honey bee foragers in the field and at the hive entrance (pollen and nectar) and stored food items (bee bread and nectar) with significant lower in-hive residues (sampled from honey bee and bumble bee colonies).

Residues on pollen sampled from solitary bee hives are difficult to be interpreted since results are based on only four of five study fields and on a limited number of samples due to methodological limitations in this test system. The residues on pollen were < LOD in three study fields at all samplings dates and very low at DAT 4 in one study field in comparison with honey bees and bumbles bees at the respective sampling date.

The highest residues in bee-relevant matrices were found in pollen (maximum 1.75 mg/kg). Decline of residues in pollen was observed for all samples. Dissipation time (DT50) was < 4 days. No residues or residues close to the LOQ (0.01 mg/kg) were found in nectar samples. The sugar content was determined to be 81.5 %.

No other attractive crops that flowered during the course of the study were detected. Therefore, the obtained data reflect a worst-case scenario under realistic conditions (trials conducted in agricultural landscapes).

The selected application rate (60 g a.i./ha) covers the maximum single application rate according to GAP. Based on the highest residues, found in the bee-relevant matrix pollen, the 90th percentile was determined to be 1.61 mg/kg at the first sampling after application (honey bee foragers) with an average value of 1.15 mg/kg.

4.9 Exposure by nesting material? – Investigation of potentially suitable methods for higher tier studies with solitary bees

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The registration processes and risk assessment of plant protection products (PPPs) on bees resulted in an increasing need for experiments with non-apis pollinators to assess potential side effects of PPPs on this relatively new group of test organisms. Recently, numerous studies have been performed but there is still a wide range of ongoing challenges. One of the challenges is the risk from insecticide exposure to solitary bees (especially at larval stages) by contaminated nesting material (e.g. mud partitions – mason bees). In 2017, an experiment was performed with the horn-faced mason bee *Osmia cornuta* (Hymenoptera, Megachilidae) under modified field conditions. The aim of the experiment was to develop a suitable test method for higher tier risk assessments with solitary wild bees exposed to treated nesting material. The potential effect of an insect growth regulator (IGR) to bees and their brood was examined. The reproduction capacity and brood termination rate were observed in the study as endpoints. Furthermore, hatching success and flight activity were recorded as additional information at several occasions.The present results provide no evidence that the exposure has an effect on the development during the larval stages of *Osmia cornuta*, neither in pollen mass nor in the nesting material.

Introduction

Pollination plays as ecosystem service[1] an important role in maintaining the global biodiversity and food production[2,3]. Over the last decades the global pollinator diversity decreased[4] and consequently the status of the bees moved in the focus of public interest. As a result, the registration processes and risk assessment of plant protection products (PPPs) on bees proposed

requirements including experiments with non-Apis pollinators to assess potential side effects of PPPs on this relatively new group of test organisms[5].

The honey bee has been investigated as a surrogate species for bees in the current risk assessments up to now, but to which extent an extrapolation of the honey bee data on wild bee species is reasonable as currently postulated is further unclear. Regarding the different life-history-traits, nesting activities and foraging behaviours the sensitivity to pesticides may vary among these organisms[6,7,8] and result in differences to be exposed to PPPs. The identified exposure routes include contact exposure (spray deposits, seed treatments and granules) and oral exposure (consumption of pollen/nectar and contaminated water, accumulative toxicity and risks from metabolites).

In this experiment, the in the past unnoticed exposure route of contaminated soil by agrochemicals to a solitary bee and their brood is tested. It is unclear up to date if the contamination (e.g. soil deposition during furrow applications, product drift of spray deposits and seed treatment) may result in effects on adults or larvae from contact exposure. No standardized techniques are currently available as required for registration procedures or risk assessments[9,10,11]. The aim of this research work was to investigate a suitable test method for higher tier risk assessments with solitary wild bees exposed to treated nesting material within an experiment by determining certain parameters.

Materials and methods

The experiment was performed with *Osmia cornuta* (Hymenoptera, Megachilidae), six replicates per treatment group and two independent replications (1st and 2nd application) in an 7-day interval at two comparable locations in Northern Germany (Southeast Lower Saxony).The IGR diflubenzuron (product: Dimilin 80 WG) was tested at two concentrations (T1:1ppm; T2: 5ppm), assuming 0.3 g to be the average pollen mass in every cell [12], based on the LC_{50} values for *Bombus terrestris* [13] and *A. mellifera* [14]. The experimental trial was adopted from the research performed by Sgolastra et al. (2015) and was adjusted according to given field conditions.

In the field, cells were selected and the test solution (20 µl) was pipetted into the pollen provision (*exposure route* "P") after making a longitudinal hole by using a needle. Representative for the nesting material (*exposure route* "N") the rear mud walls were wetted. The potential effects to bees and their brood were examined in both treatments (T1, T2) and compared to a water treated control (C). The brood development was observed as endpoint in regularly time intervals from egg laying (beginning of April) until cocoon spinning (mid June). At the beginning of the test emerging and flight activity was occasionally recorded to assess the dispersal rates and to ensure a sufficient nesting acceptance.

