



DRIPPING POISON

**AN ANALYSIS OF NEONICOTINOID
INSECTICIDES IN THE GUTTATION FLUID OF
GROWING MAIZE PLANTS**

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GREENPEACE

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SUMMARY



Growing maize plants in
Pest county, Hungary

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The European Food Safety Authority (EFSA) has carried out reviews of the neonicotinoid pesticides thiamethoxam, imidacloprid and clothianidin in order to assess the possible risks posed by these systemic insecticides to bees. These reviews helped underpin the decision by the European Commission to ban the three active ingredients from certain applications for a period of two years. In particular, the reviews identified shortcomings and gaps in the available data which prevented an holistic and exhaustive risk assessment from being carried out. One key uncertainty identified by EFSA in each case related to the role of guttation fluid exuded by commercial crop plants as a potential source of the chemicals to bees when they used it as a water source for themselves or for the colony as a whole.

The use of neonicotinoid insecticides as seed treatments and granules applied to soil is known to lead to these chemicals being present in the guttation fluid of various crop plants. Although the literature on this subject is sparse, the research carried out to date indicates that the neonicotinoids may be present at high concentrations. In order to investigate this phenomenon further, Greenpeace undertook a study of guttation fluid produced by maize plants grown in field conditions in Hungary, which according to the farmer had been treated with two different commercial seed treatment products. One field had been planted with seeds treated with Poncho®, with clothianidin as the active ingredient, while the other had been planted with seeds treated with Cruiser®, with thiamethoxam as the active ingredient. Samples of guttation fluid were sampled from each field over a number of days and analysed using UPLC-MS/MS techniques.

The results of the analyses revealed significant concentrations of neonicotinoid pesticides present in guttation fluid. Up to 11 709 µg/l of clothianidin was present in the fluid from the Poncho-treated seeds, while up to 55 260 µg/l of thiamethoxam was present in the fluid from Cruiser-treated seeds. In addition, the Cruiser-treated plants also exuded up to 9651 µg/l of clothianidin, most likely as a degradation product of the primary active ingredient used on the seeds.

The higher values of neonicotinoid pesticides in guttation fluid reported here are equal to or exceed the concentrations of active ingredient recommended for use in commercial formulations of sprayed insecticide. Significantly, even after a growth period of a month, the plants were found still to exude concentrations of the pesticide able to deliver the acute oral LD₅₀ quantity (or more) of pesticide to individual bees as a result of a single water-foraging event.¹ This was calculated on the basis of the same methodology used by EFSA in its assessments and using the limited data available for foraged water volumes in bees.

These findings, and their potential toxicological significance to bees both on an individual and whole colony level, suggests that, not only is the current restriction on the three neonicotinoid insecticides wholly justified, but that it should be maintained at least until the potential significance of guttation fluid as a water resource for bees is fully characterised, and until the other identified areas of uncertainty and missing information identified by EFSA are resolved. The scale and scope of the necessarily small-scale study conducted here needs to be expanded to include the full spectrum of crops grown using neonicotinoid seed dressings. In addition, the significance of guttation as a toxicological exposure route for bees needs to be investigated not only for a variety of crops but also under the full variety of growing conditions encountered for these crops across the European Community, in order to extend the currently highly limited information base available.

1 Acute oral LD₅₀ (median lethal dose) is a statistically derived dose of (in this case) a pesticide active ingredient that can cause death in 50% of bees within a maximum period of 96 hours after a single oral dose is administered.

INTRODUCTION



Growing maize plants in Pest county, Hungary

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Systemic pesticides can be defined as pesticides which, when they are applied to a plant or animal, move from the area to which they were originally applied to reach untreated tissues. It is possible, for example, to use systemic pesticides to control internal and external parasite infestations in animals. In plants, the applied pesticide may have herbicidal activity and be designed to kill the plant, or alternatively it may be targeted at a fungal or insect pest that affects the plant (Ministry of Agriculture, British Columbia, Canada 2013). One particular class of systemic insecticide used on plants – the neonicotinoids – has recently attracted considerable attention in relation to the potential impact on pollinators, in particular domestic honeybees. Much of the research into this topic has involved the Western or European honeybee (*Apis mellifera*), as researchers have sought to unravel the complex interaction of the diverse factors that may be responsible for declining pollinator populations and Colony Collapse Disorder (CCD). Greenpeace has recently produced an overview of the potential contributors to pollinator decline including the possible involvement of systemic and other insecticides (Tirado *et al.* 2013).

