

1,4-Dichlorobenzene (DCB) and 2,6-dichlorobenzonitrile (DCBN) were tested in ranges of 3 concentrations: chemical activities of 0.3, 3 and 30% for DCB and chemical activities of 0.1, 1 and 10% for DCBN. A control was added with 0% of chemical activity. These concentrations were selected from a range of concentrations tested on *T. aestivum* (data not shown) for having non-lethal effects, using a logarithmic scale ensuring substantial differences between concentrations and for being realistic concentrations occurring in the field (cf. paragraph below).

To measure the actual exposure concentrations during the test, three loaded medium samples per concentration were chemically analysed using a Dionex high-performance liquid chromatography system consisting of a Vydac 201TP reverse-phase column (C18; 5 µm, 4.6 x 250 mm) with a Water Spherisorb ODS2 Guard Column (C18; 5 µm, 4.6 x 10 mm) connected to a fluorescence detector (model FP-1520; Jasco, UK) and a diode-array UV detector (UVD 320, Dionex, Germering, Germany). As solvent, a mixture of demineralised water and acetonitrile (99.9%, Sigma-Aldrich) was used. Calibration curves were established for both compounds to calculate actual concentrations. The average concentrations and standard errors of 0.3,

3 and 30% DCB were 210 ± 17.0, 3307 ± 187 and 38458 ± 5546 µg.L^{-1}, respectively. For 0.1, 1 and 10% of chemical activity of DCBN, concentrations were 3.3 ± 0.5, 78.8 ± 8.4 and 1085 ± 280 µg.L^{-1}, respectively. No traces of DCB and DCBN were detected in the control.

Recent accurate measurements of DCB concentrations in the soil have not been published, making it impossible to relate our concentrations to environmental exposure conditions in the field. By contrast, DCBN is found in the environment at concentrations of up to 1320 µg.kg^{-1} with an average of 55 µg.kg^{-1} (Björklund et al. 2011). The Kd (soil/water adsorption coefficient) of DCBN varies between 0.294 and 18.1 L.kg^{-1} depending on the type of soil (Swann et al., 1983; US Environmental Protection Agency, 1998). This would result in estimated concentrations in the interstitial water between 3 and 187 µg.L^{-1} and maxima ranging from 73 to 4490 µg.L^{-1}. Consequently, our low and intermediate concentrations of DCBN are within the range of concentrations expected in the field and our highest test concentration is within the range of the maximum concentration measured in polluted soil.

Pesticide exposure using passive dosing vials

In the soil matrix the partitioning of compounds takes place into a fraction bound to soil particles and a fraction dissolved in the soil interstitial water, making the route of exposure therefore hard to assess (van Gestel et al. 1996). To circumvent this issue and to realize a constant exposure of the wheat plants, in which the toxicants are replenished constantly to equilibrium at the required concentration, passive dosing was applied (Mayer and Holmstrup 2008; Smith et al. 2010). Although this does not represent soil conditions in the field, general principles of exposure, uptake and transport through the tri-trophic system are comparable.

The passive dosing vials were prepared with a silicone coating and loaded as described in Mayer and Holmstrup (2008). The passive dosing vials used were 10 ml glass vials with a 500 mg (± 1%) cast layer of PDMS silicone, consisting of a silicone pre-polymer and catalyst (SILASTIC MDX4-4210, Velten & Hering GbR, Genthin) that were mixed according to instructions of the supplier. To load the vials, methanol (99.9%, Sigma-Aldrich) loading solutions were prepared with the required concentrations of DCB (99%, Sigma-Aldrich) and DCBN (97%, Sigma-Aldrich) by diluting methanol suspensions that contained clearly visible crystals of the compounds to ensure maximum dissolved concentrations. The controls were loaded with methanol only. The loading solutions were transferred into the vials and were left for 72 hours at room temperature in order to let the compounds partition between the loading solution and the silicon. After removing the compound solution, the silicon was dried with lint free tissue and cleaned of the methanol by rinsing three times with 3-5 ml demineralised water with a total contact time of at least 2 hours. Thereafter, the vials were dried again with lint free tissue and filled with a 0.5 concentrated Hoagland solution and kept at room temperature for at least 72 hours to allow partitioning of the compounds between the silicon and the solution (Hoagland and Arnon 1950). Subsequently, the vials were closed by Teflon® covered caps with a punctured hole (Ø = 2 mm) through which the roots of one plant were inserted.

Experimental set-up

There were three treatments per concentration: *T. aestivum* only (P) (N=10 per concentration), *T. aestivum* with *S. avenae* (PA) (N=10) and *T. aestivum* with *S. avenae* and *A. rhopalosiphi* (PAW) (N=10).

