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VOLUME 3 – Annex B

- *Beauveria bassiana* PPRI 5339 -

B.9 Ecotoxicology

Rapporteur Member State: The Netherlands

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B.9 Effects on non-target organisms

Introduction

PPRI 5339 is a naturally occurring, non-modified strain of *Beauveria bassiana*, originally isolated from the larva of a tortoise beetle, *Conchyloctenia punctata* (Coleoptera: Cassidinae) collected in South Africa. Further information on origin can be can be found in chapter B.2.1.1.2.

Beauveria bassiana is an opportunistic entomopathogenic fungus. *Beauveria bassiana* attacks its insect hosts percutaneously; the conidia of *Beauveria bassiana* adhere to the insect cuticle by means of hydrophobic interaction between the spore wall and epicuticle lipids. Germination of the conidia and the subsequent successful infection depend on a number of factors, e.g. susceptibility of the host and host stage, and certain environmental factors, such as optimal temperature and humidity. Before penetration, germ tubes may form so-called appressoria and infection pegs. The penetration process is by mechanical means and by the production of enzymes, including proteases, chitinases and lipases, which degrade the insect cuticle. Penetration is followed by invasion, which is accompanied by several host immune response activities. Further information on mode of action can be found in chapter B.2.1.2.2.

The conidia of *Beauveria bassiana* adhere to the insect cuticle, germinate and penetrate in the insect body, where they replicate as yeast-like cells (blastospores) and destroy the internal structures, causing morbidity within 36 - 72 hours. After death of the insect, the fungus starts its saprophytic growth: blastospores transform into mycelia, which emerge through the cuticle. Aerial conidia are formed on the surface of the insect cadaver, which build the characteristic white growth. *Beauveria bassiana* strain PPRI 5539 requires contact with viable host in order to proliferate.

Beauveria bassiana strain PPRI 5339 has no known sexual stage.

It is unclear if secondary metabolites are part of the mode of action or not and it appears the production of secondary molecules such as beauvericin maybe variable between strains. Additionally it is thought by some researchers that beauvericin may be produced against competitive saprophytes rather than to overcome the host immune defences. The available body of scientific literature indicates that secondary metabolites are only produced post germination of conidia on the cuticle of a suitable host. This means that secondary metabolites are only present in the cadavers of host insects. Skrobek *et al*[1]., 2008 found that destruxins (a cyclohexadepsipeptide related to the enniatins and beauvericin) were decomposed in host insects before they, with the cadaver, were released to environments, so it is unlikely to contaminate the food chains. Destruxin decomposition was presumably due to the activity of hydrolytic enzymes in the cadavers and appeared to be independent of host or soil type and biota. This may also be the fate for beauvercin in insect cadavers.

Beauveria bassiana does not multiply and is metabolically inactive in the absence of host insects, and as a consequence no presence of potentially harmful metabolites outside the infected insect to levels that might affect the environment is expected. Metabolic inactivity continues as long as the environmental conditions remain unchanged.

[1] *Skrobek A, Farooq A. Shah, Tariq M. Butt: Destruxin production by the entomogenous fungus Metharizium anisopliae in insects and factors influencing their degradation, BioControl (2008), Vol. 53 (2). Pp 361-373*

More information on the metabolites produced can be found in Vol. B1-B5, section B.2.1.8.

BAS 480 00 I is an OD formulation containing 4.0 × 10^{12} CFU/L, equivalent to 40 g *Beauveria bassiana* strain PPRI 5339/L.

The BAS 480 00 I formulation is intended for glasshouse application only, details of the proposed use patterns that will be assessed are summarised in Table 10-1.

Table 10-1 GAP of BAS 480 00 I

Crop	Application method	Maximum individual application rate (g as/ha)	Number of applications	Maximum total application rate per season (g as/ha)	Minimum application interval (days)	Application timing
Tomato Sweet pepper Aubergine Edible and processed fruits Cucumber	Spraying (SP)	100 (i.e. 1x 10^{13} CFU/ha)	122	12200 (i.e. 1.22 x 10^{15} CFU/ha)	3	BBCH 11 – 89 (January to December)
Ornamentals Non-edible / Non-food crop	Spraying (SP)	100 (i.e. 1x 10^{13} CFU/ha)	122	12200 (i.e. 1.22 x 10^{15} CFU/ha)	3	BBCH 11 – 99 (January to December)

The impact of formulants is limited to short-term effects such as formation of stable spray dispersions or to facilitate uptake by target organisms, while their influence on long-term processes, such as degradation and distribution of the active substances in the environment is considered negligible. Therefore, for the purposes of this risk assessment it is assumed that the formulants do not influence the ecotoxicological properties of the active substances in the environment and they are not considered further. Therefore, the information provided on the microbial pest control agent *Beauveria bassiana* PPRI 5339 is directly applicable to the reference product and as such no studies are submitted for the product BAS 480 00 I. Also, PPRI 5339 is 98% genetically similar to GHA + ATCC 74040, therefore all information from the peer-reviewed literature can be used for BAS 480 00 I (please refer to section B.5.1.1 in Vol. B1-B5).

B.9.1 Effects on birds (Annex IIM 8.1; Annex IIIB 10.1)

No specific studies on *Beauveria bassiana* strain PPRI 5339 have been conducted on birds. Data for other strains are presented in table 8.1-1.

Table 8.1-1: Summary of avian toxicity data

Species	Test type	MCPA	Endpoint	Reference/Source
American kestrel (*Falco sparvensis)*	Dietary field test	*Beauveria bassiana* strain GHA	LR_{50} > 5 × 10^6 spores of *Beauveria bassiana*/g body weight. No adverse effects on growth or body mass and behaviour	Althouse *et al.,* (1997)
Ring-necked pheasants (*Phasianus colchicus*)	Dietary test in which birds were fed with spores and infected grasshoppers		No significantly different weight gain and no detectable histopathological changes	Johnson *et al*., 2002
Chicken (*Gallus domesticus*)	5-day dietary toxicity	*Beauveria bassiana*	No adverse effects on mortality, behaviour or weight at 10^9 conidia of *Beauveria bassiana*/g food	Haas-Costa *et al.* (2010)

Reference 8.1/01

Report: C. M. Althouse, B. E. Petersen, L. C. McEwen. Effects on Young American Kestrels *(Falco sparverius)* Exposed to *Beauveria bassiana* Bioinsecticide. Bull. Environ. Contam. Toxicol. (1997) 59:507-512
Published

Guidelines: None

GLP: No

Abstract

Beauveria bassiana fungus is a pathogen that holds promise as a biological control material for a major rangeland grasshopper pest species *(Melanoplus sanguinipes)* (Marcandier and Khachatourians 1987; Moore and Erlandson 1988; Inglis et al. 1995).

Biological controls are useful for reducing high grasshopper populations in environmentally sensitive areas such as lake and stream borders, nest sites of endangered bird species, and other endangered species habitats where chemical insecticides are restricted. Grasshopper control with biological materials may be more target-pest specific and pose lower risk to other animal life inhabiting treated areas (USDA 1987). In this paper we report the results of field tests in which wild American kestrel (*Falco sparverius*) nestlings were exposed to acute oral dosages of *B. bassiana* spores. The young kestrels were observed through the post-fledging stage in Colorado during the 1992 breeding season.

Null hypotheses tested were: (1) mean growth parameters and survival would not differ between treated and untreated nestlings, (2) timed responses to systematic behavior/reaction tests would not differ between treated and untreated nestlings, and (3) mean distances moved and survival would not differ between treated and untreated fledglings. Juvenile kestrels were chosen for this study because the optimal timing (June) of application of *B. bassiana* spores to late instar grasshopper populations coincides with the kestrel nestling stage. Grasshoppers are important prey for kestrels and are a valuable food source for the young (Bent 1961).

Kestrels are suitable subjects for field toxicology experiments because they (1) are readily attracted to nest boxes, (2) tolerate handling for treatments and measurements, (3) are abundant and widely distributed, and (4) are cogeneric with the peregrine falcon (*F. peregrinus*) (Wiemeyer and Lincer 1987). Kestrels may also be bioindicators for other raptors, such as Swainson's hawks (*Buteo swainsoni*), that prey on grasshoppers.

Materials and methods

Thirteen kestrel nest boxes containing 55 young, with a minimum of 4 nestlings/box, were used in the study. Three birds (8-16d of age) from each nest were randomly selected and given a sub-lethal acute oral dosage of one of *Beauveria bassiana* formulation ($5x10^6$ spores/µl) or corn oil only. The fourth nestling in each box served to maintain a normal brood size and provided an untreated comparison to the dosed birds. Behavior and growth data were collected every 4 days until nestlings fledged. All dosages were 1 µL/g of body weight (bw). Nestling dosages were based on maximum potential ingestion of grasshoppers exposed to *B. bassiana* spores and U.S. Environmental Protection Agency protocols. Nestlings rather than adult kestrels were tested because young, growing birds may be more sensitive to food contaminants and also because their confinement to the nests reduced the environmental variables associated with studies of wild birds. Growth and survival results could also be compared with 4 years of baseline data from prior studies by the authors of untreated kestrels in the same nest boxes (unpublished data).

