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Ministry of Food, Agriculture and Fisheries
Danish Institute of Agricultural Sciences

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SUMMARY

In late March 1998, 30 honeybee colonies (*Apis mellifera*) in four apiaries were treated for the parasitic mite (*Varroa jacobsoni*) with either spraying or trickling of oxalic acid. Four colonies were not treated and served as controls. Prior to the treatment, eight days after the treatment, and at the first honey harvest in June one food sample was taken in each colony. Of these samples five from sprayed, five from trickled, and the four from control colonies were chosen and the oxalic acid residue level was determined by means of liquid chromatography. The results showed that the maximum residue level was found eight days after treatment in the sprayed group ($\bar{x}$=0.0062%) but also that there was no significant difference in oxalic acid concentration between the groups at any of the sampling dates.

In another apiary, the glutathione *S*-transferase (GST) activity was measured in individual pupae and adult bees from trickled and control colonies. The result showed that 15 days after treatment the GST activity in pupae and adult bees from the trickled colonies was not different from the GST activity found in non-treated colonies indicating that trickling treatment of colonies with oxalic acid does not seem to have an effect on the level of GST activity in pupae or newly emerged adult bees.

The varroa mortality was recorded after the spring treatments with oxalic acid trickling and spraying and again in the autumn after an oxalic acid trickling treatments. Furthermore, the bee colony strength and brood amount were recorded prior to the spring treatment and again a year after the treatments. A significant difference in varroa mortality was seen after the spring treatment between the treated colonies and the controls. In the trickling group the total mite drop-down per colony was in average 61.53, in the sprayed group it was 145.47 and in the control group 1.50. After the autumn treatment, no significant difference was found between the three groups and the mite drop-down ranged between 936 and 1,400 mites. In 1998, the mean bee colony strength was approximately 5.5 comb gates before the treatment. At the same time the mean brood amount ranged from 1.77 to 3.25 dm^2. During the 1998 season, no difference in colony development was observed among the three trial groups. One year after the treatments the mean colony strength ranged from 4.93 to 6.25 comb gates. The brood amount ranged from 0.89 to 1.53 dm^2. There was no significant difference between the treated groups and the control group at any time.

INTRODUCTION

Several studies have shown that formic (Fries 1991) and lactic acid (Koeniger *et al.* 1983, Klepsch *et al.* 1984, Kraus 1991, Brødsgaard *et al.* 1997) are effective in controlling infestation with the parasitic mite *Varroa jacobsoni* Oudemans on honeybees (*Apis mellifera*, L.). However, in laboratory tests oxalic acid was even more poisonous than the two former organic acids to varroa mites (Fuchs, pers. comm.). In field tests Radezki (1994) found very high mite mortality (97,3%) when spraying 3% oxalic acid on adult mite infested bees.

Though oxalic acid is found naturally in very low concentrations in e.g. spinach and rhubarb (Fassett 1973) it may be harmful to humans even in low concentrations. Oral ingestion of oxalic acid may be deadly, by dermal contact it may cause damage to the skin and tissue and by inhalation it may cause damage to the mucous membranes. Therefore, it is necessary that precautions are taken while spraying oxalic acid (appropriate use of mask, gloves, and glasses) (Radezki 1994).

To avoid oxalic acid in aerosol form, the application of oxalic acid has been further developed in Italy by Nanetti and Stradi (1997). They found that one application of oxalic acid by trickling an oxalic acid sugar solution onto the frames in the colonies provided efficacies ranging from 89,6% to 96,8% depending upon the oxalic acid concentration.

However, besides being harmful to the varroa mites, physiological effects on the bees in the colonies treated with oxalic acid by the trickling method has been suggested (W. Ritter, pers. comm., A. Imdorf, pers. comm.). The hypothesis is that the bees ingest some of the oxalic acid-sugar solution during the treatment leading to damage on tissues of the digestive system. If tissues with glutathione S-transferase (GST) activity are damaged, a possible effect could be a lowering of the level of GST activity. It has been shown that several different tissues, including gut tissues have GST activity in honeybees and other insects (e.g. Clark 1989, El-Ghareeb & Omar 1994). The GST enzymes are an important group of enzymes for eliminating harmful substances that the bees come into contact with (Smirle & Winston 1988, Yu *et al.* 1984). A lowering of the level of GST activity could make the bees more vulnerable to toxic substances in the environment. Therefore a treatment of bees that results in a lowering of the level of GST activity may influence their overall fitness.