At day 0, wheat plantlets (4 day-old germinated plants, with shoot lengths of 1-2 cm, root lengths of 3-4 cm and a 3 roots structure) were individually placed into loaded vials, which were placed in a polystyrene tray (L = 50 cm, W = 7 cm, H = 7 cm). Each vial was individually enclosed by a PVC-tube (L = 25 cm, Ø = 5 cm) covered by gauze at the top. The gaps between the caps of the vials and the polystyrene trays were filled with moist cotton wool to prevent light from reaching the roots of the wheat plants and aphids (PA, PAW) from crawling into the gap (Fig. 1). At day 3, five recently (within the last three days) matured adult aphids were placed on all the plants of the PA and PAW treatments. At day 4, the adult aphids were removed, leaving their progeny on the plants as the aphid population. At days 8 and 9, aphids on the plants of the PAW treatment were counted. At that time the aphids reached second or third instar levels, the preferred stage for parasitoid oviposition (Outreman et al., 2001a,b; Colinet et al., 2005), so parasitoids were introduced and placed on the plant in the PVC tube. The parasitoids were watched continuously to record their attack behaviour, i.e. number of stings. Exploitation of the colony of aphids by the parasitoid was ended after 30 minutes (past this time, the parasitoid would not encounter nor attack aphids) or when the parasitoid left the plant for more than 60 seconds or when all aphids were stung (Le Lann et al., 2008; 2011). At day 17 after exposure of the wheat plants, aphids and parasitoids of the P (N=70), PA (N=70) and all PAW treatments that did not contain mummies (N=37) were harvested, collected and frozen (-20°C). In order to measure the developmental time of the parasitoids, all PAW treatments containing mummies (N=33) were left until all parasitoids emerged. Emergence of parasitoids was checked every day and each newly emerged individual was frozen (-20°C) for further measurements. At day 24, all parasitoids had emerged.

Fitness trait measurements on wheat plants, aphids and parasitoids

From the 17-day exposed plants, the length of roots and shoots as well as the total fresh biomass (balance precision: 0.1 mg) were measured. The total number of aphids and aphid biomass per plant as well as their reproductive rate (number of aphid progeny per adult per plant) were quantified. The overall parasitism rate (number of parasitoids emerged at 24 days per number of aphids on a plant at 8-9 days) was calculated as a global measure of parasitoid fitness. To investigate potential changes in oviposition behaviour and failure at different stages of parasitoid development, several intermediate steps and traits were determined and analysed: attack rate (number of aphids stung per number of aphids on the plant at 8-9 days), mummification rate (number of mummies formed at 17 days per number of aphids stung at 8-9 days), emergence rate (number of parasitoids emerging at 24 days per mummy formed at 17 days), parasitism success (number of parasitoids emerging at 24 days per aphid stung at 8-9 days), development time (from oviposition to emergence), sex ratio (proportion of males) and the body mass of males. Both total aphid fresh biomass per plant and individual male parasitoid biomass were measured with a microbalance (UMT2, Mettler Toledo, Tiel, the Netherlands, d = 0.0001 mg).

Statistical analysis

WHEAT PLANTS

A two-way analysis of variance (ANOVA) was performed for the effect of the DCB concentrations (control, 0.3, 3, 30 %) and of the different treatments (P, PA, PAW) and their interaction on total plant (shoots and roots) length and biomass after 17 days exposure, followed by a *post hoc* Tukey test for pairwise comparisons.

The datasets of plant length and biomass after 17 days exposure to DCBN (control, 0.1, 1, 10 %) of the three treatments (P, PA, PAW) were not normally distributed, but had a gamma distribution and were therefore analysed using Generalized Linear Models (GLMs) assuming a gamma distribution with an inverse link function. *Post hoc* chi-square tests were performed for pairwise comparisons between the different concentrations with a correction for the false discovery rate (fdr) that corrects for multiple comparisons (Benjamini and Hochberg 1995).

APHIDS

Effects of test concentrations, treatments (PA, PAW) and their interactions on the total aphid biomass per plant (square-root transformed) and the number of aphids per plant after 17 days exposure were analysed with an ANOVA, followed by a *post hoc* Tukey test for pairwise comparisons. The effect of test concentrations, treatments (PA, PAW) and their interactions on the aphid reproductive rate was analysed using a GLM with a binomial distribution (i.e. for proportional data) with a logit link function. *Post hoc* chi-square tests with fdr correction were performed for pairwise comparisons between the different concentrations.

PARASITOID

Proportions such as the parasitoid attack rate, mummification rate, emergence rate, total developmental rate and overall parasitism rate were analysed using separate sets of GLMs for DCB and DCBN, with binomial distributions and logit link functions. *Post hoc* chi-square tests with fdr correction were performed for pairwise comparisons between the different concentrations.

Only few parasitoids developed and/or emerged from exposed aphids, therefore the effect of each compound (all concentrations combined) was tested for sex ratio, development time and parasitoid body mass. The sex ratio (i.e. proportion of parasitoid males) was analysed for each pesticide using a GLM with a binomial distribution with a logit link function. As no female emerged in the DCBN treatments and parasitoid females are on average larger than males (Godfray 1994), the effect of each compound on body mass was analysed in males to avoid a gender-correlated bias. Including the females did not change the results (data not shown). The body mass of males exposed to DCBN and the development time of individual parasitoids exposed to both compounds were analysed using ANOVA. The body masses of male parasitoids exposed to DCB were not normally distributed either, but followed a gamma distribution, hence they were analysed with a GLM using a gamma distribution and an inverse link function.