At each post-dosing visit we measured mass. Two behaviour tests of our own design were then performed on each nestling. In the first test, after crop status was recorded as full, partial, or empty, the ability of the kestrel to follow a food item (grasshopper) from left to right was recorded. Prior to the test, the bird's head was covered for one minute to create a darkened environment. Each nestling was given a 20-second maximum time period (by stopwatch) for orientation and to focus on and follow the food item. In the second test, each kestrel was placed on its back and timed on recovery to regain an upright position. This test was performed 3 times consecutively. If a bird did not respond within the specified test time of 60 seconds, that particular repetition was terminated. Birds that did not respond within the allotted time were omitted from statistical analyses. At 26-31 days of age, just prior to fledging, backpack transmitters (Holohil Systems, Woodlawn, Ontario, Canada) were attached to 38 of the kestrels. Post-fledging observations were obtained by ground-tracking the birds with small receivers and handheld antennas at random times, usually every other day, for 5-18 days post-fledging.

Data were collected on survival and movement of 28 of those birds (10 attachments failed). The 2 types of movement data collected were: (1) distance from the nest box of each fledgling and (2) distance from the previous location to the location on the next observation.

Seven treated birds (5 *B. bassiana* and 2 carrier oil), ages 31-42 days, were randomly collected after fledging to check for pathology. Three additional treated fledglings (2 carrier oil and 1 corn oil) were found dead, including one that had been partially eaten by a predator. Necropsies on all collected birds were performed.

Results and discussion

No differences were found among treatments in body mass or in central retrices and tarsus lengths. During the nestling stage, females were larger in mass than males at all stages of growth. Where significant differences were detected in growth or behaviour parameters, these results expressed sexual dimorphism and were not related to treatments.

Nestling recognition of a food item and ability to track movement of the item was compared by treatment and sex in relation to crop status. No significant differences were detected.

A nestling's ability to regain an upright position when placed on its back did not differ statistically between treated birds and controls over time (P = 0.067), although carrier oil dosed birds were slowest overall. Females exhibited a slower response (avg = 2.46 sec) than males (avg = 1.23 sec) to regain an upright position.

No differences were detected between treated birds and controls in either of the 2 types of distance measurements (P = 0.928 and P = 0.765, respectively).

Necropsies performed on collected kestrels in our current study detected no visible gross pathology. All test birds successfully fledged and survived in our study excepting 3 that died of natural causes.

Conclusion

Young American kestrels (*Falco sparvensis*) were fed with 5×10^6 spores of *Beauveria bassiana*/g body weight in field experiments. No adverse effects on growth, body mass or survival were apparent.

Comments by RMS: The results of this study suggest that dietary exposure of kestrel nestling at a dose of 5×10^6 spores of *Beauveria bassiana*/g bw do not result in mortality, growth, and behavioural changes. The absence of mortality had been linked to the inability of the fungus to grow at temperatures above 35^0 C (n.b. the avian body temperature is 39^0 C or above). It was further suggested that the spores cannot germinate in the gut, but remain viable and thus are still viable in the droppings. The RMS considers that the results of this peer-reviewed paper can be used in the risk assessment.

Reference 8.1/02

Report: Dan L. Johnson, Judit E Smits, Stefan T. Jaronski, David K. Weaver. Assessment of health and growth of ring-necked pheasants following consumption of infected insects or conidia of entomopathogenic fungi,

Metarhizium anisopliae var. *acridum* AND *Beauveria bassiana,* from Madagascar and North-America. Journal of Toxicology and Environmental Health, Part A, 65:2145–2162, 2002

Published

Guidelines: None

GLP: No

Abstract

Isolates of two fungi *(Beauveria bassiana* and *Metarhizium anisopliae* var*. acridum)* from Madagascar are being developed for control of grasshoppers and locusts, as part of a search for alternatives to environmentally detrimental chemical insecticides. The probable effects of these entomopathogens on nontarget species must be determined before operational use. Birds may become exposed to these fungi either directly, by consuming spores deposited on their food items, or secondarily, by consuming grasshoppers or locusts that have died from infection by these biocontrol agents. This article presents the results of per os challenge from fungus-infected food items. Male and female ring-necked pheasants *(Phasianus colchicus)* were exposed at 4 d of age and again at 9 d of age to challenge treatments, or 2 control treatments (18 male and 18 female birds per treatment group) . Pheasants were weighed at 9, 17, and 25 d of age, tarsal length was measured at 25 d of age, and they were observed daily for signs of adverse effects of the experimental treatments. At the time of euthanasia (25 d of age), 2 or 3 randomly selected birds from each of the groups exposed to infected grasshoppers, plus the 2 control groups, underwent complete necropsies and histopathological examination of 16 tissues from each bird. Results show that in both sexes, weight gain at both 17 and 25 d was not significantly different between challenge groups and control groups. Tarsal length at 25 d of age, an indication of structural growth, was also not markedly different among challenged and control groups. Histopathological changes were generally undetectable, mild, or moderate, and not consistently associated with any treatment.

Materials and methods

Avian Test Species

Uniform, day-old, ring-necked pheasant chicks were obtained from a farm and maintained and fed duckling starter supplemented with vitamins A, D and E.

Experimental Design

Each replication (i.e., complete set of the treatments) included 60 birds, with 6 birds (3 of each sex) in each of 10 treatment groups. The experiment was replicated 6 times, resulting in 18 males and 18 females per treatment or a total of 360 birds. Treatments were assigned randomly to birds and pens within each replication. Birds and cages were labeled with code numbers in random order so that pathology observations were conducted “blind” with regard to treatment identities. Each experimental group was housed in a newly constructed wooden pen (1.7 m × 1.2 m × 0.6 m high) equipped with two 74-W brooder lamps, water, feed trays,

and wood-chip bedding over black plastic sheets. The birds were confined and observed in the pens for 16 d after treatment.

Experimental Treatments

Each bird was fed two grasshoppers, infected or uninfected with *Beauveria bassiana* GHA (live, per capita body weight of fourth-instar nymphs was 0.21–0.25 g), on each of 2 d separated by 4 d without treatment (June 6 and 11). At the age the pheasants were being treated (4 and 9 d old), two grasshoppers were equivalent to approximately 2% of the body weight of the birds. Before being fed to the birds, the grasshopper nymphs were infected with one of the three fungal isolates, using methods that simulate direct and secondary contact between grasshoppers and sprayed vegetation in the field. The resulting dose was 1–1.5 × 10^5 viable conidia per insect.

Individual birds were weighed at 4, 9, 17, and 25 d of age and tarsus length was measured at 25 d of age. Chicks were observed daily for any clinical signs of physiological or pathological effects associated with treatment. Clinical signs to be recorded included unusual vocalization or blinking, change in gait or coordination, other neurological signs, respiratory distress, anorexia, postural changes, ruffled or denuding of feathers, prolonged sternal recumbency, weakness, or decreased responsiveness to investigator presence. Any evidence of gastrointestinal disorder (i.e., vomition or diarrhea), nasal or ocular discharge, cyanosis of skin or mucous membranes, skin lesions, emaciation, or mortality was recorded. Sixteen days after completion of the dietary exposure, samples of feces and blood were tested for the presence of *B. bassiana.* Two birds from each treatment group were selected for histological analysis. The following organ and tissue samples were collected within 60 min of death: brain, thymus, heart, lung, crop, proventriculus, ventriculus, duodenum, jejunum, cecum, colon, pancreas, spleen, liver, kidneys, and bursa of Fabricius.

Results

One female bird in the control group, which received uninfected grasshoppers, died. The birds readily consumed the infected insects during the period of exposure to the experimental diets, although in some cases the sporulated grasshoppers had to be placed in a bird's mouth via forceps before they would consume them. During the 16-d observation period surrounding treatment, there was no evidence of differences in behaviour among the treatment groups, nor were there any cases of birds that showed clinical evidence of infection or disease. There was no evidence that consumption of the infected grasshoppers affected weight gain of the male pheasants (repeated measures mixed model ANOVA, p = 0.065). No significant differences occurred among the mean weight gains for males for any period. Histopathological examination of the tissues revealed no consistent changes associated with any treatment. Five of the nine birds examined (also *M. anisopliae* var. *acridum* dosed) had lesions in at least three and up to seven tissues. The changes, however, were not atypical for normal young pheasants and could not consistently be ascribed to either the challenged or the control groups. No fungal colonies other than *Mucor* and *Penicillium* were obtained from plating the selective media with feces and blood from treated birds.

Conclusion

Male and female ring-necked pheasants (*Phasianus colchicus*) were dosed with conidia of *M. anisopliae* var. *acridum* and with *Beauveria bassiana* strain GHA infected grasshoppers. In both sexes, the weight gain at 17 and 25 days was not significantly different between treated and control groups. Histopathological changes were generally undetectable

Comments by RMS:

Based on the information provided in this peer-reviewed paper, the objective was to "to assess the potential for negative effects on the health of birds consuming mycoinsecticide-infected grasshoppers and locusts. The principal objectives were (1) to determine whether the presence of the entomopathogenic fungi in the food items had a measurable impact on the appetite, growth, development, general behavior, survival, or health of the birds, (2) whether these fungi could establish infections, and (3) whether and for how long the fungi could be detected in the feces and tissues of treated birds". The feeding rate was in accordance to the number of grasshopers found in the diet of grassland species. No adverse effects were seen in a study in which male and female ring-necked pheasants (*Phasianus colchicus*) were dosed with conidia of *Beauveria bassiana* strain GHA. This result is used for risk assessment.