As that Greenpeace report – *Bees in Decline* – makes clear, no single factor can be blamed for what is essentially an overall global decline in bee populations and in their overall health. The most important of the identified factors in play relate to diseases and parasites, and to wider industrial agricultural practices that may affect many aspects of a bee's life cycle. Underlying all these, climate change is also putting increased strains on pollinator health. This decline is undoubtedly, therefore, the product of multiple factors – both known and unknown – acting singly or in combination.

In relation to diseases, the parasitic mite *Varroa destructor* is of global significance, while the parasite *Nosema ceranae* is a regionally significant pathogen, principally in southern Europe. Other new diseases – including novel viruses – may well be identified

in the future. The ability of bees to resist diseases and parasites seems to be influenced by a number of factors, particularly their nutritional status and their exposure to toxic chemicals. Some pesticides, for example, seem to weaken the immune system of honeybees, making them more susceptible to infection and to parasitic infestation.

Chemicals in the form of biocides are routine inputs into agricultural systems under the current paradigm of industrialised agricultural production. Some of these pose a direct risk to pollinators. In addition, habitat destruction and fragmentation of natural and semi-natural habitats, expansion of monocultures, and reduction of plant diversity all play a role in pollinator health. Finally, changing climate – which may affect weather, making it more erratic or extreme – is one factor whose impact upon bees and other pollinators may prove to be huge, although it is extremely difficult to characterise and predict.

Faced with this diversity of driving factors, *Bees in Decline* concluded that one crucial first step that could be taken would be to ban the use of several pesticides with a known high toxicity to bees. The list includes imidacloprid, thiamethoxam, clothianidin, fipronil, chlorpyrifos, cypermethrin, and deltamethrin. Following evaluations of the pesticides imidacloprid, thiamethoxam and clothianidin carried out by EFSA (ICPBR 2011; EFSA 2012a; EFSA 2013a) in April 2013, a majority of EU countries supported the European Commission proposal (EC 2013) to temporarily restrict the use of these three pesticides. Partial bans of neonicotinoids were already in place in Italy, France, Germany and Slovenia. In Italy, no significant negative impacts on agricultural production were reported, but there were some reported positive effects on the health of bees (European Parliament 2012).

The EC Implementing Regulation that was put in place reflected the agreement to suspend the three neonicotinoid insecticides in question because of significant data gaps in the body of data considered to be necessary in order to conduct an holistic assessment of the risks they posed to bees. One of the key uncertainties identified in the case of each, used as a seed dressing or in granulated form, was the potential for exposure of bees to these systemic insecticides via guttation fluid. Guttation fluid is exuded by many plants, including those grown from seeds treated with neonicotinoid pesticides. Some information exists showing that the exudate can contain neonicotinoid pesticides, but the data set is far from exhaustive.

This present study was designed to investigate and document the presence of these pesticides in guttation fluid from commercial crops treated with proprietary formulations, in order to help provide more data on this aspect.



GUTTATION AND ITS RELEVANCE TO BEES



Bees and a queen bee on a honeycomb.

© Fred Dott / Greenpeace

The word guttation comes from the Latin word “gutta”, which means drop (Girolami *et al.* 2009). Guttation is a process that can occur in many vascular plants. Essentially, it is the expulsion of plant xylem sap, which then forms droplets on the tips or along the edges of leaves. This process should not be confused with the formation of dew, which also takes place under similar atmospheric conditions. Guttation typically occurs when soils are moist, the root pressure of the plant is high, and mostly when stomata are closed in the hours of darkness, resulting in low transpiration capacity (Hoffmann *et al.* 2012). Under these circumstances, fluid is exuded from specialised structures called hydathodes (Stevens 1956), and forms into droplets. The frequency of guttation events varies between different crops, with a tendency for it to occur with greater frequency in cereal crops (monocotyledons) as compared to broad-leaved crop plants (dicotyledons) (Joachimsmeier *et al.* 2011).