For both plants and aphids there was no significant effect ($p>0.05$) of the treatments (P, PA, PAW) nor for the interaction between treatment and exposure ($P>0.05$). Therefore, the "treatment" factor was removed from the models and only the effects of exposure (i.e. factor or categorical variable) on the variables are presented in the results. All statistical tests were done in R version 2.14.0 (R Development Core Team 2011).

Results

Effects of DCB and DCBN on the wheat plant T. aestivum

DCB had a negative effect on both biomass and length of *T. aestivum* (Table 1). For both traits, wheat plants were performing less at the highest concentration of DCB

(Biomass: $P < 0.05$; Length: $P < 0.001$) (Fig. 2). The biomass and length of *T. aestivum* also decreased with increasing DCBN exposure concentration (Table 1). Plant biomass was lower at the two highest concentrations (1%: $P < 0.001$; 10%: $P < 0.001$) and plant length was significantly reduced at all concentrations (Fig. 3).

Effects of DCB and DCBN on the grain aphid S. avenae

There was no effect on aphid reproductive rate nor on the total number of aphids per plant when reared on wheat plants exposed to DCB (Table 1). The total biomass of aphids per plant tended to decrease with increasing DCB concentration (Table 1), but effects were not significant (Fig. 3). Overall, DCBN negatively affected all three aphid fitness traits analysed (Table 1), but aphids only performed poorer at the highest concentration of this compound (Number: $P < 0.001$; Biomass: $P < 0.001$; Reproductive rate: $P < 0.05$) (Fig. 3).

Effects of DCB and DCBN on the parasitoid A. rhopalosiphi

Number of aphids on plants when exposed to parasitoids at 8-9 days, aphids stung, mummies formed, parasitic wasps emerged and male parasitoids counted after treatment of 1,4-dichlorobenzene, 2,6-dichlorobenzonitrile and control were used to calculate the different rates (Table 2). The overall parasitism rate decreased significantly with exposure to either DCB or DCBN (Table 1). However, the differences between the concentrations of DCBN were not significant, but the highest concentration of DCB was ($P < 0.05$) in comparison to the other concentrations and the control (Fig. 4). Parasitoid attack rate was significantly lower when foraging on plants exposed to either DCB or DCBN compared to the control (Table 1). For DCB the attack rate was lower at the highest concentration in comparison to the other

concentrations and the control ($P < 0.01$). For DCBN fewer aphids were stung at the lowest concentration (Fig. 4). For both compounds, the parasitism success and the mummification rate were not affected (Table 1). Moreover, all parasitoids emerged from their mummy thus no effect of the tested compounds was found on the emergence rate (Tables 1,2). The average (±SE) male mass (control: 0.17±0.10 mg; DCB: 0.14±0.05 mg; DCBN: 0.15±0.05 mg) and the mean development time (±SE) (control: 14.3±2.2 days; DCB: 14.2±1.7 days; DCBN: 13.6±1.3 days) of the parasitoids did not differ between concentrations of each compound and the control (Table 1). Nevertheless, the sex ratio was more male biased than in the control when exposed to DCBN but not when exposed to DCB (Table 1).

Discussion

Plant fitness was affected by both compounds, whereas the aphids were only affected at highest concentrations of DCBN. Both compounds altered parasitoid attack and overall parasitism rate and the sex ratio was altered by the lower concentrations of DCBN. Therefore, the results show that the pesticides tested affected each trophic level differently and that there was a bypass of the second trophic level at some concentrations.

Effects of DCB and DCBN on the wheat plant T. aestivum

Growth of the wheat plants was inhibited at the highest concentration of DCB. This result concurs with general plant responses to DCB as growth inhibition was demonstrated in cultured cells of three other plant species (tomato, soybean, and carrot) exposed to DCB (Wang et al., 1996). Plant biomasses and lengths were

reduced in a dose-related manner when exposed to DCBN. As DCBN is an inhibitor of seed germination, cellulose synthesis and division of meristem cells (Gonzalez-Reyes et al. 1986), our results support current knowledge on the effect of this pesticide on plants.

Effects of DCB and DCBN on the aphid S. avenae

Only at the highest DCBN concentration aphid fitness traits were negatively affected. The lower performance of aphids at the highest concentration of DCBN may be due to the phytotoxicity of the compound. However, at this concentration only, plants grew little and were discolouring. Senescent plants in particular can lead to severe stress and provoke a decrease in the body mass of aphid mothers and of their progeny (Stadler 1995). In addition, severe stress can decrease the number of aphid progeny produced due to resorption of embryos by the mothers (Ward and Dixon 1982). Consequently, the negative effects observed on the aphids may be caused by indirect effects of DCBN decreasing the quality of their diet, instead of direct toxic effects.