Reference 8.1/03

Report:	Jucelaine Haas-Costa, Luis Francisco Angeli Alves and Alaxsandra Aparecida Daros. Safety of *Beauveria bassiana* (Bals.) Vuill. to *Gallus domesticus* L. Brazilian archives of biology and technology, Vol.53, n. 2: pp. 465-471, March-April 2010 Published
Guidelines:	None
GLP:	No

Abstract

Beauveria bassiana, an important insect pathogen was used to evaluate its effect on the feeding, behavior, histology of the digestory system and anatomy *of Gallus domesticus*. The fungus (strain Unioeste 4) was administrated orally treated with chicken food. Chicken feces were collected, and the birds observed for 28 days. Also, were evaluated the weight of the birds, as well as any sign of intoxication or pathological modification. Tissue samples were

withdrawn to test lesions with the optic microscope. There was 100% of survival of the birds, and no behaviour alteration or external lesion was found. The test group presented the highest weight gain (1383.9±54.4g). Viable conidia were observed in the feces until 24 h after feeding the fungus, indicating that there was no germination inside the digestory system. No tissue lesion was observed, providing overwhelming evidence for the safety of *B. bassiana* to non-target organism *G. domesticus*.

Materials and methods

Test animals were male chickens (*Gallus domesticus*), 11 days old. The animals were fed *ad*

libitum with commercial chicken food without antibiotics, to avoid interference with the fungus viability. The fungus viability was evaluated before the experiment. The birds were separated into three groups, maintained in cages of 1.0×0.80×1.0, each group was composed by 10 birds. The groups were: (1) Active fungus: birds fed with diets containing *B. bassiana* (10^9 conidia/g chicken food); (2) Inactive fungus: fed with inactivated fungus at the same concentration and (3) Control: birds fed just with the diet.

Daily, for five days, the birds were fed with diet containing fungus. The fungus was mixed to the chicken food immediately before the administration to the birds. After the treatment period, the birds were fed *ad libitum* with the commercial ration without antibiotics.

Samples of the test group feces were collected daily, at the same daytime, from the 1st to the 9th day of experiment in order to verify the presence/absence of fungus after the passage through the animals' gastrointestinal tract.

The birds were observed for 28 days, for any clinical signs of any effects associated with the treatment. These included: uncommon vocalization, change in gait or coordination, anorexia, ruffled or denuding of feathers, weakness or decreased responsiveness to investigator presence. Any evidence of gastrointestinal disorder (vomition or diarrhea) or skin lesions were verified. The birds' weight was measured before the fungus administration and on the days 0, 7, 14 and 28 days after the ingestion (DAI). All the birds were sacrificed at the end of the evaluation period (28 DAI), and three birds from each group were selected at random and necropsied in order to observe internal pathological alterations. Samples from the lungs, heart, small intestine, kidney and liver were collected.

Results and discussion

All of the birds survived during the experiment and no kind of external lesions was found.

Histological examination of the tissues (lungs, heart, small intestine, kidney and liver) revealed no consistent changes associated with any treatment, showing no kind of visible alterations. Relating to the birds behaviour, all the individuals presented the same pattern with no deviations from the normal.

Table 1 - Survival and behavior evaluation of *Gallus domesticus* submitted to feeding of *Beauveria bassiana* in 3 treatments: active fungus, inactive fungus and control, during 4 weeks (Temperature: 25±5°C; photophase: 14 hours).

Treatments	Observed response			
	Individuals per treatment (n)	Behaviour alteration	Dead individuals	Survival (%)
Control	9	Negative	0	100a
Inactive fungi	10	Negative	0	100a
Active fungi	10	Negative	0	100a

There was no statistical difference between weight of birds at day 0 and the three following weeks among the three groups. But on the last evaluation after 4 weeks birds treated with active fungus showed higher weight compared to the other two groups.

The conidia of *B. bassiana* were present and viable in *G. domesticus* feces between the 1st and the 6th day, indicating that those went through the gastrointestinal tract rapidly, staying for not more than

24 h. This fact associated with the different pH through the gastrointestinal tract and the high temperature of the body of the birds (40ºC) is believed to have prevented the conidia to germinate and cause infection or release any kind of toxins in the birds' body.

Conclusion

The data led to the conclusion that *B. bassiana* was present and viable in the birds feces until 24 h after the ingestion, indicating that it did not germinate in the birds body. In addition, there was no negative effect upon the birds gain weight. Also, there was no bird mortality due to the fungus ingestion, nor there was any sign of behaviour alteration, external or tissue lesion in the evaluated organs.

Comments by RMS:

The results of the study suggest that *B. bassiana* fed at 10^9 conidia/g food to *Gallus domesticus* does not affect the birds in the study. The results can be used for assessment.

TOXIN/METABOLITE FROM ACTIVE INGREDIENT

No information or an additional study regarding the toxicity metabolites is provided. Metabolites/toxins can be expected to be present in the host insects which can be part of the bird's diet. In the study by Johnson *et al*. pheasants were fed infected insects from which it can be assumed metabolites, if any were produced upon infection, are present.

Reference 8.1/04

Reference:	Dan L. Johnson, Judit E Smits, Stefan T. Jaronski, David K. Weaver. Assessment of health and growth of ring-necked pheasants following consumption of infected insects or conidia of entomopathogenic fungi, *Metarhizium anisopliae* var. *acridum* AND *Beauveria bassiana,* from Madagascar and North-America. Journal of Toxicology and Environmental Health, Part A, 65:2145–2162, 2002
	Published
Guideline:	none
GLP:	no
Material and methods:	
Test substance:	*Beauveria bassiana* GHA
Test species:	ring necked pheasant
Number of test animals:	18 males & 18 females per treatment
Treatments:	1–1.5 × 10^5 viable conidia per insect
Duration:	25 days
Test conditions:	Woodpen, 74-W brooder lams, feed trays, bedding
Deviations from guideline	not relevant
Endpoint:	no effects

Observations: clinical signs, weight, feces and blood for the m.o., histopathological analysis

Results:

The birds readily consumed the infected insects during the period of exposure to the experimental diets, although in some cases the sporulated grasshoppers had to be placed in a bird's mouth via forceps before they would consume them. During the 16-d observation period surrounding treatment, there was no evidence of differences in behaviour among the treatment groups, nor were there any cases of birds that showed clinical evidence of infection or disease. Tissues were examined for changes that could have resulted from mycotoxin damage or mycotic infection. There was no evidence that consumption of the infected grasshoppers affected weight gain of the male pheasants (repeated measures mixed model ANOVA, p = 0.065). No significant differences occurred among the mean weight gains for males for any period. Histopathological examination of the tissues revealed no consistent changes associated with any treatment. Five of the nine birds examined (also *M. anisopliae* var. *acridum* dosed) had lesions in at least three and up to seven tissues. The changes, however, were not atypical for normal young pheasants and could not consistently be ascribed to either the challenged or the control groups.

Conclusion:

No adverse effects were seen in a study in which male and female ring-necked pheasants (*Phasianus colchicus*) were dosed with with *Beauveria bassiana* strain GHA infected grasshoppers and observed for 25 days. Weight gain was not significantly different and histopathological changes were generally undetectable.

Comments RMS:

Though there is a lack of any adverse effects in the study, there was no specific focus at toxic effects that may be caused by the presence of metabolites in infected insects. However, based on the description in the study pheasants were fed heavily infected grasshoppers in which metabolites involved in the mode of action must have been present. No adverse effect was seen in the study. The authors suggest that the body temperature and pH will not allow the development of mycotoxins or invasion of the fungal hyphae. This result is used for risk assessment.

B.9.1.1 Infectiveness

In the studies by Johnson *et al* (2002) hist opathological observations due to the immune response to infectious agents were not present.

B.9.1.2 Pathogenicity

No external and clinical symptoms of pathogenicity have been observed.

B.9.1.3 Summary of the studies on birds on toxicity, infectiveness and pathogenicity

Table 8.1.4.a. Summary of the studies on effects on birds treated with *Beauveria bassiana*.

Species	strain tested	Test duration	Dose range	Results/ Endpoint	Observations	Reference
TOXICITY						
American kestrel (*Falco sparvensis)*	GHA	50 days dietary field test in nestlings and fledglings	5×10^6 spores/g body weight	$>5 \times 10^6$ spores/g body weight	No adverse effects on growth or body mass and behaviour	Althouse *et al.,* (1997)
Ring-necked pheasants (*Phasianus colchicus*)	GHA	25 d (2 days of feeding, 4 day interval, 16 day observation)	$1–1.5 \times 10^5$ viable conidia per insect, $4 – 6 \times 10^5$ conidia/bird/day	$>4 –- 6 \times 10^5$ conidia/bird/day	No mortality. No significantly different weight gain differences in behaviour among the treatment groups	Johnson *et al*., (2002)
Chicken (*Gallus domesticus*)	UNIOESTE 4	5-day dietary toxicity, 28 d observation	10^9 conidia /g food	$>10^9$ conidia /g food	No adverse effects on mortality, behaviour or weight	Haas-Costa *et al.* (2010)
INFECTIVENESS						
Ring-necked pheasants (*Phasianus colchicus*)	GHA	25 d (2 days of feeding)	5×10^6 spores/g body weight	$>5 \times 10^6$ spores/g body weight	no detectable histopathological changes, no clinical signs of infection	Johnson *et al*., (2002)
Chicken (*Gallus domesticus*)	UNIOESTE 4	5-day dietary toxicity, 28 d observation	10^9 conidia /g food	$>10^9$ conidia /g food	no visible histopathological changes	Haas-Costa *et al.* (2010)
PATHOGENICITY						
Ring-necked pheasants (*Phasianus*	GHA	25 d (2 days of feeding 4	5×10^6 spores/g body weight	$>5 \times 10^6$ spores/g body weight	no pathogenicity observed	Johnson *et al*., (2002)

Species	strain tested	Test duration	Dose range	Results/ Endpoint	Observations	Reference
colchicus)		day interval, 16 day observation)				
Chicken (*Gallus domesticus*)	UNIOESTE 4	5-day dietary toxicity, 28 d observation	10^9 conidia /g food	>10^9 conidia /g food	no signs of pathogenicity	Haas-Costa *et al.* (2010)

Table 8.1.4.b. Summary of the studies on effects on birds treated with toxin/metabolite

Species	Test duration	Dose range	Results/Endpoint	Observations	Reference
TOXICITY					
Ring-necked pheasants (*Phasianus colchicus*)	25 days	1–1.5 × 10^5 viable conidia per insect	no effects	mortality, weight, histopathology for mycotoxin damage or mycotic infection	Johnson *et al*., (2002)

B.9.1.4 Risk assessment for birds active micro organism

The intended use for BAS 480 00 I is for control of pests of glasshouse crops, exposure of foraging birds to BAS 480 00 I would therefore be negligible.