This study includes preliminary results on the use of GST activity as a biological marker for possible physiological effects on bees from oxalic acid treated colonies.

Furthermore, residue levels in honey have only been studied after oxalic acid treatment in autumn and early winter (Radezki 1994, Nanetti & Stradi 1997) and in Denmark the need for an efficient spring treatment is profound. Therefore, a further aim of this study has been to examine the residue levels of oxalic acid after treatment of bee colonies in the spring time with either spraying or trickling of oxalic acid. To get an impression of differences in the treatments the varroa mortality was monitored shortly after the spring treatment and again in the autumn after a second single treatment with oxalic acid trickling.

MATERIALS & METHODS

Oxalic acid treatment

In spring 1998 (23 March), totally 35 honeybee colonies in five apiaries in Denmark were treated with oxalic acid. The colonies were naturally infested with varroa mites. In both the first and the second apiary four bee colonies were sprayed, four were trickled and one untreated colony served as control. In the third apiary three bee colonies were sprayed, four were trickled, and one untreated colony served as control. In the fourth apiary four bee colonies were sprayed, three were trickled, and one untreated colony served as control. The treated colonies were selected randomly within the apiaries. The colonies in the last apiary were used for assaying GST activity in the bees (see below). In this apiary five randomly selected bee colonies were trickled and five untreated colony served as controls.

For spray treatment an oxalic acid solution was made of 30 g oxalic acid crystals (oxalic acid dihydrate) dissolved in 1 l demineralised water (Radetzki 1994). 3-4 ml per comb side were sprayed (Imdorf *et al.* 1996) with a hand operated atomiser (Ginge®, 0.5 l, no. 20-05-00, adjusted to the finest atomisation).

Trickling with oxalic acid sugar solution using 1 part (weight) oxalic acid dihydrate, 10 part demineralised water, 10 parts sucrose (Nanetti & Stradi 1997). 3 ml were trickled with a syringe per fully occupied comb gate. E.g. totally, 30 ml were used

for a weak bee colony, 40 ml for a medium strong bee colony and 50 ml for a strong colony (Imdorf *et al.* 1998).

Climatic measurements

The outdoor temperature was above 5°C and the oxalic acid solutions were approximately 10°C before the treatment. Outdoor temperature and relative air humidity (RH) were recorded in the apiaries at the time of the treatment by Tiny Talk® data loggers at 17 mins interval and continued for three weeks.

Varroa mortality

In the colonies in the apiaries 1-4 the varroa mortality was recorded in specially designed wooden inserts covering the entire bottom board every second day for four weeks after treatments. The inserts were emptied each sampling date. Furthermore, the varroa mortality was calculated in all bee colonies after one trickling treatment with oxalic acid in the autumn 1998. At the time of the autumn treatment no brood was present in the colonies. The results were analysed statistically by means of Kruskal-Wallis Multiple Comparisons (K-W) (Siegel and Castellan, 1988).

Bee colony strength

In the apiaries 1-4 the amount of brood was estimated in dm^2 by visually dividing the capped brood into squares. The bee colony strength was estimated as the number of comb gates occupied by the bees when looking into the colony without removing the combs. The estimations were carried out before the treatment and again one year after the treatment. The results were analysed statistically by means of Kruskal-Wallis Multiple Comparisons (K-W) (Siegel and Castellan, 1988).

Oxalic acid residues

In the apiaries 1-4 one food sample was taken in each of the colonies before treatment. Eight days after the treatment one sample of unsealed food or honey were taken in each of the colonies. Furthermore, one sample of the first honey harvest (early June) were taken in each of the colonies. The samples were stored in darkness at -20°C until processing. In the apiaries 1-4, five sprayed, five trickled, and four control colonies were randomly chosen and analysed for oxalic acid residues by liquid chromatography at Instituto Zooprofilattico Sperimentale delle Venezie, Legnaro, Italy. The results were analysed statistically by means of Kruskal-Wallis Multiple Comparisons (K-W) (Siegel and Castellan, 1988).