DCB is known to be toxic to a wide range of insects when directly exposed (Linnie and Keatinge 2000). The bean aphid *Aphis rumicis* had an increased mortality of up to 24% under direct exposure by spraying at a concentration of 10 g/L (Mills 1935). This concentration however, largely exceeds DCB solubility in water (0.105 g/L at 20° C). In our study, no deleterious effect of DCB on aphids was found. This may be because we used lower concentrations (up to 0.038 g/L) and exposed phytophages indirectly through the plants. Another possible explanation is that aphids might sequester these pesticides or hydrolyse them as with organic plant toxins, causing high concentrations of these compounds in their bodies that repel or decrease the fitness of parasitoids and predators (Pasteels 2007; Pentzold et al. 2014).

Effects of DCB and DCBN on the parasitoid A. rhopalosiphi

Previous experiments that exposed *A. rhopalosiphi* directly by spraying or submerging individuals into pesticide solutions focussed mainly on lethal effects and found negative effects on hatching success, searching behaviour and survival (Borgemeister et al. 1993; Jansen et al. 2010). We observed several sublethal effects including a decrease in the overall parasitism success of *A. rhopalosiphi* through indirect exposure to DCB or DCBN via plants and aphids. By looking at multiple stages between the choice to sting and emergence of the parasitoid (Tables 1, 2) we wanted to identify the most susceptible stage. Of these stages only the attack rate was significantly affected, suggesting that oviposition is the critical stage.

Other studies also observed negative effects of pesticides on long-range oviposition behaviour of parasitoids, for example reductions in chances of finding suitable hosts and oviposition sites (Longley and Jepson 1996; Umoru et al. 1996). In addition, a parasitoid species that is closely related to our species, *Aphidius ervi*, had a significantly reduced short-range oviposition activity, like antennal examination and number of stings, when exposed to a sublethal concentration of the insecticide lambda-cyhalothrin (Desneux et al. 2004). We also observed such short-range effects. There are two possible explanations for negative short-range effects of pesticides: a) they can disrupt the chemical cues that enable the parasitoid to locate its host (Vet and Dicke 1992; Schneider et al. 2004) b), or they can potentially alter the external cues of the host. Parasitoid females are able to assess the quality of their hosts externally by antennal tapping or from a short distance, as they possess gustatory/olfactory sensilla at the extremities of their antennae (van Baaren et al. 2007, 2009; Le Lann et al.

2011). These sensilla could possibly pick up the pesticide or changes induced by it that may indicate a lower host quality.

Apart from the negative effects on attack rates, a male-biased sex ratio was observed for *A. rhopalosiphi* when offered aphids on plants exposed to DCBN. Multiple pesticides have been reported to alter the sex ratio of predators or parasitoids (Desneux et al. 2007). In general two major causes could lead to biased sex ratios: a) sex specific egg-developmental failure caused by exposure to the toxicant, and b) switch in offspring sex allocation by the mother (Idris and Grafius 1993). Since, the number of parasitoid eggs laid inside the host cannot be determined without killing it, developmental failure cannot be excluded within our experimental set-up. However, if developmental failure would have occurred, a decrease in the parasitism success (emerged wasps per aphids stung) would have been expected, which we did not see. *Aphidius rhopalosiphi* is a parasitoid with haplo-diploid sex determination, enabling females to choose the sex of their progeny by fertilizing their eggs (females) or not (males) according to host quality. They prefer to lay female eggs in high quality hosts whereas male eggs are often deposited in lower quality hosts (Godfray 1994). In addition, *Aphidius* species including *A. rhopalosiphi* (Le Ralec and Rabasse 1988) also possess sensilla on their ovipositors allowing them to determine host quality internally (van Baaren et al. 2007, 2009). Therefore, parasitoid females may decide to lay a male egg instead of a female egg if they perceive bad quality aphids with their gustatory/olfactory sensilla. Similar to our results, Joseph et al. (2011) also observed a decrease in the number of attacked aphids and a male-biased sex ratio, while the adult emergence rate and the developmental time stayed the same compared to the control, when aphids supplied with Plenum-contaminated artificial food were offered to *A. ervi*. Thus it seems that parasitoid sex ratio, attack rate and parasitism success can be

negatively affected by indirect exposure to pesticides and that this is most likely caused by external and internal detection of the compounds and/or changes provoked by the compounds inside and outside the host.

Effects of DCB and DCBN on tritrophic interactions

In this study, negative effects of indirect exposure to the pesticides on the parasitoid *A. rhopalosiphi* were observed at concentrations at which their hosts were not significantly affected. Similar bypasses of the intermediate trophic level were found in studies on the effects of natural plant defence chemicals on parasitoids and hyperparasitoids (Harvey et al. 2003). These bypass effects might be caused by the sequestering of organic toxins by aphids, which is a well-documented strategy in response to plant toxins (Pasteels 2007; Pentzold et al. 2014). Hence, these effects might be more general in food chains including parasitoids. In our test system, such a bypass may lead to a decreased top-down effect of the parasitoids on the aphids. Since parasitoids represent the most important regulators of aphid populations (Schmidt et al. 2003), this may have great consequences for the population size of the aphids in a natural environment and create an imbalanced system.