According to the working document to the environmental safety evaluation of microbial biocontrol agents[2] (hereafter referred to as 'the working document') for indoor greenhouse use microbial biocontrol agents (mBCAs) emissions due to spray drift from permanent structures via open windows and openings can be considered negligible.

B.9.1.5 Risk assessment for birds of toxins/metabolites

The intended use for BAS 480 00 I is for control of pests of glasshouse crops. Exposure of foraging birds to infected insect as a result of the use of BAS 480 00 I would therefore be negligible.

[2] OECD (2012): Working Document to the Environmental Safety Evaluation of Microbial Biocontrol Agents ENV/JM/MONO(2012)1. The working party on chemicals, pesticides and biotechnology. SANCO/12117/2012 – rev. 0 September 2012

As a conservative approach and assuming that 0.1% of the product would drift from the greenhouse during a spraying event, the maximum external exposure would be 1×10^{10} cfu/ha. In fact, the major route of exposure from *Beauveria bassiana* to birds would be through consumption of infected insects. The predicted maximum external exposure outside the greenhouse would not be high enough to infect sufficient insects and so it can be concluded that the oral exposure of birds to insects infested with *Beauveria bassiana* strain PPRI 5339 would be negligible.

The exposure via drinking water is considered relevant. According to the EFSA bird mammal guidance document (EFSA Journal 2009; 7(12):1438), the worst-case for drinking water is a small granivorous bird with a body weight of 15.3 g, with a drinking rate of 7.0 mL/day, equivalent to 0.46 L/kg bw/d. Based on the worst-case PECsw of 5.8×10^5 CFU/L, gives a daily dose of 4060 CFU/day or 265 CFU/g body weight/day. This dose is insignificant compared to, for example, the dose of 5×10^6 cfu/g body weight administered in the American kestrel test or the diet containing 1×10^9 cfu feed to chickens. It can be concluded that exposure to spores via drinking water is an acceptable risk to birds.

Similarly, for secondary metabolites it would be considered that the highest exposure would be due to consumption of insect cadavers, exposure via surface water would necessarily result in a highly diluted dose. As exposure to insect cadavers had no effect it is not likely that exposure via drinking water would have any effect.

B.9.2 Effects on aquatic organisms (Annex IIB 8.2; Annex IIIB 10.2)

The applicant submitted aquatic toxicity studies with organisms exposed to *Beauveria bassiana* strain PPRI 5339. The summaries and evaluations of these studies are presented below.

B.9.2.1 Effects on fish (Annex IIM 8.2.1; Annex IIIB 10.2)

B.9.2.1.1 Toxicity

ACTIVE INGREDIENT

Reference 8.2.1/01

Report: [redacted] **(2015).** *Beauveria bassiana (PPRI 5339):* Toxicity, infectivity and pathogenicity to Rainbow trout (*Oncorhynchus mykiss*) during a 28 day test. [redacted] Study Code: CEMR-6525

Guidelines: Microbial Pesticide Test Guidelines OPPTS 885.4200 Freshwater Fish Testing Tier 1

OECD Guideline for Testing of Chemicals. 'Fish, Acute Toxicity Test' No.203 (Adopted 17 July 1992)

GLP: Yes

Deviations: The fish mean length was 6.5 cm and the mean weight 2.57 g which meant the fish numbers per treatment exceeded the loading rate.

The Tween 80 used in the bioassay was supplied by Sigma Aldrich, Lot Number: MKBQ6327V rather than Acros Organics, Lot Number: A0341586. A new batch of Tween 80 was purchased before starting the biological phase of the study.

Summary

This study was designed to assess the toxicity, infectivity and pathogenicity of the microbial pest control agent (MPCA) test substance, *Beauveria bassiana* PPRI 5339 to the freshwater fish species, *Oncorhynchus mykiss* (Rainbow Trout), under semi-static (weekly renewal) conditions for 28 days. Exposure solutions were prepared at a loading rate of 0.1 mg/L, equivalent to 1.91×10^7 CFU/L. Due to its hydrophobic properties test item solutions were prepared with Tween 80 with a final concentration of 0.0001% v/v, except the untreated control. Thirty fish were exposed to 0.1 mg *B. bassiana* PPRI 5339/L solutions and ten fish were exposed to 0.1 mg sterile *B. bassiana* PPRI 5339/L (attenuated), Tween 80 and untreated controls.

Exposure of rainbow trout to PPRI 5339 at a nominal concentration of 0.1 mg/L equivalent to 1.91×10^7 CFU/L (mean measured 2.46×10^6 CFU/L in freshly prepared solutions and 4.27×10^5 CFU/L in 7 day old samples) resulted in no mortality or sub-lethal effects. *Beauveria bassiana* strain PPRI 5339 was demonstrated not to be infective to *Oncorhynchus mykiss.*

Materials and methods

Start of laboratory phase:	4th July 2014
End of laboratory phase:	31st March 2015

Test item

Name:	*Beauveria bassiana* PPRI 5339 concentrate
Batch No.:	S826
Physical State:	Light cream powder
Purity:	1.91×10^{11} CFUg
Storage conditions:	Refrigerated

Attenuated test item

Name:	*Beauveria bassiana* PPRI 5339 concentrate – sterile
Batch No.:	S826
Treatment:	*Beauveria bassiana* (PPRI 5339) concentrate autoclaved at 121°C for at least 20 minutes at CEMAS
Storage conditions:	Refrigerated

Treatments

Test concentrations:	Dilution medium, vehicle control (Tween 80 with a final concentration of 0.0001% v/v), attenuated control (0.1 mg sterilised PPRI 5339/L with

	0.0001% v/v Tween 80) and nominal test item loading rate of 0.1 mg PPRI 5339/L, equivalent to 1.91×10^7 CFU/L (mean measured loading rates were 2.46×10^6 CFU/L in freshly prepared solutions and 4.27×10^5 CFU/L in 7 day old samples).
Vehicle:	Tween 80 with a final concentration of 0.0001% v/v
Positive control:	None
Analysis of test concentrations:	Yes, samples plated in triplicate on Rose Bengal Agar and number of CFUs counted.

Test organism

Species:	*Oncorhyncus mykiss*
Source:	[redacted]
Acclimation period:	At least 12 days
Feeding:	Nutra Plus 02 (Skretting, Cheshire, UK),1% body weight per day during the exposure period
Weight and length of fish at start of exposure period:	0.69 – 1.41 g, mean 0.97 g 4.3 – 5.3 cm, mean 4.8 cm
Loading rate:	0.6 (for 15 L vessels) – 0.3 g/L (for 30 L vessel)

Test design

Test vessels:	Glass aquaria, containing 15 L test solution (control vessels) or 30 L for the test item vessel
Test medium:	De-chlorinated, tap water
Replication:	Each test group had one replicate test vessel. Each control test vessel contained 10 fish, and the test item vessel contained 30 fish.
Exposure regime:	Semi-static, 7 day renewal period
Aeration:	Constant aeration
Duration:	28 days

Environmental conditions

Test temperature:	13.3 to 14.6 °C
pH:	Freshly prepared solution: 7.6 – 7.8 Aged solutions: 7.3 – 7.5
Dissolved oxygen:	78 to 107 % saturation
Lighting:	16:8 hour light : dark photoperiod was maintained with a 1 hour dawn

dusk period at the start and end of the cycle

A stock solution of PPRI 5339 was prepared by adding 0.5 mL Tween 80 to 50 mg of PPRI 5339. This was mixed together to form a homogenous paste and transferred to 500 mL de-chlorinated tap water. This was stirred for approximately 5-20 minutes until it formed a homogenous suspension. An aliquot of 30 mL of this stock was added to a glass tank holding 30 L of de-chlorinated tap water to give a final concentration of 0.1 mg test item/L with 0.0001% v/v Tween 80. The attenuated control was prepared in the same way however the PPRI 5339 used was sterilised in an autoclave for 20 minutes at 121°C prior to use. The study design consisted of a single replicate test vessel for each test concentration and the dilution medium control. The vehicle control was diluent with Tween 80 added at a rate of 0.0001% v/v, the control was untreated diluent only.

Ten rainbow trout were transferred into each control vessel and 30 into the test item vessel. The study design consisted of a single replicate test vessel for the test item concentration and controls. Exposure solution were renewed every 7 days, fish were fed daily.

Mortality assessments were conducted after approximately 15 minutes, 1, 2, 3 and 4 hours and then at least once per day for the 28 day exposure. Other observations were made at additional time points; and were allocated to the nearest 24 hour time point. Sub-lethal observations of visible abnormalities and abnormal behaviour (e.g. loss of equilibrium, abnormal swimming behaviour) or physiological (pigmentation change, respiratory function) were also noted at observation points.