Guttation was found to occur under varying conditions of relative humidity in the crops tested in this study. Early work established that guttation fluid contained sugars together with inorganic chemicals (Goatley & Lewis 1966). Later work has shown that amino acids and proteins may also be present, and although the function of hydathodes is poorly understood, it is widely accepted that they also play a role in recovering solutes from the guttation stream (Pilot *et al.* 2004). Guttation drops are most easily seen at dawn, and are quickly evaporated by sunshine or wind, sometimes leaving a whitish residue. They may also be subject to re-uptake by the plant.

In addition to the normal organic and inorganic solutes that may be present in guttation fluid from plants, systemic pesticides may also be translocated within the plant and exuded in this way. Guttation fluid from plants grown from neonicotinoid-treated seeds has been shown to contain significant concentrations of these insecticides (Girolami *et al.* 2009; Tapparo *et al.* 2011). In the Girolami *et al.* (2009) study, concentrations up to 100 mg/l were found for thiamethoxam and clothianidin, and up to 200 mg/l for

imidacloprid, in maize leaf guttation fluid from plants grown from neonicotinoid-coated seeds. In the Tapparo *et al.* study, neonicotinoids were detected in the guttation fluid of maize plants grown from seeds coated with neonicotinoids and with fipronil. The insecticides were detected at up to 346 mg/l in the case of imidacloprid, at 102 mg/l for clothianidin, and 146 mg/l for thiamethoxam. The neonicotinoid concentrations in the guttation fluid progressively decreased during the first 10-15 days after the emergence of the plant from the soil. Fipronil was not detected in guttation fluid. Rock melon plants grown in soils treated with a liquid application of imidacloprid were also found to exude the insecticide in guttation fluid at similar concentrations (Hoffmann & Castle 2012). The researchers reported imidacloprid at maximum concentrations of 4.1 mg/l in guttation fluid collected three days after a soil application, and at 37 mg/l one day after another soil application of insecticide at the highest concentration recommended by the manufacturer on the product label.

Guttation is highly relevant to honeybees because the fluid may be collected by them as a source of water. Water is collected for two reasons, both of which are weather related. Collected water is used to cool the brood on hot days using evaporative cooling, while on days where nectar collection is limited by cool or wet weather, water may be used to dilute stored honey which is then used for feeding of the brood (Nicolson 2009). The International Commission for Plant-Bee Relationships Bee Working Group has assessed the risks posed by ingestion of guttation fluid to honeybees and identified a number of factors that can be influenced to mitigate potential risk from systemic insecticides via this route (ICPBR 2011). Although this working group was relatively positive about the prospects of mitigating exposures via this route, and identified maize as the highest risk crop, this outlook contrasts somewhat with the views published by the EFSA (EFSA 2012a; EFSA 2012a; EFSA 2012b) in risk assessments for the three systemic insecticides clothianidin, thiamethoxam and imidacloprid. These risk assessments concluded that there were insufficient data to fully evaluate the risks to bees posed by these insecticides in guttation fluid. However, on the basis of observational data alone, the experts involved considered that the risks from some crops could be low, but they were unable to reach definitive conclusions. The uncertainties over the potential risks associated with guttation fluid, together with other key uncertainties in the available data, contributed considerably to the decision to formulate and impose the European Commission Implementing Regulation 485/2013 (European Commission 2013) to ban these three insecticides from use in seed treatments for a period of two years.

Accordingly, Greenpeace undertook this pilot study of guttation fluid produced by maize crops, with a view to adding to the available data on the topic of the content of neonicotinoid pesticides in this fluid.