Conclusions

The novel test system introduced in this study showed suitable for detecting carryover effects of pesticides with different modes of action on non-target organisms, while mimicking exposure via the interstitial water. Our results support the hypothesis that pesticides can be more toxic to parasitoids than to their hosts (Croft and Brown 1975). A bypass of the effects of sublethal concentrations in the plant growth medium of both DCB and DCBN on the second trophic level was found leading to negative

effects on the oviposition behaviour of the third trophic level. Thus, the unintentional spread of persistent pesticides in the environment could have a much larger effect upon non-target organisms than previously presumed. Alterations in parasitoid population dynamics, through biased sex ratio and reduced host attack rate, could drastically reduce parasitoid pressure on their hosts and alter natural ecosystem functioning, although field experiments will be necessary to confirm this.

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Compliance with Ethical Standards

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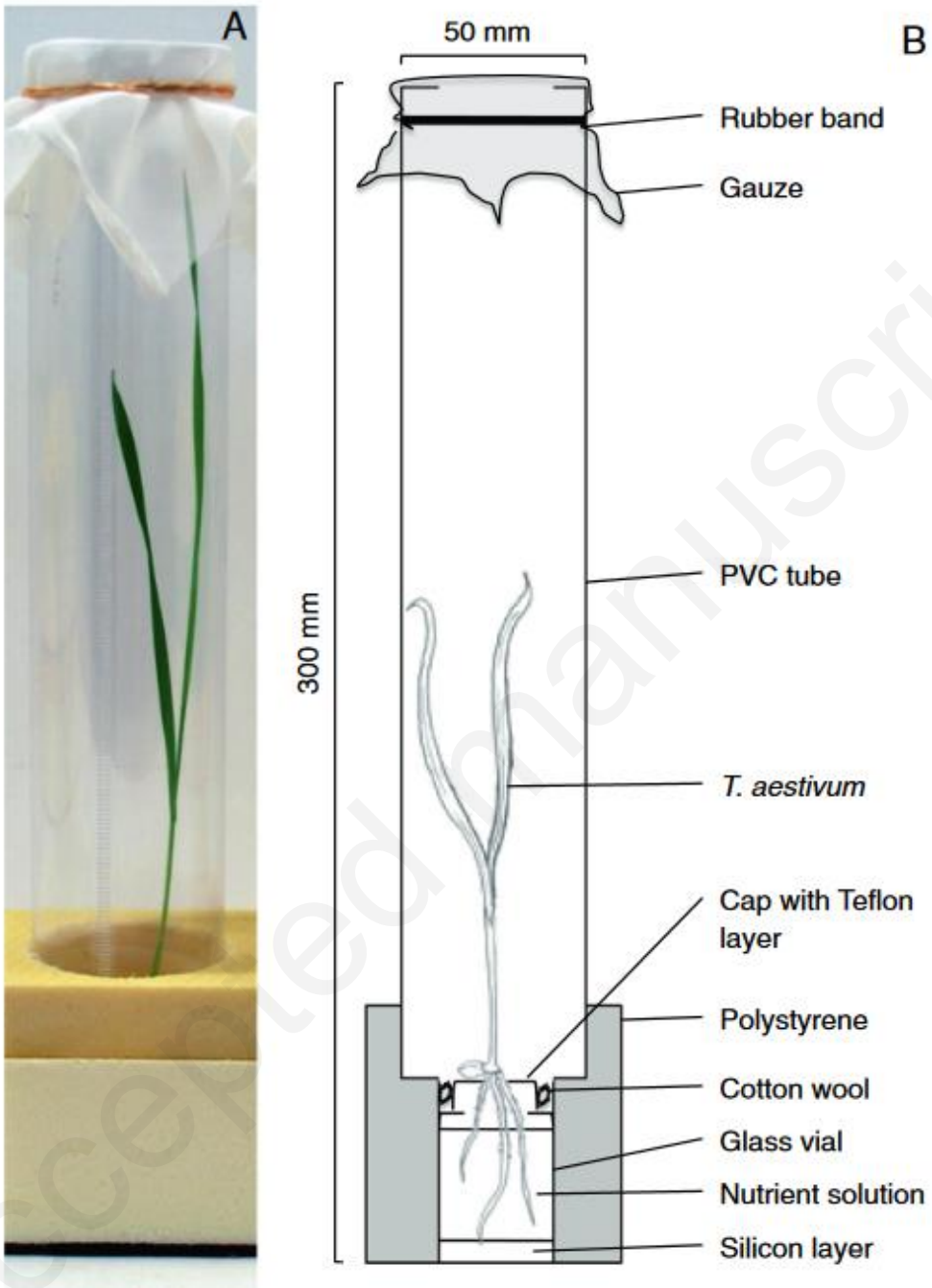

Figure 1: Photograph of the experimental set-up used for determining the effect of pesticides on a tritrophic system of a plant (*Triticum aestivum*), aphids (*Sitobion avenae*) and a parasitoid (*Aphidius rhopalosiphi*) (A). Schematic representation of the experimental set-up with annotated compartments (B).