From the day of the application (0 DAA) the photo recording took place every three days until day nine and afterwards once a week. During the experimental time, the following end-points were recorded: developmental period (number of days of the different stages egg-larvae, larvae without defecation-larvae with defecation, larvae-cocoon); brood termination (number of bees not developed during larval stages) and termination date (point of time when development is terminated).

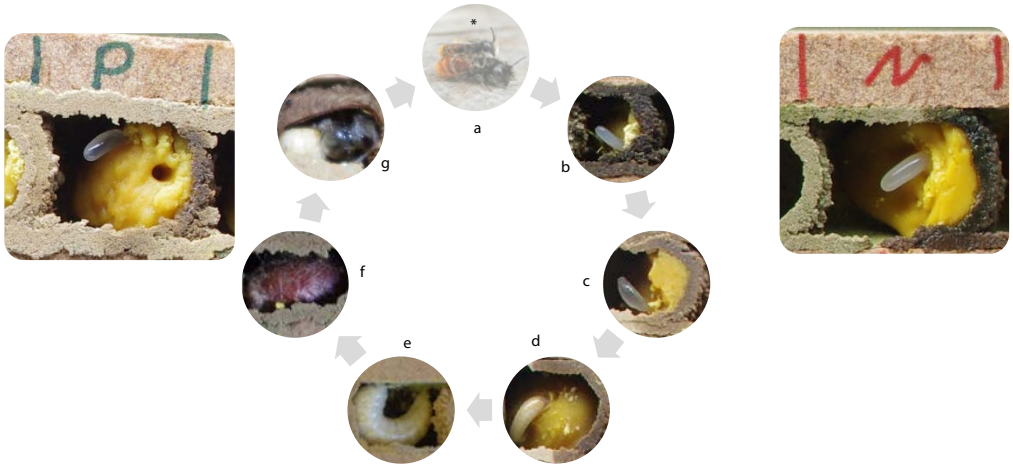


Fig. 1 brood cell with treated pollen ("P")
Fig. 2 brood cell with treated soil ("N")
Fig. 3 life cycle with times of observations

(a: mating, b: egg, c: hatched larvae, d: young larvae, e: old larvae with defecation, f: cocoon, g: development to adult bee)

* image extracted and edited from Stiftung Natur und Umwelt Rheinland-Pfalz (2017) http://bienen-rlp.de/index.php?id=476.

Normal distribution of the data was checked; for normally distributed data multifactorial ANOVA models and for not normally distributed data a Kruskal-Wallis-test/Post-hoc test was used. The statistical analysis was performed with the software R (version 3.4.0, 2017).

Results

Unsuitable cells

Nearly one fifth of all treated cells were excluded from the dataset for both applications as a result of an insufficient data quality (application failure, systematic errors of the photographic evaluation, methodological and biological errors).

Table 1 Unsuitable cells per treatment and application

	1st application			2nd application			total		
	total cells	unsuitable cells	unsuitable cells [%]	total cells	unsuitable cells	unsuitable cells [%]	total cells	unsuitable cells	unsuitable cells [%]
C	78	12	15.4	97	27	27.8	175	39	22.3
T1	99	10	10.1	87	21	24.1	186	31	16.7
T2	95	17	17.9	103	21	20.4	198	38	19.2
total	272	39	14.3	287	69	24.0	559	108	19.3

Developmental period

During the experiment the duration of the stages 1 (*egg - larvae without defecation (larvae I)*), 2 (*larvae without defecation - larvae with defecation (larvae II)*), 3 (*larvae - cocoon)* and the total developmental period were recorded (tab. 2).

Table 2 Developmental time of the stages per treatment and application

		egg – larvae I	larvae I – larvae II	larvae II - cocoon	total
1st application	C	9.5	18.4	13.4	41.3
	T1	9.5	18.2	15.0	42.7
	T2	9.6	19.2	11.8	40.6
	mean	9.6 ± 1.2	18.6 ± 4.6	13.5 ± 6.3	41.4 ± 4.3
2nd application	C	6.5	13.6	17.8	37.9
	T1	6.4	13.5	19.3	39.2
	T2	6.1	12.8	18.3	37.2
	mean	6.4 ± 1.6	13.3 ± 4.6	18.5 ± 5.1	38.2 ± 3.4

Both applications show nearly equal developmental periods regarding the total period and the individual durations of the larval stages. Nevertheless, the statistical analysis reveals significant differences ($p > 0.05$) between T1 and T2 for both applications. On average, the total development as well as the individual stages of T1 lasts longer than the durations of T2. Therefore, a sublethal effect on developmental duration seems to be indicated by our data but the results are insufficient and characteristic of the effect are marginal for seeing the assumption as given.

