1.8 Lethal and sublethal effects of several formulations of azadirachtin on IPM Impact R&D colonies of the bumblebee Bombus terrestris (Hymenoptera: Apidae)

Guido Sterk [1], Julie Cuylaerts [2], Paraskevi Kolokytha[1]

[1] IPM Impact, Gierkensstraat 21, 3511 Hasselt, Belgium

[2] Thomas More Institute, Department of Agro-and Biotechnology, Geel

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Abstract

The effects of different dose rates of the most important commercially available formulations of azadirachtin and technical powder of azadirachtin were tested on *Bombus terrestris*, using a new laboratory method on full standardised IPM Impact R&D colonies, starting with a mother queen and 20 callows. The maximum field recommended concentration (MFRC) was applied in the first series of tests through topical, oral pollen and oral sugar water treatment. A sequential dilution testing scheme was used, by decreasing the dose rate each time with 1/10 of the concentration of the previous trial, if triggered, until no significant effects were recorded any more. The survival of the mother queen and initial workers, the total number of formed workers/drones at the end of the test and the number of new born gynes and queen brood were determined as the most important end points. For the evaluation of the results the data were calculated and categorized in the IOBC side-effect classes, used for laboratory trials.

This study confirms the practical experience and the previous laboratory trials that no negative toxic or sublethal effects may occur in practice with legally registered formulations of azadirachtin on *Bombus terrestris* while spraying this botanical insecticide at the recommended and authorised dose rates.

Furthermore, during this research study it was found that an illegal formulation of azadirachtin, based on a naphta petroleum which has been withdrawn several years before the study was carried out, was used in the study of Barbosa, W.F., De Meyer, L., Guedes, R.N.C. and Smagghe, G. (2015). Analysis of two samples of this applied formulation, in EU and USA laboratories, proved that only a limited amount of azadirachtin -about half of the indicated amount- was contained, while a chlorpyrifos contamination was traced in the formulation.

Keywords: azadirachtin, *Bombus terrestris*, bumble bees, Barbosa *et al.* (2014, 2015)

Introduction

Azadirachtin, an extract from the neem tree (*Azadirachta indica*) which belongs to the Meliaceae (mahogany) family, known as margosa or Indian lilac, is widely used against several pest species all over the world. It has long been recognized not only for its insecticidal and acaricidal properties, but also having a positive effect on human health. The tree itself is an attractive broad leaved evergreen. The fruits are formed in clusters and consist of a shell and 1-3 kernels which contain azadirachtin and its homologues. Trees can produce up to 2 kg of seed per year. The tree is now commercially grown in plantations for the production of the active ingredient for compounds which have toxic, antifeedant and repellent effects against insects and mites (1).

Azadirachtin, a complex tetranortri-terpenoid limonoid from the neem seeds, is the main component responsible for both antifeedant and toxic effects on insects (1).

There have been several international conferences on neem to date, the first taking place in Germany in 1980, and there is a vast amount of scientific literature which reveals both the antifeedant effects of neem and the more important physiological effects (as far as crop protection is concerned). Proceedings of the 3rd International Neem Conference in Nairobi in 1987 by Schmutterer and Ascher (2) and an important volume book entitled 'The Neem Tree' edited by Schmutterer in 1995 summarizes knowledge of the tree (3). The International Neem Conference, organized by the Neem Foundation, takes place regularly with updates on research and experiences with derivatives from the neem tree. The most recent one took place in Nagpur in India in 2012 (4).

Commercialisation

There are several manufacturer of azadirachtin on the world market, mainly from India. In Europe three companies, Certis USA, Trifolio-M GmbH) and Sipcam Oxon , formed a task force for Annex I registration for azadirachtin technical powders Registration on Annex III was achieved in a number of member states countries against a wide range of pests, such as the western flower thrips, *Frankliniella occidentalis*, in sweet pepper and ornamentals, the rosy apple aphid, *Dysaphis plantaginea*, in apple orchards, the greenhouse whitefly, *Trialeurodes vaporariorum*, in protected crops, the Colorado beetle *Leptinotarsa decemlineata*, in potatoes and the two-spotted spider mite, *Tetranychus urticae*, besides numerous other pest species. Products based on azadirachtin are also widely used in organic growing.