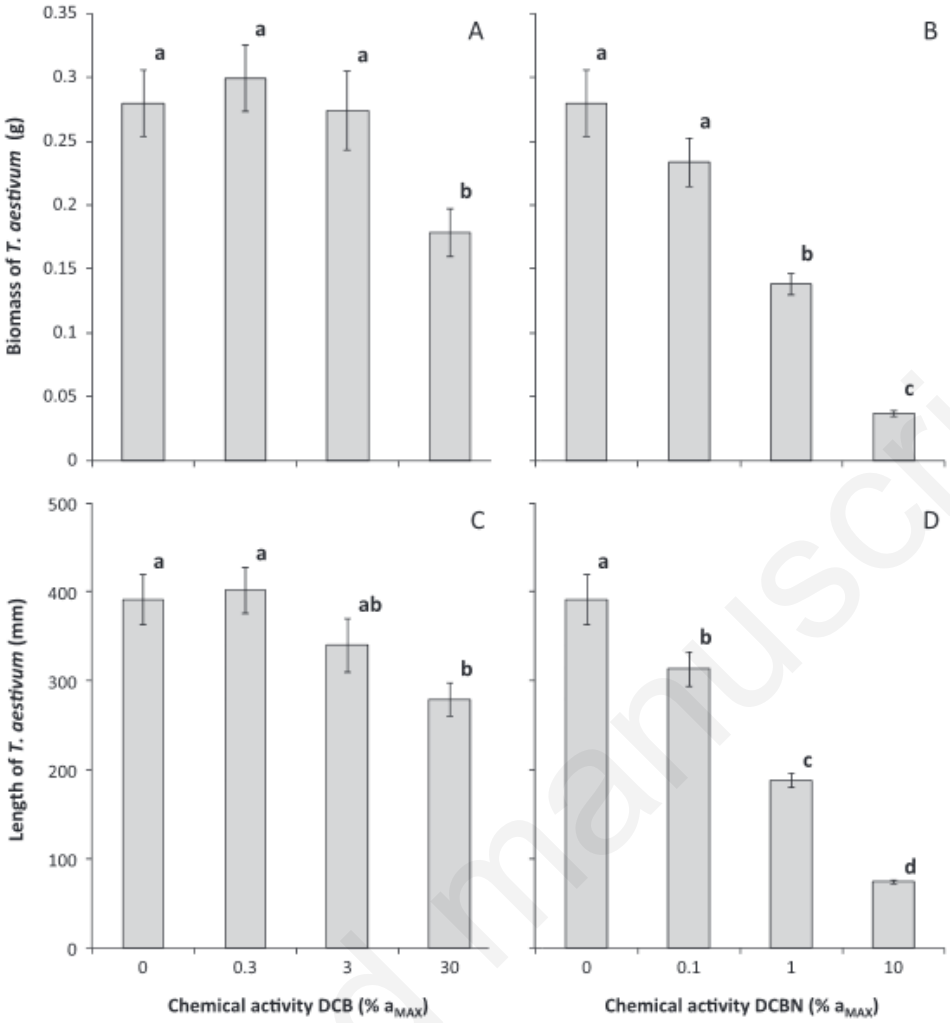

Figure 2: Effect of 17 days exposure of 1,4-dichlorobenzene (DCB) (A, C) and 2,6-dichlorobenzonitrile (DCBN) (B, D) on fresh biomass (g) and length (mm) of *Triticum aestivum* wheat plants. Exposure concentrations are expressed in percentages of maximum chemical activity (% a_{MAX}). Bars represent mean ± SE and different letters indicate significant differences ($p < 0.05$) (DCB: ANOVA, Tukey pairwise comparisons; DCBN: GLMs with gamma distribution, χ^2 pairwise comparisons).