The concentrations of the test item were determined the start and end of each renewal period. Triplicate 20 mL samples were taken from the test item vessel and control groups. Samples taken at 7, 14, 21 and 28 days after exposure were sent to the analytical laboratory on the day of sampling. Samples from Day 0 were refrigerated until they were sent to the analytical laboratory with the Day 7 samples for analysis. Upon receipt at the analytical laboratory samples were thoroughly agitated by hand and then vortex mixed, 1 mL was transferred into 9 mL of sterile phosphate buffered saline (PBS) to produce a 1×10^{-1} dilution. The dilution series was continued, agitating samples in a vortex mixer, until a final dilution of 1×10^{-10} was achieved.

100 µL of the neat sample solution and each dilution were plated in triplicate on Rose Bengal Agar. Plates were incubated at 28 ± 2°C for approximately 96 hours. Plates with the highest number of easily distinguishable colonies were selected for enumeration. Counts were made manually on a pressure-sensitive colony counter and the results read directly from the display.

At the end of the test all surviving fish from each treatment group were assessed for the infectivity potential of PPRI 5339. Colony growth from the fish from PPRI 5339 treatment group was compared with that from fish from the control groups. Whole fish were washed in three PBS, weighed, and homogenised in an equal volume per weight of to make a two-fold dilution, this was considered the primary dilution; 1ml of this was serially diluted down to a 1×10^{-10} dilution using 9 ml of PBS each time. An aliquot of 0.1 mL of each dilution was inoculated onto each of three Rose Bengal Agar plates and spread with a sterile plate spreader. Plates were incubated upright at 28 ± 2 °C and read after incubation of approximately 96 hours.

The pH, dissolved oxygen, temperature and total hardness were recorded at the start, each renewal

and at the end of the test from each treatment group.

Statistical analysis of mortality after 28 days exposure was performed (using ToxCalc v.5.023) for each control and the test item group. The Tween 80 control, attenuated control and test item groups were compared to the untreated control group using Fishers Exact Test (two-tailed, α = 0.05) to ascertain any significant differences.

Results and Discussion

Measured test item concentrations ranged from 7.3 – 19.5 % at the start of each renewal period, dropping to 0.2 – 5.6 % at the end of each renewal period (see Table 8.2.1/01-1).

Table 8.2.1/01-01: Analytical results

Treatment Group	**Mean Spore Count (CFU/L)**							
	Day 0	**Day 7**		**Day 14**		**Day 21**		**Day 28**
	Fresh	**Fresh**	**Aged**	**Fresh**	**Aged**	**Fresh**	**Aged**	**Aged**
Untreated Control	ND	ND	ND	ND	ND	ND	ND	ND
Tween 80 Control	ND	ND	ND	ND	ND	ND	ND	ND
Attenuated Control	ND	ND	ND	ND	ND	ND	ND	ND
Test Item	2.03×10^6 (10.6%)	2.7×10^6 (14.1%)	2.1×10^5 (1.1%)	3.72×10^6 (19.5%)	3.8×10^5 (2.0%)	1.4×10^6 (7.3%)	4.7×10^4 (0.2%)	1.07×10^6 (5.6%)

ND: not detected

Values in parenthesis = % theoretical nominal 1.91×10^7 CFU/L

The measured *B. bassiana* concentration in the 0.1 mg *B. bassiana* concentrate/L exposure solution was lower than the target dose in fresh and aged samples. The accurate enumeration of microbial substances from aquatic systems is notoriously difficult due to the hydrophobic nature of most spores, their heterogeneous distribution throughout the medium, and the reduced viability of aerobic spores after immersion in water. Furthermore, interference from contaminant organisms, particularly faster-growing fungi, can make the enumeration of the test item from aged medium particularly difficult. This would explain the low concentrations of *B. bassiana* that were measured in the aged 0.1 mg *B. bassiana* concentrate/L exposure solution.

Water quality throughout the test was within acceptable limits. During the exposure period the pH ranged from 7.3 to 7.8, the dissolved oxygen ranged from 78 to 107 % saturation and temperature ranged from 13.3 to 14.6 °C.

No mortality or sub-lethal effects were noted after 28 days exposure in all the control vessels and the PPRI 5339 0.1 mg/L test vessel.

The results of whole-body macerations of washed fish from the treatment groups showed colony growth of varying amounts on the plates made from eight out of ten plates made from the ten individual fish that had been exposed to the test item treatment. This growth was consistent with the

white fluffy growth characteristics of colonies of *B. bassiana*. No colony growth was visible on the plates made from the individual fish that had been exposed to the untreated control water.

The highest colony count recorded from macerated fish was 3.32 × 10^3 CFU/100 µL of plated fish solution, which equates to 3.32 × 10^4 CFU/g of fish, which is 4.45 x 10^4 CFU per mean weight of an individual fish. The nominal loading rate of PPRI 5339 was 1.91 × 10^7 CFU/L which equates to 1.91 × 10^4 CFU/mL of water. The highest colony counts per gram of fish and per mean weight of an individual fish are similar to the nominal value for PPRI 5339 per gram of water, indicating that *Beauveria bassiana* is not infective to *Oncorhynchus mykiss*, taking into account the variability of this hydrophobic MPCA in water.

Validity Criteria

The study was considered valid as the following criteria was met:

- Mortality of ≤ 10% fish was observed in the control treatments
- Dissolved oxygen concentration was >60 % saturation throughout the test

Conclusions

Exposure of rainbow trout to PPRI 5339 at a nominal concentration of 0.1 mg/L equivalent to 1.91 × 10^7 CFU/L (mean measured 2.35 × 10^6 CFU/L in freshly prepared solutions and 4.27 × 10^5 CFU/L in 7 day old samples; geometric mean of 1.00 × 10^6 CFU/L) resulted in no mortality or sub-lethal effects. *Beauveria bassiana* strain PPRI 5339 was demonstrated not to be infective to *Oncorhynchus mykiss.*

Comments by RMS: According to the study authors the study was conducted according to the OECD and US EPA guidelines. Based on the US OPPTS 885.4200 the test duration should be at the minimum of 30 days, while in the current study this duration was slightly lower of 28 days, which is considered acceptable. According to the same guideline testing should be performed at a concentration in the test medium of at least 10^6 units/ml (i.e. 10^9 CFU/L) or at least the 1000x the maximum calculated pesticide concentration in 6-in (i.e. 0.15 m) layer of water whichever is greater attainable. The current calculated PECsw in a water depth of 0.3 m is 5.8 x 10^5 CFU/L which is equivalent to 1.2 x 10^6 CFU/L in 0.15 m layer of water. The current test concentrations were mean measured 2.46 × 10^6 CFU/L in freshly prepared solutions and 4.27 × 10^5 CFU/L in 7 day old samples which makes them not in the range of the maximum hazard dose as suggested by US OPPTS guidelines. The RMS is of opinion that testing at maxium hazard dose will ensure that effects, if any, will be certainly captured, and therefore this is required for the test to be acceptable.

Furthermore, the authors mentioned that the fish loading was higher than the OECD recommended 1 g fish/L. According to the “Deviation 1” of July 2015 larger tanks were not available and “the study was terminated within 24 h due to high fish mortality and therefore there was no impact on the test”. The RMS considers this last statement somewhat contradictory.

Regarding the infectivity, the RMS doe not agree that by comparing the CFU/g fish with those of water will conclude that the infectivity did not occur. The fact that the fish were washed 3 times in PBS should exclude the presence of fungi on the fish. Thus the CFU measured were present in the fish. The RMS is of opinion that the results basically demonstrate that *B. bassiana* is able to survive in fish.

In the case infectivity and pathogenicity should be tested then a histopathological report needs to be available.
No pathogenicity was reported.
Based on these deviations, the RMS considers this study **unreliable**.

Reference 8.2.1/02

Reference:	Fred J. Genther and Douglas P. Middaugh. Effects of *Beauveria bassiana* on Embryos of the Inland Silverside Fish (*Menidia beryllina*). APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Sept. 1992, p. 2840-2845 Vol. 58, No. 9.
	Published
Guideline:	none
GLP:	no

Material and methods:
B. bassiana (UF1 5789) was cultured at 25°C on glucose-yeast extract-basal salts agar medium. At 10-14 days conidia were harvested and stored frozen. Numbers of conidiospores were determined by plate counts. Inland silverside embryos were obtained from adults that spawned naturally in the laboratory 5 promile salinity and 25°C. The photoperiod was 16 h of light and 8 h of darkness. Exposures were initiated by washing blastula-stage embryos five times with sterile moderately hard water. For embryo exposures, a 1-mg/ml conidiospore suspension was prepared in sterile moderately hard water. This suspension yielded a culturable count of approximately 10^8 CFU/ml.
This concentrated stock was used to prepare 10-fold dilutions in moderately hard water. Serial dilutions containing 10, 1, and 0.1 ug of conidiospores per ml were used for testing. Thirty tubes, each containing a single embryo, were filled with 6 ml of each of the three dilutions. This design yielded four treatments, a control and three 10-fold dilutions of conidiospores, with 30 replicate embryos for each treatment. In additional tests, embryos were exposed, as described above, to conidiospores (10 whether µg/ml) that had been sterilized in an autoclave (20 min, 15 lb/in^2), experiment 5.
Short-term attachment experiments were performed to determine whether a detergent influences attachment and whether the number of conidiospores attached per embryo was dependent upon the concentration of conidiospores in the water. In the first attachment experiment, two treatments (10 µg of unwashed and detergent-treated conidiospores per ml) were used. In the second attachment experiment, three concentrations (10, 1, and 0.1 µg/ml) of untreated conidiospores were used.
After 18 h, embryos were removed from exposure tubes and washed to remove unattached spores. Three groups of five embryos from each treatment were homogenized. The numbers of conidiospores attached per embryo were determined by plate counts.
Results:
There was substantial variability in the observed effects of *B. bassiana* on embryonic *M. beryllina* This variability precluded among test comparisons of embryonic responses. Individual responses

within a given treatment were not mutually exclusive. For example, many embryos that exhibited advanced hyphal growth did not rupture, and ruptured embryos often completed development and successfully hatched. Table 9.2.1.1-01 summarizes the results.