GREENPEACE SAMPLING: METHODOLOGY AND RESULTS

Sampling

Sampling area and timing

The areas selected for a study on neonicotinoid concentration in guttation fluid were located in Pest County, central Hungary, north of Budapest, and comprised two actively and conventionally cultivated open maize fields located close to one another on similar alluvial sandy-humic soil types. According to the farmer, both fields had been sown with maize seed that had been treated with neonicotinoid seed treatments. One field – Field A – had been sown on 24 April 2013 with seed treated with Poncho (having clothianidin as its active ingredient). The other – Field B – had been sown on 1 May 2013 and treated with Cruiser (having thiamethoxam as its active ingredient). Sampling began approximately three weeks after sowing, and after the emergence of the first three leaves from the plumule. At the start of sampling, plants were between approximately 8 and 12 cm in height, and at the conclusion of sampling between approximately 25 and 35 cm in height, depending upon the date of planting in the respective fields.

Sampling was planned for every second day, but the programme was kept flexible in order to allow for adverse weather conditions, including rain or wind, which made meaningful sampling impossible. In these cases sampling was conducted on the following day. All samples were taken at dawn. Guttation fluid had generally evaporated between the first and second hour after dawn. Field A was sampled on seven occasions (17 May-2 June). Samples were taken on five occasions from Field B (21 May-2 June); the number of samples taken was limited by a major flooding event on the River Danube, which rendered the field inaccessible.

The number of samples taken on each occasion is shown below:

	Number of samples	
	Field A	Field B
17 May 2013	3	-
19 May 2013	3	-
21 May 2013	3	3
23 May 2013	1	2
25 May 2013	3	2
29 May 2013	3	3
2 June 2013	3	3

Sampling method

Sampling was carried out from three parallel planting rows in each of the two fields. A row was chosen in the centre of the field and marked together with the fifth and tenth rows adjacent to it for replicate samples. The centre of the field was chosen in order to eliminate any possible field edge effects upon growing plants, and also in order to minimise the possibility of cross-contamination with other seed treatments previously

used in the seed drill. Samples were taken with Gilson micropipettes fitted with 50 µl or 10 µl tips as appropriate to the droplet size. Droplets from the tip or the edge of the leaves were chosen in order to minimise the possibility of sampling drops of dew.

The pipette contents were discharged into 5 ml disposable polypropylene cryogenic vials (Corning Life Sciences) until approximately 1.5-2.5 ml of exudate had been collected. The tubes were closed with the threaded caps and seals. Sealed tubes were wrapped in aluminium foil after sampling, and kept cool in a cool box or refrigerator at 3-4°C until analysis. In sunny or windy conditions, sample volumes were sometimes less than the target amount of 1.5-2.5 ml. Repeat samples in all cases were collected from plants in exactly the same rows. In addition, approximately 1kg of samples of the seeds planted in the respective fields were obtained direct from the farmer, for use in any confirmatory analyses.

Sample preparation and analysis

After collection, the 32 samples were filtered through a 13 mm diameter 0.45 micron pore size teflon syringe filter (Whatman), to remove particulates and bacteria. Samples were then transported in a cool box at 3-4°C to the analysing laboratory. Samples were analysed as follows, based upon direct injection of an aliquot of the filtered sample into an LC-MS/MS system. The system used was a Waters Acquity UPLC coupled with a Waters Xevo TQS Mass Spectrometer run in the electrospray ionisation positive mode.

Samples were diluted to the appropriate working range of between 0.001 and 5.0 µg/l, and an internal standard of deuterated clothianidin added. An aliquot of 20 µl of this was injected into the instrument for analysis. Two mobile phases were used. Mobile Phase A comprised 95% H₂O and 5% MeOH (methanol) in 0.25 mM NH₄Ac (ammonium acetate) and 0.01% HAc (acetic acid). Mobile Phase B comprised 100% MeOH in 0.25 mM NH₄Ac. The UPLC column used was a 50 mm-length Kinetex 2.6 µm particle size, C8 phase, 100A pore size x 2.1 mm internal diameter reverse phase column (Phenomenex).

The phase gradient programme was as tabulated below:

Minutes	Percentage (%)	
	Mobile Phase A	Mobile Phase B
0	80	20
1	80	20
2	10	90
3.4	10	90
3.6	100	0

Total run time was 5 minutes with a flow rate of 0.4 ml/min.

The Mass spectrometer was run with the electrospray ionisation (ESI) in the positive mode.