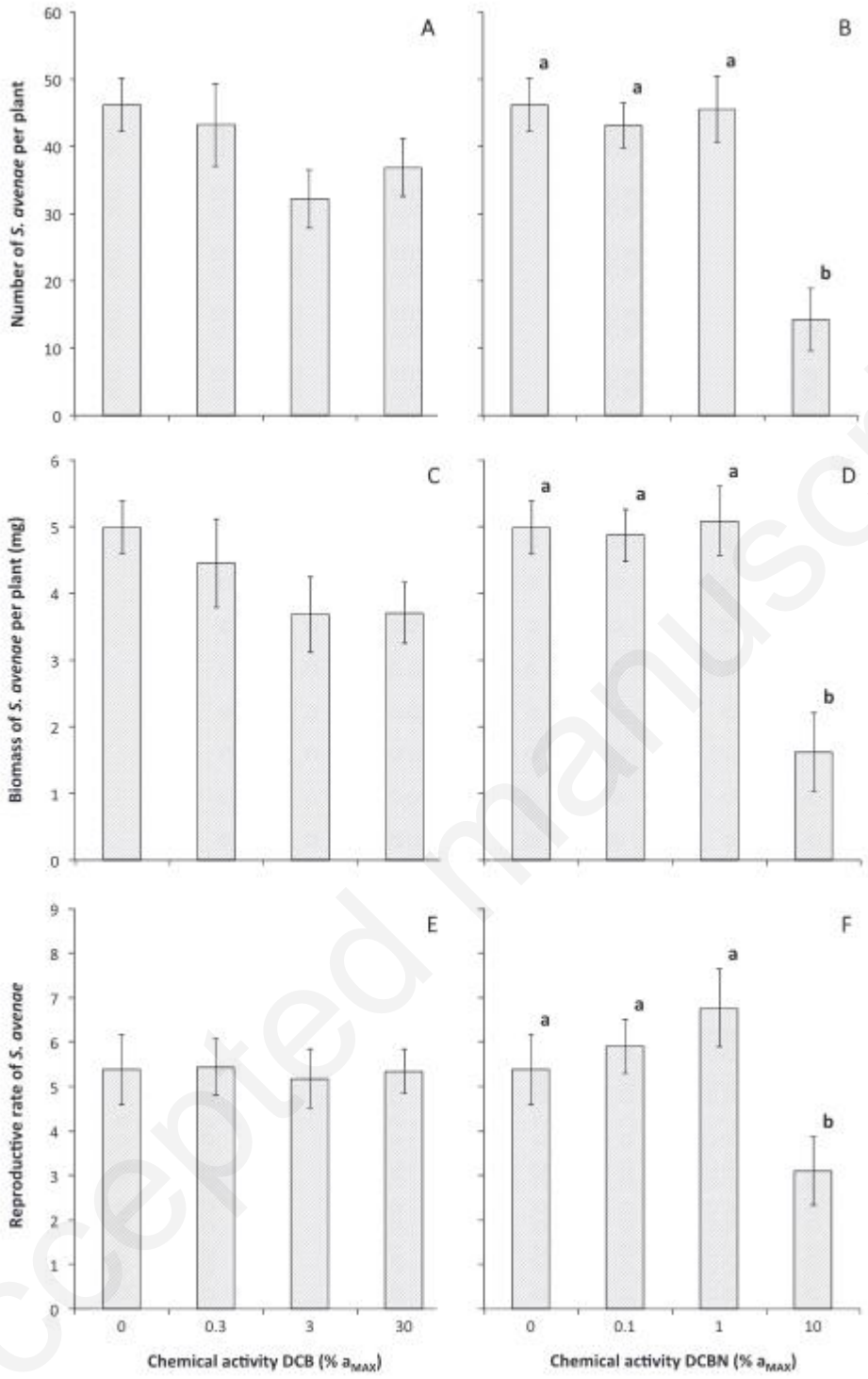

Figure 3: Effect of 17 days exposure of 1,4-dichlorobenzene (DCB) (A, C, E) and 2,6-dichlorobenzonitrile (DCBN) (B, D, F) on the number of aphids (*Sitobion avenae*) per plant, aphid biomass per plant and reproductive rate. Exposure concentrations are expressed in percentages of maximum chemical activity (% a_{MAX}) in the plant growth

medium. Bars represent mean ± SE and different small letters indicate significant differences (mass and number: ANOVA, Tukey pairwise comparisons; amount of progeny: GLMs with binomial distribution, χ^2 pairwise comparisons).