Table B.9.2.1.1.-01: Responses of embryonic *M. beryllina* exposed to *B. bassiana* in experiments 1 through 4

Expt	Treatment (spores/ml) (n = 30)	Observed response (%)[a]			
		Embryo adv gr	Embryo rupture	Embryo death	Larval hatch
1	Control (0)	0	0	10	90
	8.3×10^3	0	13	13	87
	8.3×10^4	0	53	63	37
	8.3×10^5	0	0	90	10
2	Control (0)	0	0	7	93
	4.4×10^3	0	1	7	93
	4.4×10^4	10	10	10	87
	4.4×10^5	10	27	27	73
3	Control (0)	0	0	7	93
	9.6×10^4	33	67	3	93
	9.6×10^5	33	77	13	87
	9.6×10^6	3	3	10	87
4	Control (0)	0	0	0	100
	1.2×10^5	37	23	3	77
	1.2×10^6	17	17	7	83
	1.2×10^7	17	0	0	100

[a] The sum of values in each row may exceed 100% because individual responses were not always mutually exclusive. adv gr, advanced hyphal growth on chorion surface.

The Friedman ANOVA revealed significant differences ($P \leq 0.05$) among the treatments within each experiment. Post hoc analyses showed statistically significant responses in treatments in which conidiospore concentrations were $\geq 8.3 \times 10^4$ or $\leq 1.5 \times 10^6$/ml. Spore concentrations of $\leq 4.4 \times 10^4$ or $\geq 9.6 \times 10^6$/ml failed to produce responses that were different from those of the controls (Table B.9.2.1.1.-02).

Table B.9.2.1.1.-02: Summary of the Friedman ANOVA and post hoc analyses of data for experiments 1 through 4

Expt	χ^2	df	ANOVA *P*	Post hoc analyses		
				Treatment (spores/ml)	Σ rank	*P*
1	4.9	3	≤0.01	Control (0)	49.5	
				8.3×10^3	58.0	NS[a]
				8.3×10^4	85.0	≤0.01
				8.3×10^5	107.5	≤0.01
2	8.8	3	≤0.05	Control (0)	67.5	
				4.4×10^3	66.0	NS
				4.4×10^4	74.0	NS
				4.4×10^5	92.5	≤0.05
3	42.4	3	≤0.01	Control (0)	50.0	
				9.6×10^4	96.0	≤0.01
				9.6×10^5	100.0	≤0.01
				9.6×10^6	54.0	NS
4	15.7	3	≤0.01	Control (0)	57.0	
				1.2×10^5	94.0	≤0.01
				1.2×10^6	81.5	≤0.05
				1.2×10^7	67.5	NS

[a] NS, not significant.

Responses attributable to spore viability and attachment were investigated in experiment 5 by exposing embryos to conidiospores (1.5 x 10^6/ml) that had been (i) untreated, (ii) killed by heat sterilization in an autoclave, or (iii) pretreated with the biological detergent Triton X-100. Heat-killed or detergent-treated conidiospores at a concentration sufficient to elicit a significant pathogenic response (P ≤ 0.05) with untreated, viable spores failed to cause statistically significant responses in this experiment (table 9.2.1.1-03).

Table B.9.2.1.1.-03: Responses of embryonic *M. beryllina* exposed to *B. bassiana* in experiment 5

Treatment[a] (n = 30)	Observed response[b] (%)			
	Embryo adv gr	Embryo rupture	Embryo death	Larval hatch
Control	0	0	3	97
Viable spores	43	3	7	93
Killed spores	0	0	3	97
Detergent-treated spores	0	0	0	100

[a] In each treatment with spores, 1.5×10^6 spores were used. In the control, no spores were added.
[b] See footnote *a* of Table 1.

z

Pretreatment of conidiospores with Triton X-100 significantly decreased the number of conidiospores found attached per embryo. The number of conidiospores attached per embryo was directly related to the concentration of conidiospores in the water. *B. bassiana* was consistently isolated from washed embryos that had been exposed to spores at all concentrations. *B. bassiana* was not isolated from unexposed embryos or larvae. *B. bassiana* was isolated from all newly emerged larvae exposed as embryos to 4.4 x 10^6 spores per ml and from 60% of the newly emerged larvae exposed as embryos to 8.3 x 10^3 spores per ml.

Conclusion

Our finding that the number of conidiospores found attached per embryo was directly proportional to the concentration of conidiospores in the water supports the dose response data. Specifically, conidiospore concentrations of ≥.8.3 x 10^4 were required to show a significant effect.

The next step in the infection process is spore germination. Nutrient limitation may be one reason why spore concentrations of ≥9.6 x 10^6 failed to show an effect in this study.

The proteinaceous nature of the chorion probably rendered the inland silverside embryo susceptible to digestion by the extracellular enzymes of this fungus. The ruptured chorion is a phenomenon never before observed with this test system. Since heat-killed spores did not cause any adverse effects, we conclude that germination and penetration were responsible for rupture of the chorion and the increased incidence of embryo death. We suspect that even if the fungus was effectively inhibited by the defense mechanisms of the embryo, the penetration of the chorion by hyphae was sufficient to cause a rupture. Although several toxic compounds have been identified and/or isolated from culture filtrates or mycelium of *B.bassiana* analysis of a nonpolar extract from the conidiospore inoculum failed to detect beauvaricin, the most studied of the toxic metabolites produced by this organism (data not shown).

Comments by RMS:

There was substantial variability in the observed effects in experiments 1 through 4. The authors have no definitive explanation of this occurrence. The RMS sees that effects were measured but agrees that because of the variability some of the effects are hard to address without any relation to the dose in a static exposure test at 8.3 x 10^3 to 1.2 x 10^7- conidia per millilitre.

TOXIN/METABOLITE FROM ACTIVE INGREDIENT

Presence of spores in recirculation water in the greenhouse cannot be excluded (especially for non-soil bound use), which in turn can result in exposure of surface water. The metabolites however, will be limited to the host insects and the chance of infected insects being present in the reciculation water in the greenhouses is low. *Beauveria bassiana* does not multiply nor shows metabolic activity in the absence of hosts insects, and therefore presence of the metabolites in the aquatic environment as result of insect's exposure in the greenhouses is highly unlikely. Nevertheless, all the information available from the peer-reviewed literature will be added in this document.

B.9.2.1.2 Infectiveness

In the study by Genther and Middaugh infectivity to *Menidia beryllina* embryos was shown. There was substantial variability in the observed effects in experiments where the authors have no definitive explanation of this occurrence.

B.9.2.1.3 Pathogenicity

Based on the results of the study by Genther and Middaugh a potential pathogenicity to fish embryos of *B. Beauveria* can be seen.

B.9.2.2 Effects on freshwater invertebrates (Annex IIM 8.2.2; Annex IIIB 10.2)

B.9.2.2.1 Toxicity

ACTIVE INGREDIENT

Reference 8.2.2/01

Report: Forbes T (2015a). *Beauveria bassiana* (PPRI 5339): Toxicity, infectivity and pathogenicity to *Daphnia magna* (Straus) during a 21 day test. CEM Analytical Services Limited (CEMAS) Imperial House, Oaklands Business Centre, Oaklands Park, Wokingham, RG41 2FD, UK. Study Code: CEMR-6526

Guidelines: Microbial Pesticide Test Guidelines OPPTS 885.4240 Freshwater Aquatic Invertebrate Testing Tier 1

OECD Guideline for Testing of Chemicals. '*Daphnia* sp., Acute Immobilisation Tests' No.202 (Adopted 13 April 2012)

OECD Guideline for Testing of Chemicals. '*Daphnia magna* Reproduction Test' No.211 (Adopted 02 October 2012)

GLP: Yes

Deviations: Daphnids were fed every other day and on each media renewal during the bioassay instead of every day. This was to reduce build-up of algae and general debris between medium renewals.

The hardness exceeded the 140-250 mg $CaCO_3$/L range as stated in the Study Plan in the aged test item group at 7, 14 and 21 days exposure and the untreated control group at 7 days exposure (up to 278 mg $CaCO_3$/L). This was due to the hardness of the water in the area where the laboratory is situated.

The temperature of the test solutions at 21 days exposure dropped below 18°C (down to 17°C). These temperatures were recorded outside of the test room after the observations.

The Tween 80 used in the bioassay was supplied by Sigma Aldrich, Lot Number: MKBQ6327V rather than Acros Organics, Lot Number: A0341586. A new batch of Tween 80 was purchased before starting the biological phase of the study.

Summary

This study was designed to assess the toxicity, infectivity and pathogenicity of the microbial pest control agent (MPCA) test substance, *Beauveria bassiana* PPRI 5339 to the freshwater invertebrate

species, *Daphnia magna*, under semi-static (weekly renewal) conditions for 21 days. Exposure solutions were prepared at a loading rate of 0.1 mg/L, equivalent to 1.91×10^7 CFU/L. Due to its hydrophobic properties test item solutions were prepared with Tween 80 with a final concentration of 0.001% v/v, except the untreated control. Fifty daphnids distributed between 10 replicate vessels were exposed to 0.1 mg *B. bassiana* PPRI 5339/L solutions, 0.1 mg sterile *B. bassiana* PPRI 5339/L solutions, together with Tween 80 and untreated controls.