For each pesticide two specific ion transitions were monitored:

Clothianidin: 250 -> 169 Thiamethoxam: 292 -> 211
 250 -> 132 292 -> 181

Limits of detection (LOD) and limits of quantitation (LOQ) were 5 µg/l and 10 µg/l respectively for both clothianidin and thiamethoxam.

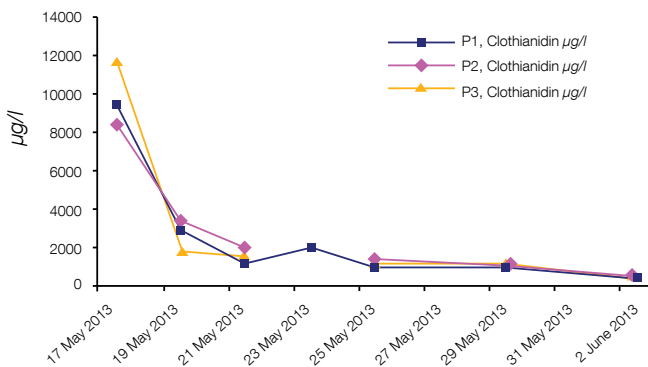
Results

Clothianidin was detected in all samples of the guttation fluid taken from maize grown in Field A and where seeds were treated with a declared coating of 1.25 mg clothianidin per seed (APENET 2011). Analysis of three replicate samples taken on six occasions, and of a single sample taken on one further occasion, together yielded concentrations ranging between 391 and 11 709 µg/l. Concentrations of thiamethoxam observed in the replicated guttation fluid samples in Field B (seeds treated with Cruiser at a declared concentration of 0.6 mg of thiamethoxam per seed) (APENET 2011) were much higher, ranging between 678 and 55 260 µg/l. In these samples significant concentrations of clothianidin were found, ranging between 167 and 9651 µg/l. This may be explained by the fact that clothianidin is not only an active substance in its own right but also occurs as a metabolite of thiamethoxam (EFSA 2012b), the primary active ingredient of Cruiser.

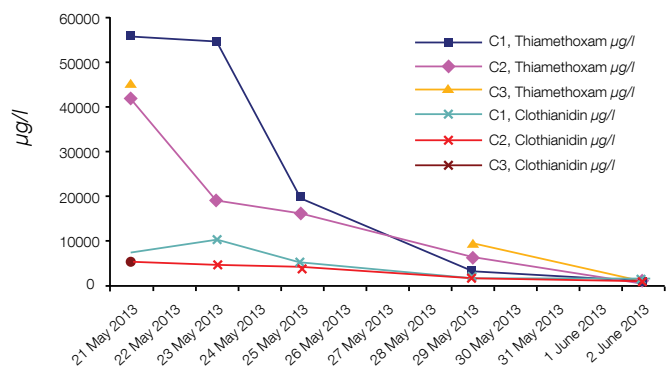
Concentrations of the two neonicotinoid pesticides in guttation fluid progressively fell over the duration of the sampling period, as shown in Figures 1 & 2. The clothianidin concentration from plants in Field A decreased rapidly, from an initial high value of 9.6 mg/l, and then appeared to stabilise somewhat at a lower concentration of around 0.5-1.0 mg/l in all replicate samples. Decreases in concentrations of thiamethoxam for Field B were similarly observed, but these appeared to be less rapid than for clothianidin. Recorded thiamethoxam residues were initially in excess of 50 mg/l (50 ppm) and fell to around 0.8 mg/l over the period of sampling. It is noteworthy that the initial concentrations found were equal to, or even exceeded, concentrations of active ingredient normally used in prepared spray formulations. For example, for spraying on paprika, tomato and lettuce, Syngenta recommends dilutions of 10–40 g Actara® per 100 l water, which is equivalent to between 25-100 mg of the active ingredient thiamethoxam/l of water.²

2 See: <http://www3.syngenta.com/country/hu/hu/cp/Termekeink/Rovarolo-szerek/Pages/Actara-25-WG.aspx>

Clothianidin in Poncho-treated maize guttation drops



Thiamethoxam and metabolite Clothianidin in Cruiser-treated maize guttation drops