Glutathione *S*-transferase (GST) activity

Honeybees

In the fifth apiary, pupae and newly emerged adult worker honeybees were collected from the colonies. Pupae were collected immediately before oxalic acid trickling treatment (as mentioned above) in five colonies and again 15 days after the treatment. Newly emerged adult bees were collected 15 days after the oxalic acid treatment. As control, individuals were collected simultaneously from five non-treated colonies at the same locality. After collection the individual pupae and adults were stored at -80°C until assayed.

Chemicals

Reduced glutathione (GSH) and 1-chloro-2,4-dinitrobenzene (CDNB) were purchased from Merck (Darmstadt, Germany), 1-phenyl-2-thiourea (PTU) was purchased from Aldrich (Milwaukee, WI) and ethylenediaminetetraacetic acid (EDTA) was purchased from Sigma Chemical Co. (St. Louis, MO). The Bradford dye reagent and bovine serum albumin (fraction V) were purchased from Pierce (Rockford, IL). All other chemicals were of analytical quality and purchased from commercial suppliers.

Homogenisation of pupae or adult bees for **in vitro** *analysis*

Whole pupae or whole adult bees were homogenised individually in 0.1 M sodium phosphate buffer (pH 6.5) containing 10 mM GSH, 1 mM PTU and 1 mM EDTA. The individuals were homogenised manually in microtubes held on ice. The homogenate was centrifuged at 10,000 g for 20 min at 4°C and the supernatant was used in the assays.

GST and protein assays

All measurements in the GST and protein assays were made at an ambient temperature (20-25°C) and absorbance was recorded using a Multiskan MCC/340 microplate reader (Labsystems, Finland).

GST activity was measured with CDNB as substrate as described by Habig *et al.* (1974) and modified for a microplate. Assays were done on homogenate diluted with homogenisation buffer to an appropriate concentration. Diluted homogenate (20 μl) was added per well in a microplate and 180 μl of GSH (10 mM final concentration) and CDNB (1.0 mM final concentration) in 0.1 M sodium phosphate buffer (pH 6.5) was added to each well. The microplate was left 5 min to equilibrate and then

absorbance at 340 nm was recorded continuously. Reaction rates were calculated by linear regression analysis on the linear portion of the curve after plotting absorbance against time. Duplicate determinations were made on each individual.

Protein content in homogenates was determined by the method of Bradford (1976), as modified in the Pierce standard assay for microplates, with bovine serum albumin as a standard.

The GST activity was measured in individuals from non-treated colonies and in individuals from colonies trickled with oxalic acid. Eight individuals were assayed from each colony. GST activity was assayed 15 days after oxalic acid trickling treatment of the colonies. Concerning the pupae, GST activity was also measured in individuals collected immediately before treatment.

RESULTS

Climatic measurements

On the day of the oxalic acid treatments the outdoor day temperature was 17.0 °C. In the following four weeks the temperature ranged between -0.6 °C at night and 26.3 °C in daytime with an average of 7.0 °C. On the day of the treatments the relative humidity (RH) was 28.6 %. In the following 4 weeks the RH ranged between 21.9% and 100.0% with an average of 86.2%.

Varroa mortality

After the spring treatment, the mean varroa mortality increased in the treated groups reaching a maximum daily mite drop-down (±S.E.M.) of 24.0±5.52 per colony in the trickled group and of 64.93±30.51 in the sprayed group 4 days after treatment. At that time the control group had a mean drop-down of 0.25±0.25 varroa mites. Hereafter, the daily drop-down in the treated groups decreased continuously to 0.47±0.24 in the trickled group and 0.27±0.21 in the sprayed nearly reaching the control level of 0.00, four weeks after the treatment (FIGURE 1). The total mean varroa drop-down (±S.E.M.) in the trickled group in the spring sampling period was 61.53±15.42 mites, in the sprayed group it was 145.47±66.68 and in the control group 1.50 ±0.29. The result of the control differed significantly from the treated colonies (K-W, $p<0.05$).

After the autumn trickling treatment, the group treated with trickling in spring had a mean varroa drop-down (±S.E.M.) of 1,250.00±333.33. In the sprayed group the mean drop-down (±S.E.M.) was 935.80±391.53. The group serving as control in spring had a mean varroa drop-down (±S.E.M.) of 1,400.00±377.49 (FIGURE 2). The differences in varroa mortality were not significant (K-W, $p>0.05$).