Exposure of *Daphnia magna* to PPRI 5339 at a nominal concentration of 0.1 mg/L equivalent to 1.91×10^7 CFU/L (mean measured 1.43×10^7 CFU/L in freshly prepared solutions and 6.10×10^6 CFU/L in 7 day old samples) resulted in no significant immobilisation or reduction in reproductive capacity. *Beauveria bassiana* strain PPRI 5339 was demonstrated not to be infective to *Daphnia magna.*

Materials and methods

Start of laboratory phase:	14th October 2014
End of laboratory phase:	14st November 2014

Test item

Name:	*Beauveria bassiana* PPRI 5339 concentrate
Batch No.:	S826
Physical State:	Light cream powder
Purity:	1.91×10^{11} spores/g
Storage conditions:	Refrigerated

Attenuated test item

Name:	*Beauveria bassiana* PPRI 5339 concentrate – sterile
Batch No.:	S826
Treatment:	*Beauveria bassiana* (PPRI 5339) concentrate autoclaved at 121°C for at least 20 minutes at CEMAS
Storage conditions:	Refrigerated

Treatments

Test concentrations:	Dilution medium, vehicle control (Tween 80 with a final concentration of 0.001% v/v), attenuated control (0.1 mg sterilised PPRI 5339/L with 0.001% v/v Tween 80) and nominal test item loading rate of 0.1 mg PPRI 5339/L, equivalent to 1.91×10^7 CFU/L (mean measured 1.43×10^7 CFU/L in freshly prepared solutions and 6.10×10^6 CFU/L in 7 day old samples).
Vehicle:	Tween 80 with a final concentration of 0.001% v/v

Positive control:	None
Analysis of test concentrations:	Yes, samples plated in triplicate on Rose Bengal Agar and number of CFUs counted.

Test organism

Species:	*Daphnia magna* (< 24 hours old)
Source:	In house culture
Feeding:	*Pseudokirchneriella subcapitata* solution every two days at a rate of 0.1 mL/daphnid/day for the first five days and 0.5 mL/daphnid/day thereafter.
Culture medium:	Elendt M4 medium

Test design

Test vessels:	250 mL glass beakers, containing approximately 100 mL of test solution
Test medium:	Elendt M4 medium (hardness 242 – 278 mg $CaCO_3$/L)
Replication:	Each test group had five replicate test vessels. Each vessel contained 10 daphnids.
Exposure regime:	Semi-static, 7 day renewal period
Aeration:	No
Duration:	21 days

Environmental conditions

Test temperature:	17.1 to 20.4 °C
pH:	Freshly prepared solution: 7.4 – 7.9 Aged solutions: 7.4 – 9.0
Dissolved oxygen:	8.4 to 12.9 mg/L
Lighting:	16:8 hour light : dark photoperiod

A stock solution of PPRI 5339 was prepared by adding 5 mL Tween 80 to 50 mg of PPRI 5339. This was mixed together to form a homogenous paste and transferred to 500 mL Elendt M4. This was stirred for approximately 30-50 minutes until it formed a homogenous suspension. An aliquot of 1 mL

of this stock was added to 1000 mL Elendt M4 to give a final concentration of 0.1 mg test item/L with 0.001% v/v Tween 80. The attenuated control was prepared in a similar way however the PPRI 5339 used was sterilised in an autoclave for 20 minutes at 121°C prior to use. The study design consisted of five replicate test vessels for each test concentration and the dilution medium control, each containing 10 daphnids. The vehicle control was diluent with Tween 80 added at a rate of 0.001% v/v, the control was untreated diluent only.

A volume of 100 mL of control or test item solution was added to five 250 mL glass beakers per treatment. Five neonate daphnids were added to each beaker (20 per treatment). Exposure solution were renewed every 7 days, daphnids were fed with *Pseudokirchneriella subcapitata* solution every two days at a rate of 0.1 mL/daphnid/day for the first five days and 0.5 mL/daphnid/day thereafter.

Immobility of daphnids in each test vessel was assessed once a day during the 21 day exposure period. Immobilisation was classified as those daphnids not able to swim within 15 seconds after gentle agitation, even if they could still move their antennae. Any other abnormal behaviour or morphology was also noted at observation points. The day of juvenile production and number produced was recorded to assess the effects, if any, on daphnid life cycle. Juvenile production was assessed once a day during the 21 day exposure period.

The concentrations of the test item were determined the start and end of each renewal period. Triplicate 20 mL samples were taken from the test item vessel and control groups. Samples taken at 0 and 21 days exposure were sent to the analytical laboratory on the day of sampling. Other samples were refrigerated until they were sent to the analytical laboratory at the end of the test. Upon receipt at the analytical laboratory samples were thoroughly agitated by hand and then vortex mixed, 1 mL was transferred into 9 mL of sterile phosphate buffered saline (PBS) to produce a 1×10^{-1} dilution. The dilution series was continued, agitating samples in a vortex mixer, until a final dilution of 1×10^{-10} was achieved.

100 µL of the neat sample solution and each dilution were plated in triplicate on Rose Bengal Agar. Plates were incubated at 28 ± 2°C for approximately 96 hours. Plates with the highest number of easily distinguishable colonies were selected for enumeration. Counts were made manually on a pressure-sensitive colony counter and the results read directly from the display.

At the end of the test surviving daphnids from each treatment group were assessed for the infectivity potential of PPRI 5339. Colony growth from the daphnids from the PPRI 5339 treatment group was compared with that from daphnids from the control groups. Daphnids were washed in three changes of PBS with gentle vortex mixing to remove surface spores. Six daphnids from each treatment group were placed individually on Rose Bengal Agar plates and squashed and spread with a sterile glass rod. Plates were incubated at 28 ± 2 °C and read after incubation of approximately 96 hours.

The pH, dissolved oxygen, temperature and total hardness were recorded at the start, each renewal and at the end of the test from each treatment group.

Statistical analysis of mortality after 21 days exposure was performed (using ToxCalc v.5.023) for each control and the test item group. The Tween 80 control, attenuated control and test item groups were compared to the untreated control group using Fishers Exact Test (two-tailed, α = 0.05) to ascertain any significant differences.

Results and Discussion

Measured test item concentrations ranged from 68.6 – 84.7 % at the start of each renewal period, dropping to 23.4 – 40.5 % at the end of each renewal period (see Table 8.2.2/01-1).

Table 8.2.2/01-01: Analytical results

Treatment	**Mean Spore Count (CFU/L)**					
	Day 0	**Day 7**		**Day 14**		**Day 21**
	Fresh	**Fresh**	**Aged**	**Fresh**	**Aged**	**Aged**
Untreated Control	ND	ND	ND	ND	ND	ND
Tween 80 Control	ND	ND	ND	ND	ND	ND
Attenuated Control	ND	ND	ND	ND	ND	ND
Test Item	1.4×10^7 (74.9%)	1.6×10^7 (84.7%)	7.7×10^6 (40.5%)	1.3×10^7 (68.6%)	6.1×10^6 (32.1%)	4.5×10^6 (23.4%)

ND: not detected

Values in parenthesis = % theoretical nominal 1.91×10^7 CFU/L

The measured *B. bassiana* concentration in the 0.1 mg *B. bassiana* concentrate/L exposure solution was lower than the target dose in fresh and aged samples. The accurate enumeration of microbial substances from aquatic systems is notoriously difficult due to the hydrophobic nature of most spores, their heterogeneous distribution throughout the medium, and the reduced viability of aerobic spores after immersion in water. Furthermore, interference from contaminant organisms, particularly faster-growing fungi, can make the enumeration of the test item from aged medium particularly difficult. This would explain the low concentrations of *B. bassiana* that were measured in the aged 0.1 mg *B. bassiana* concentrate/L exposure solution.

Water quality throughout the test was generally within acceptable limits. During the exposure period the pH ranged from 7.4 to 9.0, the dissolved oxygen ranged from 8.4 to 12.9 mg/L and temperature ranged from 17.1 to 20.4 °C.

Table 8.2.2/01-02: Immobilisation

Treatment group	**Immobilisation (%)**	**Difference to the untreated control (%)**	**Total number of juveniles produced per surviving daphnid**	**Difference to the untreated control (%)**
Untreated Control	8	-	258	-

Tween 80 Control	20	13	303	+17
Attenuated Control	48*§	43	250	-3
Test Item	6	-2	296	-15

* High variation was noted between replicates. Additionally, after the sample of PPRI 5339 was sterilised the physical properties appeared different (i.e. colour, smell and consistency). The sterilised test item did not mix well with the Tween 80 during solution preparation and required sonication to make an apparent homogenous suspension. It is thought that the sterilising process caused a change in the test item properties causing interference with their mobility

§: Significant difference when compared with the untreated control (Fishers Exact Test, two-tail, $p < 0.05$)

The daphnid macerations from each control or treatment group when plated resulted in no colony growth on the plates, indicating that *Beauveria bassiana* is not infective to *Daphnia magna*.

Validity Criteria

The study met all the validity requirements as outlined in the Study Plan. 3 The percentage immobilisation in the untreated control group did not exceed 10 % (actual value 8 %) and the daphnids in the control group showed no other signs of abnormal behaviour or effects.