Side-effects on Bombus terrestris

Despite its extensive use, no negative effects on commercial hives of the large earth or buff-tailed bumblebee, *Bombus terrestris*, were ever reported, and, based on this experience and some previous laboratory trials of IPM Impact, most side-effects lists only recommend to close the hive during the spraying of azadirachtin and to open the colony again after the drying up of the residue (9, 10, 11). This approach has been used during the last decennia to the great satisfaction of the grower. However, according to Barbosa *et al.* article first published in the proceedings of the 12th International Symposium of the ICP-PR group (2014)(5) and later on in Ecotoxicology (2015)(6) several repulsive, toxic and sublethal effects, even at very low concentrations, were reported. According to Barbosa *et al.* methodology, microcolonies, without a queen, but with a worker becoming dominant and taking the role of pseudoqueen and producing only drones were used. The authors tested dose ranges of azadirachtin above and below the MFRC of 32 mg L^{-1}. A strong repellence at the highest tested dose rates was found with a median repellence concentration of 504 mg/ L^{-}1 and only survival of bumblebee workers above 50 % at a dose rate of 3.2 mg/L^{-1} or lower. Furthermore, a negative effect on bumblebee production was recorded where no male offspring was produced in microcolonies exposed to azadirachtin concentrations above 6.4 mg/ L^{-1}. Moreover, a reduction in the body weight of the male progeny treated by azadirachtin compared with the control was noticed. In the same articles was mentioned that the length of the ovaries of the dominant workers was decreased as the tested concentration of azadirachtin increased. Finally, in a separate bio assay, strongly reduced reproduction, even at the lowest tested azadirachtin dose rate, if including foraging behaviour was observed.

Discussion on the Wagner Faria Barbosa, Laurens De Meyer, Raul Narcisco, C. Guedes and Guy Smaghhe (2015) article

- The observations of the authors are in great contrast with recorded experiences, both in laboratory and practice, with different formulations and concentrations of azadirachtin.
- The formulation of the azadirachtin compound that was tested in the trials was not mentioned but the authors indicated only the commercial name of the test product: Insecticida Natural Neem from the company Bioflower in Tàrrega, Spain.
- According to the website from the Spanish Government Registro de Productos Fitosanitarios (Minsterio de Agricultura, Pesca y Alimentación)(12), the tested compound is not a legal product on the market in Spain.
- The formulation turned to be a naphta petroleum formulation (CAS 64742-94-5) (information derived after personal communication with one of the authors). No other products, with azadirachtin as active ingredient, are available on the market and are formulated with this solvent.
- Also on the label it was indicated that the product was formulated and sold under the registration number of the company Sipcam. It became immediately clear that they were unaware of this compound being sold on the Spanish market and did not give

authorisation for the use of their registration number. Steps were immediately taken by Sipcam to stop these fraudulent sales.

It may be concluded that the research done by Barbosa *et al.* was carried out with an illegal compound, based on a naphta petroleum formulation which was withdrawn from the Spanish market several years ago.

Due to the article of Barbosa *et al.* (2014, 2015) a new series of trials were designed with all different formulations of azadirachtin that are legally on the market in the EU and the USA, and in comparison with technical azadirachtin powder. This way not only the real lethal and sublethal effects of azadirachtin on bumblebee colonies' survival and development could be measured, but also the role of the formulation could be determined.

Materials and method

The test method, developed by Biobest, Belgium in the nineties and later on (7, 8) and used by Barbosa *et al*. (2014, 2015), is not considered relevant anymore for testing the effects of plant protection products on bumblebees. There's now a general consensus that the most important end point is the formation of the new born queens, as only these will hibernate and start a new colony the next spring. Therefore a new testing method was developed by IPM Impact, starting with standardised hives, with an equal number of 20 callows, all born within the same day, and queens from the same hibernating batch. All materials was delivered by Koppert NV. and harmonised by IPM Impact.

The bumblebees were fed with commercial sugar water (Attract, Koppert) and honey bee collected pollen from different sources (Koppert).

8 replicates were used for each object in this trial.

Three different application methods were tested:

1. A topical application with approximately 50 ml water solution spayed on the whole colony, mimicking the exposure of adult bumblebees during their flight to a spraying treatment. A Birchmeier hand spraying equipment with a pressure of 2 bars was used. The control hives were sprayed with tap water. Untreated pollen and sugarwater were provided after the treatment.
2. An oral sugarwater application, representative of treatment to crops that produce ample nectar. 1 litre of spiced sugar water, prepared in the same way as a spraying solution with the same concentration, was placed in each colony. Plain sugar water was used as a control treatment. This method is comparable with the method used by Barbosa *et al*. Untreated pollen was provided from day 0 onwards.
3. An oral pollen application, representative of treatment to crops that produce lots of pollen, such as tomatoes. 200 grams of pollen in the form of a ball, saturated with the test compound was given to each hive. The control hives were given tap water treated pollen. Untreated sugarwater was provided from day 0 onwards.

Rather than taking an unrealistic exposure time like in the Barbosa *et al.* trials, only untreated pollen and sugarwater were given to all objects after 4 weeks.

The bumblebee colonies were maintained in a room at 28°C and 60-70% relative humidity (RH) and continuous darkness.

All registered commercial formulations from azadirachtin were tested at comparable concentrations of active ingredient, and compared with a water treated control.