Bee colony strength

The mean bee colonies strength (±S.E.M.) was 5.5±0.96 comb gates in the control group, 5.4±0.30 comb gates in the sprayed and 5.53±0.45 comb gates in the trickled group before the treatment. There was no significant difference between the results (K-W, $p>0.05$). The mean brood amount (±S.E.M.) in the above mentioned groups was 3.25±0.75 dm^2, 2.07±0.30 dm^2 and 1.77±0.51 dm^2, respectively. The results did not differ significantly (K-W, $p>0.05$). During the 1998 season, the oxalic acid treated colonies seemed to develop as well as the control colonies. One year after the treatment the mean colony strength (±S.E.M.) was 6.25±0.48 comb gates in the control group, 4.93±0.33 in the sprayed and 5.00±0.33 in the group trickled in spring 1998. The brood amount in the respective groups at the same time was 1.50±0.61 dm^2, 1.53±0.67 dm^2 and 0.89±0.24 dm^2. There was no significant difference between the treated groups and the control (K-W, $p>0.05$).

Oxalic acid residues

Just before the spring treatments the mean natural concentration of oxalic acid (±S.E.M.) was measured to be between 19.56±0.83 ppm and 35.85±5.96 ppm in the three experimental groups (FIGURE 3). Eight days after the treatment the oxalic acid concentration was increased in the treated groups compared to before treatment but only the increase in the sprayed group to 62.84±15.88 was significant (K-W, $p<0.05$). At the first honey harvest in June the mean natural concentration of oxalic acid (±S.E.M.) was measured to be 37.78±5.55 ppm in the sprayed group, 41.56±8.54 in the trickled group and 57.70±7.95 ppm in the control group. There was no significant difference in oxalic acid concentration between the groups at any of the sampling dates (K-W, $p>0.05$).

Glutathione *S*-transferase activity

The results for the GST activity in pupae are summarised in TABLE 1 and the results for the newly emerged adults are summarised in TABLE 2.

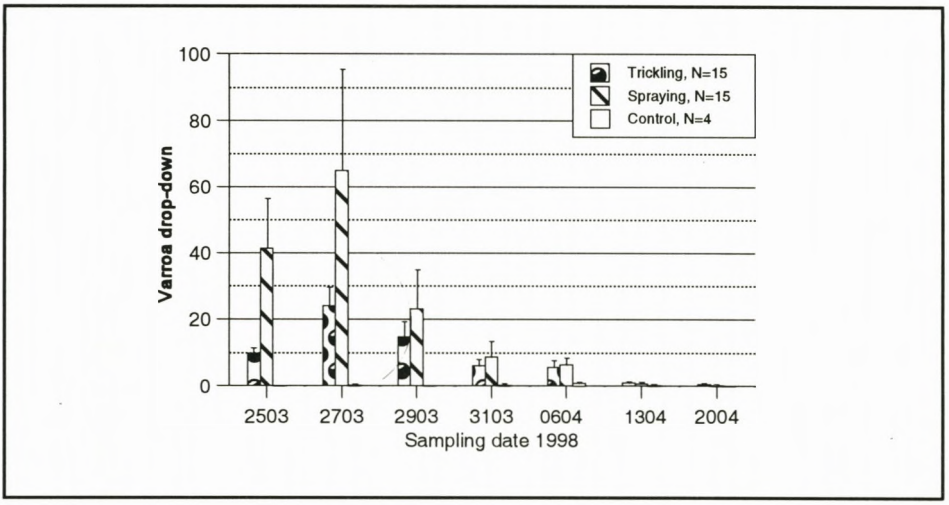

FIGURE 1. Varroa drop-down on wooden inserts after trickling or spraying treatment with oxalic acid. The treatment was carried out 23 March 1998.