Implications of results for honeybees

EFSA (2013b) provided a model calculation method in order to evaluate the potential risk to bees from the pesticide content of guttation fluid. This is based upon a comparison of oral route acute LD₅₀ values with pesticide intake, and based upon estimated intakes of contaminated guttation fluid. The EFSA (2013b) report assumes an average of 46 trips a day for honeybees engaged in foraging for water. The amount carried on each trip in the crop of the bee ranges between 30-58 µl, amounting to a total of between 1.4 ml and 2.7 ml of water each day. Even though most of this water is not retained by the bee, on the basis that its carriage represents a possible day-long period of exposure to any contaminants present, then the concentrations of pesticides present in guttation fluid as found in this study may pose a very significant risk to honeybees, even if guttation fluid forms only a small proportion of the water foraged.

The calculation is made working on the basis that the acute oral LD₅₀ of thiamethoxam to honeybees is 0.005 µg active substance per bee, and that the acute oral LD₅₀ of clothianidin is 0.00379 µg/bee following the broad calculation methods used in the EFSA (2013b) assessment. The highest value recorded for thiamethoxam in Cruiser-treated samples from Field B was 55 260 µg/l, with an associated content of 6794 µg/l of clothianidin as a degradation product. On the basis of the thiamethoxam content alone, a bee would need to consume as little as 0.09 µl of guttation fluid in order to ingest the acute oral LD₅₀ value of active ingredient. Considering the clothianidin content alone at 6794 µg/l, an individual bee would need to consume only 0.558 µl of water to ingest the acute oral LD₅₀ value. Considering the thiamethoxam and clothianidin together, and assuming a simple additive model of toxicity in proportion to the respective LD₅₀ values (consequent thiamethoxam “equivalent” concentration of 64 223 µg/l), the ingested water volume would need to be 0.078 µl. If similar calculations are made for the highest clothianidin concentration in guttation fluid from Field A's Poncho-treated crop at 11 709 µg/l, then a bee would need to consume 0.324 µl of the fluid to ingest the acute oral LD₅₀ value.

The average concentration values over the three replicate samples, recorded 12 days after the experiment was started, were 828 µg/l of thiamethoxam in the Cruiser-treated samples (2 June 2013, 32 days after sowing) and 1050 µg/l of clothianidin in the Poncho-treated samples (29 May 2013, 34 days after sowing). These data suggest that, approximately one month after sowing, guttation fluid produced by the maize crops could still deliver a quantity of pesticide to a foraging bee equivalent to a lethal dose (acute oral LD₅₀) in as little as 6.04 µl (for thiamethoxam) and 3.61 µl (for clothianidin) respectively. Given that an individual bee is estimated to ingest a maximum of 30-58 µl fluid in a single foraging trip, then it is clear that even a single visit to a Poncho or Cruiser-treated guttating maize plant could result in an exposure well in excess of the acute oral LD₅₀.

There are a number of uncertainties attached to these calculations, and to the assumptions on which the calculations are based. Nonetheless, the required consumption of guttation fluid is, in some cases, several orders of magnitude below the volumes that bees are estimated to ingest while foraging for water. Even given the possibility that they are not exposed to such concentrations over the whole of the foraging period due to guttation fluid disappearing through evaporation etc. early in the day, these figures suggest that guttation fluid could nonetheless pose a very significant toxic risk to bees. It is not possible to estimate the risk posed by water carried into the hive and evaporated for cooling purposes. This could result in pesticide carried in the fluid being deposited over brood cells.

CONCLUSIONS



Growing maize plants in Pest county, Hungary

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The results from this study have shown that neonicotinoid insecticides applied as seed treatments to commercially available maize seed can be found in the guttation fluid of plants grown in a conventional agricultural system. The concentrations found suggest that guttation fluid could pose a serious toxic hazard to bees if used as a source of water by water foragers. The concentrations found are high enough in some samples that it is not necessary to assume that all, or even a majority, of the possible 1.4-2.7 ml of water potentially carried by an individual bee in the course of a days' foraging is derived from guttation fluid. Even a single foraging event could result in a bee ingesting into its crop doses far higher than the published acute oral LD₅₀ for both clothianidin and thiamethoxam. Even in guttation fluid being produced by plants after 12 days, the volumes that need to be ingested to deliver the acute oral LD₅₀ dose were still well below the theoretical maximum volume which could be ingested by a bee during an individual foraging event.