Product	Formulation	Concentration offormulated product	PPM
Neemazal T/S	010 EC	0.330 %	33.0
Azatin US(Neemix)	045 EC	0.075 %	33.8
Azatin EU	026 EC	0.140 %	36.4
Sipcam Nafta	032 EC	0.100 %	32.0

The Sipcam Petroleum formulation was especially formulated by Sipcam for this trial and is identical to the product tested by Barbosa *et al*.

Furthermore, technical azadirachtin was tested in three different solvents: water, ethanol and acetone. Each time 32 ppm was tested.

In case a high effect was found, a sequential dilution series going from 1/1 (MFRC) up to 1/1000 was triggered.

The lethal and sublethal effects were classified according to the IOBC classification for laboratory side-effects.

IOBC Class	Range % effect (mortality, reproduction)	Evaluation category
1	<30	Harmless
2	30-79	Slightly harmful
3	80-98	Moderately harmful
4	>98	Harmful

Results

Although several parameters were withheld in the assessments, only the most important ones are taken into consideration. These are the toxicity for the mother queen, the formation of new born adults (workers and drones) and the formation of gynes (new born queens). The numbers in brackets for the control are the total numbers from the 8 replicates.

1. Formulations
   a. 1/1 dose rates (MFRC)
      i. Topical application

Product	Dilution	Number of living mother queens	% reduction adults (workers and drones)	% reduction new born gynes
Control	1/1 (MFRC)	4	(1069)	(411)
Neemazal T/S	1/1 (MFRC)	6	48.1	64.5
Azatin US	1/1 (MFRC)	7	45.8	75.9
Azatin EU	1/1 (MFRC)	7	43.4	75.9
Sipcam Nafta	1/1 (MFRC)	5	52.2	81.3

ii. Oral sugar water application

Product	Dilution	Number of living mother queens	% reduction adults (workers and drones)	% reduction new born gynes
Control	1/1 (MFRC)	7	(748)	(169)
Neemazal T/S	1/1 (MFRC)	1	1.7	95.3
Azatin US	1/1 (MFRC)	5	5.1	86.4
Azatin EU	1/1 (MFRC)	0	4.4	95.3
Sipcam Nafta	1/1 (MFRC)	7	0.4	88.8

iii. Oral pollen application

Product	Dilution	Number of living mother queens	% reduction adults (workers and drones)	% reduction new born gynes
Control	1/1 (MFRC)	6	(1069)	(220)
Neemazal T/S	1/1 (MFRC)	8	7.1	35.0
Azatin US	1/1 (MFRC)	6	6.5	-5.9
Azatin EU	1/1 (MFRC)	8	8.8	-9.5
Sipcam Nafta	1/1 (MFRC)	6	4.6	44.5

Conclusion 1/1 MFRC

- There were only harmless to slightly toxic effects of the commercially available azadirachtin formulations after a topical application directly applied onto the colony. The Sipcam Nafta formulation was the only moderately toxic compound for the formation of new born gynes, but as mentioned above, this formulation was only prepared for this trial. The next dilution series of 1/10 is not triggered.
- The sugar water treatment had severe effects on the survival of the mother queen for two formulations and for the formation of the new born queens for all azadirachtin formulations. The next series of dilutions is triggered.
- No effects at all were observed with the pollen treatment of all azadirachtin formulations. The next dilution series is not triggered.

b. 1/10 dilution sugar water application

Product	Dilution	Number of living mother queens	% reduction adults (workers and drones)	% reduction new born gynes
Control	1/10	8	(1238)	(257)
Neemazal T/S	1/10	8	25.4	96.5
Azatin US(Neemix)	1/10	8	-9.3	90.3
Azatin EU	1/10	7	26.2	97.3
Sipcam Nafta	1/10	8	43.1	95.7

Conclusions 1/10 dilution

At 1/10th of the MFRC, no toxicity was observed on the mother queens any more. Furthermore, there was no or only a limited reduction in the formation of adults (workers and drones). There was still a high reduction in the number of new born queens, so the 1/100 dilution series was triggered.

c. 1/100 dilution sugar water application

Product	Dilution	Number of living mother queens	% reduction adults (workers and drones)	% reduction new born gynes
Control	1/100	8	(1584)	(315)
Neemazal T/S	1/100	7	-30.0	45.7
Azatin US	1/100	8	13.0	24.7
Azatin EU	1/100	7	5.0	31.0
Sipcam Nafta	1/100	8	-12.0	5.1

Conclusion 1/100 dilution

At 1/100th of the MFRC, no or only limited effects were observed on the survival of the mother queen and the formation of adults. Also there was no or only slight reduction in the number of new born queens.