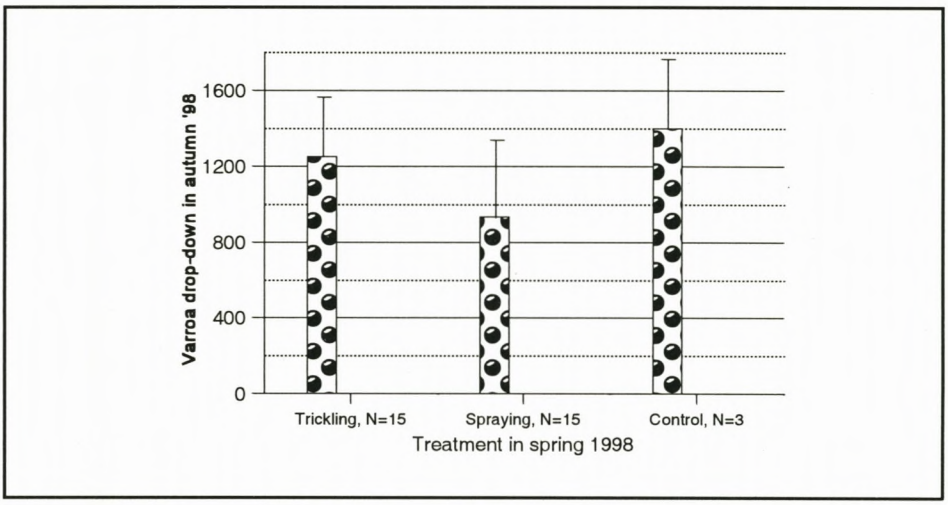

FIGURE 2. Varroa drop-down on wooden inserts after trickling treatment with oxalic acid. The colonies were treated in spring '98 with trickling, spraying or no treatment, respectively. The autumn treatment was carried out in September.

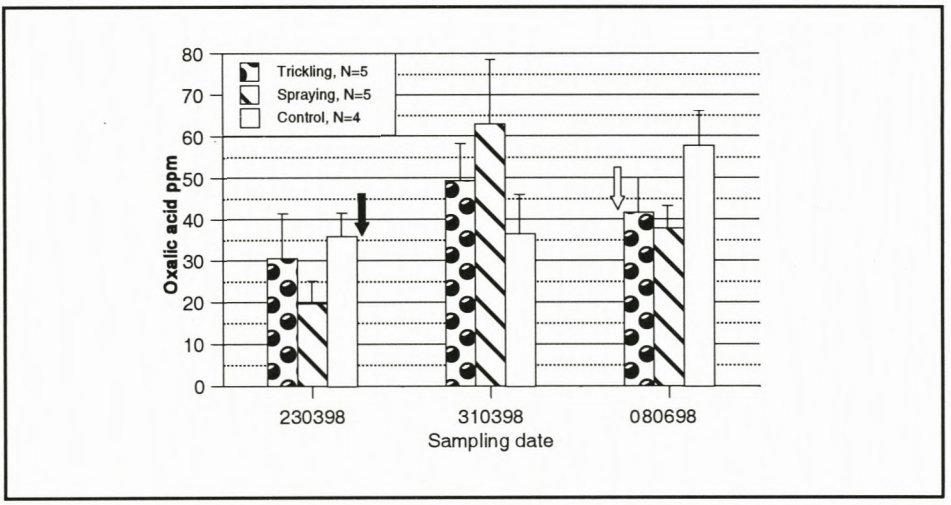

FIGURE 3. Concentration of oxalic acid residues in honey after spring treatment of bee colonies with oxalic acid by trickling. Black arrow indicates treatment, white arrow indicates honey harvest.

The data from the GST assays on the pupae were analyzed by one way analysis of variance with multiple comparison (Tukey's test, Zar 1996). The only groups that were significantly different ($P<0.05$) were pupae from non-treated colonies collected before treatment (184.4±5.8 nmol min^{-1} mg $protein^{-1}$) and pupae from colonies treated with oxalic acid (207.0±4.2 nmol min^{-1} mg $protein^{-1}$). Thus, 15 days after treatment the GST activity in pupae from the treated colonies was not significantly different from the GST activity in pupae from the same colonies before treatment and not significantly different from the GST activity in pupae from non-treated colonies collected at the same time.

The data from the GST assays on the newly emerged adult bees was analysed by *t* test (Zar 1996) and the test showed that the two groups were not significantly different ($P=0.14$). Thus, 15 days after treatment the GST activity in the adults from the oxalic acid trickled colonies were not different from the GST activity found in adults from non-treated colonies.

TABLE 1. Glutathione S-transferase (GST) activity in honeybee pupae collected before treatment and 15 days after trickling treatment with oxalic acid (OS).

Collection of pupae for GST assays	Pupae	n	GST activity[a] (nmol min^{-1} mg $protein^{-1}$)
Collection before treatment			
	Pupae from five non-treated colonies	40	184.4±5.8
	Pupae from five colonies *to be* trickled with OS after collection	40	194.1±5.5
Collection 15 days after treatment of the colonies			
	Pupae from five non-treated colonies	40	191.5±4.8
	Pupae from five colonies trickled with OS	40	207.0±4.2

[a] Mean activity ± SEM.