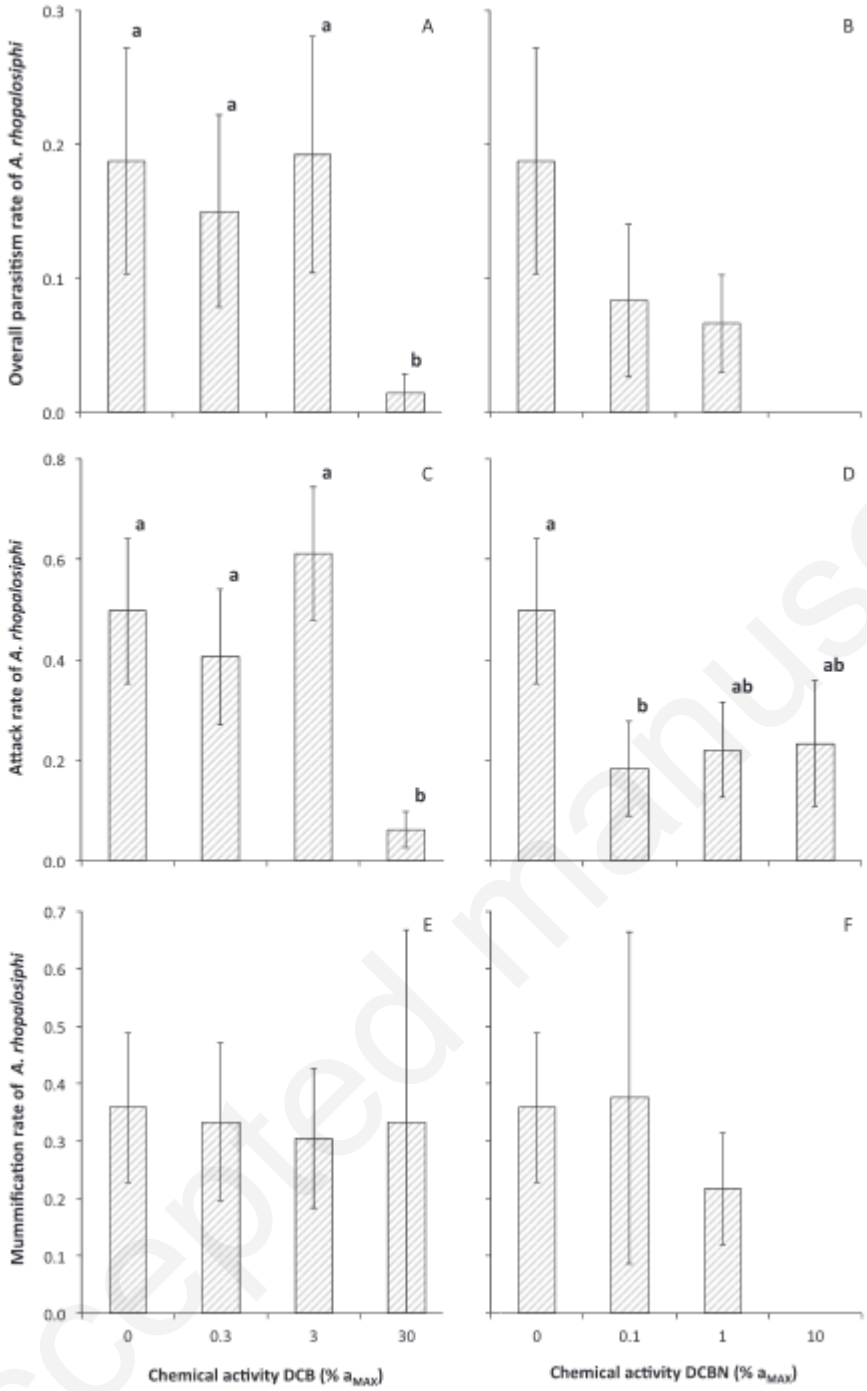

Figure 4: Effect of 1,4-dichlorobenzene (DCB) (A, C, E) and 2,6-dichlorobenzonitrile (DCBN) (B, D, F) on the attack rate, mummification rate and overall parasitism rate of the parasitoid *Aphidius rhopalosiphi* attacking aphids on wheat plants. Exposure concentrations are expressed in percentages of maximum chemical activity (% a_{MAX})

in the plant growth medium. Bars represent mean ± SE and different letters indicate significant differences (GLMs, χ^2 pairwise comparisons).

Table 1: Influence of 1,4-dichlorobenzene (DCB) and 2,6-dichlorobenzonitrile (DCBN) on fitness traits of wheat plants (*Triticum aestivum*), aphids (*Sitobion avenae*) and parasitoids (*Aphidius rhopalosiphi*) exposed through the plant growth medium. The table shows GLMs and LMs models with χ^2 and F-values (Value), degrees of freedom (d.f.) and corresponding probability values (*P).* Significant *P*-values are shown in bold.

Organism	Parameter	DCB				DCBN			
		Test	d.f.	Value	*P*	Test	d.f.	Value	*P*
Triticum aestivum	Biomass	F	3	5.16	**< 0.01**	χ^2	3	17.55	**< 0.001**
	Length	F	3	5.61	**< 0.01**	χ^2	3	7.95	**< 0.001**
Sitobion avenae	Number per plant	F	3	1.54	0.22	F	3	13.74	**< 0.001**
	Biomass per plant	F	3	2.57	0.07	F	3	12.55	**< 0.001**
	Reproductive rate	χ^2	3	58.66	0.62	χ^2	3	94.52	**< 0.01**
Aphidius rhopalosiphi	Overall parasitism rate	χ^2	3	39.67	**< 0.01**	χ^2	3	32.31	**< 0.05**
	Attack rate	χ^2	3	55.94	**< 0.001**	χ^2	3	70.36	**< 0.01**
	Parasitism success	χ^2	3	16.55	0.94	χ^2	3	8.83	0.32
	Mummification rate	χ^2	3	16.55	0.94	χ^2	3	8.84	0.32
	Emergence rate	χ^2	3	0	1	χ^2	3	0	1
	Male mass	χ^2	1	3.67	0.29	F	1	0.21	0.66
	Development time	F	1	0.05	0.83	F	1	0.64	0.44
	Proportion of males	χ^2	1	24.02	0.17	χ^2	1	11.46	**< 0.05**

Table 2: Total number of aphids on wheat plants (*Triticum aestivum*) when exposed to parasitoids at 8-9 days, aphids (*Sitobion avenae*) stung at 8-9 days, mummies formed at 17 days, parasitic wasps (*Aphidius rhopalosiphi*) emerged and male parasitoids counted after 24 days exposure to three concentrations (expressed as chemical activity) of 1,4-dichlorobenzene (DCB) and 2,6-dichlorobenzonitrile (DCBN) in the plant growth medium. Also given are responses recorded for the control.

Chemical	Chemical activity (%a_{MAX})	Aphids on plant	Aphids stung	Mummies formed	Parasitoids emerged	Male parasitoids
Control	0	57	25	9	9	6
DCB	0.3	49	18	8	8	7
	3	54	27	9	9	8
	30	78	4	1	1	1
DCBN	0.1	63	5	2	2	2
	1	74	19	6	6	6
	10	31	6	0	0	0