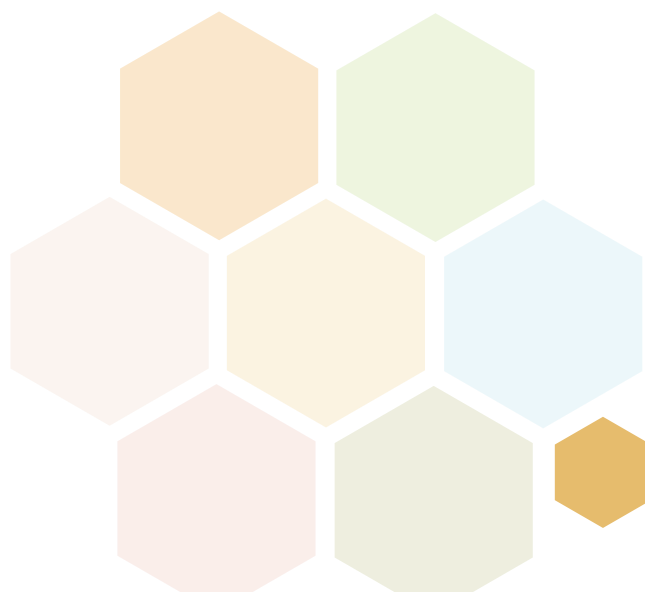
The results raise further questions that require urgent investigation in order to more thoroughly understand the significance of this route of insecticide exposure to bees, both in relation to impacts on individual foragers and on the colony as a whole. A key question that needs addressing is the degree to which bees may rely on guttation fluid and under what circumstances. Further information is required on what proportion of any pesticide in guttation fluid ingested by the bee is retained, and what proportion might be carried into and transferred into the colony, and in turn to what uses this fluid may be put. In the case of evaporative cooling, then the potential exists for any insecticide to be spread through the whole colony. In the case of diluting honey for brood feeding, then the potential risk is transferred to larval bees. Both aspects require targeted research to elucidate them fully.

Until all the outstanding questions are resolved, a precautionary approach should be fully adopted and used as a basis to inform development of agricultural policy and bee-protective farming practices. In addition, EU regulations on the use of potentially bee-harming substances should be emplaced, rigorously following the precautionary principle. These regulations should not only incorporate current scientific evidence concerning potential harm to, and overall vulnerability of, honeybees, but also extend precautionary regulation to other wild pollinators in light of their crucial role in securing pollination services now and in a highly uncertain future. In short, urgent action is required to protect the essential ecosystem service of pollination.

Recommendations

Honeybees and wild pollinators play a crucial role in agriculture and food production. However, the current industrial chemical-intensive farming model is threatening both, and putting European food at risk. This report provides additional evidence of a potential pathway through which neonicotinoids could pose significant risk to cultured honeybees and could be contributing to the overall decline and ill health of bee colonies. In consequence, policy makers should:

- 1) Make the ban on the usage of the bee-harming pesticides imidacloprid, thiamethoxam, clothianidin and fipronil permanent, and extend the ban to products that are currently authorised in the EU containing other bee-harming pesticides such as chlorpyrifos, cypermethrin and deltamethrin.
- 2) Adopt Bee Action Plans that include the monitoring of the health of bees and other pollinators. Improve the conservation of natural and semi-natural habitats around agricultural landscapes, as well as enhancing biodiversity within agricultural fields.
- 3) Increase funding for research and development on ecological farming practices that move away from reliance on chemical pest control towards biodiversity-based tools to control pests and enhance ecosystem health. EU policy makers should direct more funding for ecological agriculture solutions research under the auspices of the CAP (direct payments) and Horizon 2020 (EU research framework).



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