TABLE 2. Glutathione S-transferase (GST) activity in newly emerged adult honeybees collected 15 days after trickling treatment with oxalic acid (OS).

Adult bees	n	GST activity[a] (nmol min^{-1} mg $protein^{-1}$)
Adults from four non-treated colonies	32	211.5±6.4
Adults from four colonies treated with OS trickling	32	226.2±7.4

[a] Mean activity ± SEM.

DISCUSSION

At the time of the oxalic acid treatments the bee colonies' strength and brood amount were average for Danish conditions. Neither eight days after the March treatment, nor at the first honey harvest in June a significant difference could be detected in oxalic acid concentration in food or honey between the treated groups and the control group. Eight days after the treatment the maximum level of oxalic acid was found in the sprayed group with a mean concentration of 0.0062%. For comparison, the natural concentration of oxalic acid (oxalates) based on fresh weight in spinach is 0.3-1.2%, in rhubarb 0.2-1.3%, in tea 0.3-2.0% and in cocoa 0.5-0.9% (Fassett 1973). Since oxalic acid is not fat-soluble no residues will build up in the wax in the treated colonies (Imdorf *et al.* 1998). Thus, residues in honey and wax after spring treatment with oxalic acid seems not to be problematic.

If a difference in GST activity from treated *vs.* non-treated colonies was found it could be an indication of a physiological effect on individuals in the treated colonies. In this study trickling treatment of colonies with oxalic acid does not seem to have a prolonged negative effect on the level of GST activity in pupae or newly emerged adults as no difference in GST activity in treated *vs.* non-treated colonies could be demonstrated. However, a lack of effect on the level of GST activity does not rule out that individuals in oxalic acid treated colonies were physiologically affected by the treatment.

During the season 1998, the treated colonies seemed to develop normally compared to the control colonies. Other Danish trials using the same methods in springtime confirm this finding (Hansen 1999). Only one of the treated colonies in the present study did not survive the winter 98/99. In spring 1999, the colonies in the present study had strength ranging from 4.93 to 6.25 comb gates and a brood amount of 0.89 to 1.53 dm^2 which corresponds with the average in bee colonies at that time the country.

The results of this study do not give a direct measure of the efficacy of the two treatments. Oxalic acid spraying does not have an effect on varroa mites in the sealed brood (Radetzki 1994). Also trickling is only recommended in broodless colonies as experiments have suggested that the efficacy of one treatment in colonies with brood was too poor and several treatments weakened the colonies (Imdorf *et al.* 1998). Because of the brood present in our colonies, we assume that the efficacy of

these spring treatment is lower than the approximately 98% found by Radezki (1994) and Imdorf *et al.* (1998) for spraying and trickling, respectively. In spring in Denmark, it is not possible to cut out the brood to increase the efficacy as it is done in the autumn by lactic acid treatment (Brødsgaard *et al.* 1997). Removing the brood in spring would most likely weaken the colonies. A proper efficacy test should of course be followed by a total count of mites in the colonies or a treatment with a pesticide with a well-documented effect. But since there are no pesticides registered for use in Denmark the honey, wax and equipment from the treated colonies would have to be destroyed and this was not possible in this preliminary study. Nevertheless, assuming that the varroa infestation was evenly distributed between the colonies there seems to be a tendency that the spraying treatment was more efficient than the trickling based on the observed varroa drop-down although the difference was not significant (FIGURE 1). The lack of difference in efficacy between trickling and spraying treatment corresponds with the findings of Imdorf *et al.* (1998) who found no differences when treating broodless colonies in autumn with the two methods. The varroa drop-down after the trickling treatment in the autumn showed that the varroa population in the colonies treated in spring seemed to have developed as well as in the control. That result could be explained by a poor efficacy of the spring treatment, reinvasion from the control colonies to the treated colonies combined with a very short persistence of oxalic acid or the very few control colonies.

Neither the residues of oxalic acid in honey, the GST activity, nor the colony development after spring treatment with either trickling or spraying with oxalic acid seem to indicate any problems. However, before the use of oxalic acid as spring treatments is recommended in Denmark it is necessary to put more effort into efficacy tests with a large number of control colonies in field trials.

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