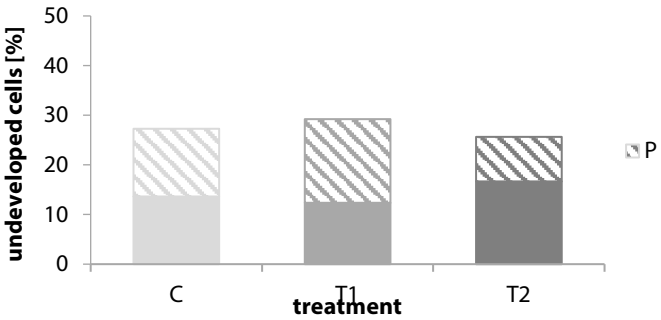
Termination date

Half of the undeveloped cells for both applications terminated within the first days after application (DAA; 0 DAA - 9 DAA) after application without any differences in the exposure routes. Furthermore, a moderate increase of termination on 23 DAA was observed over the course.

Brood termination (i.e. collapsed eggs or deformed larvae)

Both exposure routes showed no differences and were consequently presented as sum.

1st application



A quarter of all treated brood cells showed no hatching or further development, regardless of the treatments and different exposure routes (C 27.3%, T1 29.2%, T2 25.6%)

Fig. 4 Brood termination rate at the 1st application per treatment and material

2nd application

In contrast to the 1st application the treatments of the 2nd application showed differences; whereas in C only 10.0% and in T1 16.7% of all cells did not develop further, an abort rate of 39.0% was determined in T2.

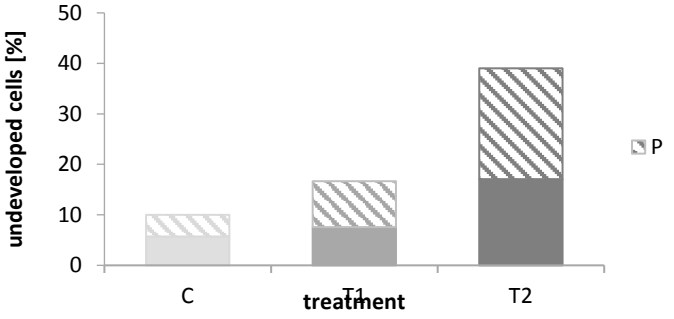


Fig. 5 Brood termination rate at the 2nd application per treatment and material

The results of the brood termination rate show huge variations between the 1st and 2nd applications. During the experimental time, undeveloped brood in the treated cells have shown diverse changes of the phenotype (collapsed eggs, complete dissolution of the brood, deformation, protrusions and discoloration of larvae). All developed larvae spun a regular cocoon at the end of the observation period. The difference of effects between the exposure routes – pollen/nectar and nesting material/soil – is small for both applications. The fact whether pollen or nesting material is contaminated seems to have no significant influence on the brood termination.

Conclusion and perspectives

This experimental work investigated the potential exposure route of contaminated soil by agrochemicals to a solitary bee (*Osmia cornuta*) and their brood. Previous studies on the effects of PPPs to solitary bees and their brood have concentrated mainly on the effect of contaminated pollen or nectar.

The brood termination was against the expectations relatively low. So far it is unclear if the low extent of observed effects is mainly caused by a low toxicity of the active substance towards *Osmia* larvae or if methodological improvements are needed. There are a lot of studies which confirm a high vulnerability of closely related species[13,14,15,16] and there are already initial findings of a sensitivity of *Osmia* species to the IGR[17]. The majority of all undeveloped brood cells were terminated in the first days after application and suggested that particularly the first larval instars seemed to be highly vulnerable to the agent. These observations were consistent with the findings of mortality patterns with species of Bombus and the honey bee[13,18,19]. The increase of termination later is probably based on an effect of application method due to a uniform distribution of the test item. A diffusion of the product from the treated nesting material into the pollen mass would explain why larvae, which should not have been in direct contact with the product and the pollen, show mortality at a similar level as the variant with directly treated pollen. More probable is certainly a higher residue in the rear part of the provision which stays in contact with the treated mud wall thus the mortality of the brood increases over experimental time. During the experimental time as well as the evaluation of the data a series of errors arose. These errors may occur directly during the application (absorption of test item, diffusion of concentration, shortage of persistence) and by photographic data acquisition (light conditions, position of egg/larvae, nesting material over the cells).

In summary, our investigation revealed against initial expectations no differences regarding the exposure routes pollen "P" and nesting material "N" as well as the concentration of the IGR on the brood of *Osmia cornuta*. Our data show a high variability so that the statistical significance has to be critically evaluated, however a trend towards higher brood mortality in T2 and a developmental delay in T1 (only at 2nd application) was assumed.

The development in the cocoon is not examined until now therefore further tests with the cocoons of our study will be performed in the next spring to assess emergence, weight, sex and

phenotypical variations. Finally, the method of our investigation principally seems to be suitable for tests with solitary bees, but some methodological limitations remain and up to today it is uncertain, if these can be overcome, which will be investigated in future tests.

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