Conclusion on the trials with formulated azadirachtin

At 1/100th of the MFRC in spiced sugar water no important effects were observed anymore on the colonies. Considering that the bumblebees were exposed to extreme laboratory trial conditions, and that this concentration will hardly be found after a spraying in practice, it may be concluded that all tested formulations of azadirachtin can be used without any problems, both in the commercial use of bumblebees and in an ecotoxicological point of view.

2. Technical azadirachtin

This trial was accomplished in two steps: first using a solution of water and technical azadirachtin, secondly with solutions of azadirachtin in ethanol and acetone.

a. 1/1 (MFRC)

Product	**Dilution**	**Number of living mother queens**	**% reduction adults (workers and drones)**	**% reduction new born gynes**
Control 1	1/1 water	7	(748)	(169)
Water solution	1/1	7	11.8	36.7
Control 2	1/1 ethanol and acetone	8	(1584)	(315)
Ethanol solution	1/1	8	38.8	78.3
Acetone solution	1/1	7	36.9	84.5

Conclusion 1/1 MFRC

Technical azadirachtin was appeared to be undiluted in water, therefore no transport from the sugar water compartment to the colony and hence no toxicity was observed. In the second series of trials, azadirachtin was found to be very soluble in both ethanol and acetone. There was no abnormal mortality observed on the mother queens. Moreover, a limited reduction in the number of adults, as well as a significant reduction in the number of new born queens were recorded for both solvents. Dilution series of 1/10th and 1/100th were, therefore, triggered.

Product	**Dilution**	**Number of living mother queens**	**% reduction adults (workers and drones)**	**% reduction new born gynes**
Control		7	(1752)	(229)
Ethanol	1/10	8	31.1	31.0
Ethanol	1/100	8	11.6	-4.4
Acetone	1/10	8	35.6	70.7
Acetone	1/100	8	12.4	19.2

Conclusion 1/10 and 1/100 dilutions

There was no effect of azadirachtin in all solvents and at all dilutions on the survival of the mother queen or on the formation of adults. There was a reduction on the numbers of adults and gynes comparing to untreated colonies for both solvents at the 1/10th concentration, but limited effects at the 1/100th dilution.

3. General conclusion

The observations of the survival of the mother queen, the formation of adults and gynes were comparable for both the formulated products and the technical azadirachtin. There was no or very limited effect from the formulations, not even from the naphta petroleum formulation, which might be expected to be repulsive.

4. Analysis of the Bioflower Insecticida Natural Neem

As this study has proven so far, both technical and formulated azadirachtin have only limited effects on the mother queen and the gynes, even at very high dose rates and if applied through sugar water, which mimics the concentration of azadirachtin in nectar after a treatment. The concentrations where an effect was recorded, being the full and the 1/10th dose rate, are unrealistic in practice. So the question remains of where the high toxicity and numerous sublethal effects that the authors of the Barbosa *et al.* article described were coming from. Therefore two samples of the Insecticida Natural Neem were taken from different sources and were sent for analysis to one European and one American laboratory.

The results from the analysis showed that in both samples the amount of azadirachtin was much lower than indicated on the label. The measured concentration of azadirachtin was approximately 1.8% while the label claimed 3.2%. Furthermore both laboratories recorded a contamination with the very toxic organophosphate chlorpyrifos.

5. Final discussion on the Barbosa, De Meyer, Guedes and Smagghe article, Ecotoxiclogy 2015

The article of Barbosa *et al.* may be severely criticised on a scientific level:

- The authors did not start the trials with harmonised and standardised hives with bumblebee workers from the same age, as described by Sterk *et al.* (1995) (7) and Merck (2002) (8), but at random collected workers from commercial hives. These workers have different ages, origins, backgrounds, possible diseases and might even belong to different subspecies.
- The formulation of the compound used in the trials was not mentioned. This might be due to the fact that it turned out to be a naphta petroleum formulation, which might have given the impression that it itself influenced the results, rather than the azadirachtin, and therefore was voluntarily left out of the article.
- The exposure time of 11 weeks or more is extremely long. Such an artificial situation will never occur in practice.
- The content or possible contamination of the test sample was never been checked.
- The authors did not check if the compound was a legal one and representative for all formulations of azadirachtin.
- Recorded data on the safe use of azadirachtin together with bumblebees over decennia were not taken into consideration.

Definitely Barbosa *et al.* made extremely frivolous mistakes on the design, methodology and conclusions of their research. However, the consequences of the publication of the Barbosa *et al.* (2015) article were severe:

- On June the 17th 2015, the Times published an article on it, claiming that organic farms are using pesticides lethal to bees (13)
- On 11th of June 2015 on the website of the European Commission was mentioned that bumblebee survival and reproduction was impaired by the pesticide azadirachtin, even at recommended concentration (14)
- Several recent scientific articles on side-effects on pollinators are referring to Barbosa *et al.* (2015).

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Foto
Pieter A. Oomen (Bumble bee *Bombus lapidarius* on thistle)

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