Conclusions

Exposure of *Daphnia magna* to PPRI 5339 at a nominal concentration of 0.1 mg/L equivalent to 1.91×10^7 CFU/L (mean measured 1.43×10^7 CFU/L in freshly prepared solutions and 6.10×10^6 CFU/L in 7 day old samples; geometric mean of 9.35×10^6 CFU/L) resulted in no significant immobilisation or reduction in reproductive capacity. *Beauveria bassiana* strain PPRI 5339 was demonstrated not to be infective to *Daphnia magna.*

Comments by RMS: According to the study authors the study was conducted according to the OECD and US EPA guidelines. Based on the US OPPTS 885.4240 testing should be performed at a concentration in the test medium of at least 10^6 units/ml (i.e. 10^9 CFU/L) or at least the 1000x the maximum calculated pesticide concentration in 6-in (i.e. 0.15 m) layer of water whichever is greater attainable. The current calculated PECsw in a water depth of 0.3 m is 5.8×10^5 CFU/L which is equivalent to 1.2×10^6 CFU/L in 0.15 m layer of water. The current test concentrations mean measured 1.43×10^7 CFU/L in freshly prepared solutions and 6.10×10^6 CFU/L in 7 day old sampless which makes them not in the range of the maximum hazard dose as suggested by US OPPTS guidelines.

The OECD guideline states that feeding should be at 0.1 and 0.2 mg C/Daphnia/day in order to achieve the required number of lving offspring to meet the test validity criteria. The report does not state what was the concentration in mg C/Daphnia/day instead it is stated that the feeding with algae was with 0.1 mL/daphnid/day for the first five days and 0.5 mL/daphnid/day thereafter. Therefore, the RMS cannot verify if the OECD recommendations were respected.

Regarding the validity criteria, the OECD 211 states that the mean number of living offspring produced per parent animal surviving at the end of the test is ≥ 60. According to the study authors, each treatment had 5 repplicates with 10 daphnids per replicate. The table below is as per the study report.

Table 1 Survival and Juvenile Reproduction at 21 Days Exposure

Treatment Group	Replicate	% Survival	Total Number of Juveniles Produced/Surviving Daphnids
Untreated Control	1	100	54
	2	90	52
	3	90	50
	4	80	53
	5	100	50

Based on these data, the validity criterion regarding the mean number of living offsprings was not fulfilled.

In the view of the comments above, the RMS evaluated the study as unreliable.

Reference 8.2.2/02

Reference: Genthner, FJ, Foss, SS, Fisher, WS.
Testing of the insect pest control fungus *Beauveria bassiana* in grass shrimp *Palaemonetes pugio*.
Diseases of Aquatic organisms, Vol. 20: 49-57, 1994b
Published

Guideline: none

GLP: no

Abstract:

Embryos, larvae and adult grass shrimp *Palaemonetes pug10* were exposed to spores of the insect-control fungus *Beauveria bassiana.* Conidiospores attached to embryos held by gravid females and remained with the egg mass for at least 6 d. In the first experiment where individual developing embryos contained in test tubes were exposed to conidiospores of *B. bassiana,* 2 of the 75 embryos became infected and died, and a third was abnormal at hatch. In Expt 2 a repeat experiment, no adverse effects were observed. In subsequent experiments, attempts were made to increase the probability of infection or abnormal development for embryos exposed to *B. bassiana.* Strategies for these attempts included lowering the salinity, adding a carbon source for the fungus to the test water,

or rendering embryos free of potentially protective symbiotic bacteria with an antibacterial agent. All attempts were unsuccessful in promoting infection or abnormal development *B. bassiana* did not cause any adverse effects adult shrimp larval development. In adult shrimp lethal infections only occurred in specimens injected with either conidiospores or blastospores of *B. bassiana.*

Material and methods:

Corn earworm *Helicoverpa zea* were exposed to *Beauveria bassiana* strain GH by dipping 2nd instar worms into a concentrated conidospore suspension. Five exposed worms were placed in a petri dis hand incubated at 25°C for 3 days. Dead worms displaying external conidiogenesis were hogenised in sterile water and incubate onto a GYBS/nalidixic acid plate. Isolated colonies were selectedd to produce conidiospores for the experiment. Beauvena bassiana was recovered from exposed embryos, larvae and adult shrimp by washing them (twice in 100 m1 sterile water) to remove the spores carried over in water from the exposure tubes. Tissues were then homogenized and dilutions spread onto the surface of GYBS + Nx plates. Bacteria on the chorion were enumerated by spreading dilutions of embryo homogenates onto the surface of NYGS agar plates.

Embryo tests

single embryos, at the blastospore/tissue cap stage (age 2 to 4 d after oviposition), were randomly placed into glass tissue culture tubes containing 6 ml of filtered (0.22 µm) sea water. Tubes were incubated for 12 d at 27 °C with slow shaking (60 rpm). Individual embryos were examined daily with an inverted microscope for stage of development, viability and extent of fungal growth on the chorion. For embryo exposures a stock suspension of *Beauveria bassiana* conidiospores was diluted in sea water to attain approximate densities of 10^6, 10^5 and 10^4 spores/ml. Six ml of these suspensions were added to each exposure tube. A heat-killed control was prepared by sterilizing a conidiospore suspension (ca 1 X 10^6 ml^{-}) in an autoclave and a no-exposure controlwas included.

Larval test

In the larval test larvea were exposed to conidiospore densities of approximately 10^6, 10^5 and 10^4 conidiospores ml-l. Each treatment was performed in triplicate with 10 larvae in glass culture bowls that contain 150 ml sea water. Larvae were fed freshly hatched *Artemia salina* nauplii daily. Every third day larvae were transferred to clean culture bowls containing fresh sea water and re-exposed to the appropriate density of conidiospores. Daily records of mortalities and infections were maintained until metamorphosis to post-larvae occurred.

Adult test

Adult shrimp were exposed to *Beauveria bassiana* by addition of conidiospores to the test water or through intrahemocoelic injection of conidiospores and blastospores. In 2 aerated aquaria 6 adult shrimps were exposed to conidiospores at a density of approx. 10^6 in a static renewal test, one aquarium served as uninocculated control. Shrimp were observed daily for mortalities; the experiment was terminated after 7 d.

In a separate experiment to determine the persistence of conidiospores adhering to embryos, 6 gravid females were placed into a small aerated aquarium containing 3 L of sea water. The aquarium was inoculated with *Beauverja bassiana* conidiospores to a density of ca 10"l-l. After 4 h the shrimp were transferred to a 60 1 aquarium equipped with a flow-through, filtered (30 µm), sea water delivery

system maintained at a flow rate of 1 L min^{-1}. A single gravid female shrimp was removed from the aquarium immediately and at 24, 48, 72 and 144 h. Oviposited embryos were removed (N = 100), separated and placed into a sterile ground glass homogenizer. The eggs were homogenized and. numbers of *B.bassiana* conidiospores in the homogenates were determined by plate count.

Injected conidiospore and blastospore dosages (2.0 µLof *Beauveria bassiana* containing 5.0 x 10^5 spores) were injected under the carapace at the postenor margin of the cephalothorax. Controls were injected with either 2.0 µL of sterile saline-solution or 2.0 µL of a filtrate produced by passing the conidiospore stock suspension through a 0.22 µm filter. Injected shrimp, 15 or 20 per treatment, were held in small, aerated aquaria for 2 h before surviving shrimp were transferred to static holding tanks. Shrimp were observed daily formortality. Moribund shrimp were either examined microscopically as a fresh-squash of the internal organs or processed for histological examination.

Results:

Embryo test

Embryos of *Palaemonetes pugio* were exposed to conidiospores of *Beauvena bassiana* in 4 separate experiments. The results of the most relevant experiments are included below.

Expt 1 provided the only data that indicated *B. bassiana* could cause adverse effects on developing embryos. In all tests only 2 exposed embryos were infected by *B. bassiana*. One embryo treated at the low conidiospore density (7.4 x 10^3 ml^{-1}) was found dead 9 d after exposure with fungal hyphae protruding from the chorion (Table 1). Another embryo, treated at the medium conidiospore density (7.4 x 10^4 ml^{-1}) exhibited a slow heart beat and possessed extensive fungal growth on the surface of the chorion 8 d after conidiospore exposureand died. Histological sections of this embryo showed extensive mycelial proliferation throughout the tissue. The highest density of conidiospores in Expt 1 produced 1 abnormal larva that died shortly after hatch. Microbiological analysis revealed that this larva harbored 2.4 x 10^3 colony-forming units (cfu) of *B. bassiana*. In contrast, 3 apparently normal larvae from the treatment also receiving the highest density of conidiospores were void of *B. bassiana.*

Table B.9.2.2.1-01 *Palaemonetes pugio*. Responses to conidiospores of *Beauveria bassiana* (GH) in Expts 1 & 2

Conidiospores ml^{-1} Viable count	Direct count	Sample size	Dead embryos	Dead or abnormal larvae
Expt 1 (20‰ salinity)				
0 (control)	0 (control)	25	0	0
$7.1 \pm 0.7 \times 10^5$	$7.4 \pm 1.4 \times 10^5$	25	0	1 abnormal[a]
$7.1 \pm 0.7 \times 10^4$	$7.4 \pm 1.4 \times 10^4$	25	1[b]	0
$7.1 \pm 0.7 \times 10^3$	$7.4 \pm 1.4 \times 10^3$	25	1[c]	0
0 (control, heat-killed)	$7.4 \pm 1.4 \times 10^5$	25	1	0
Expt 2A (20‰ salinity)				
0 (control)	0	25	0	0
$1.3 \pm 0.1 \times 10^6$	$1.6 \pm 0.2 \times 10^6$	25	0	0
$1.3 \pm 0.1 \times 10^5$	$1.6 \pm 0.2 \times 10^5$	25	0	0
$1.3 \pm 0.1 \times 10^4$	$1.6 \pm 0.2 \times 10^4$	25	1	0
0 (control, heat-killed)	$1.6 \pm 0.2 \times 10^6$	25	2	1 dead at hatch
Expt 2B (5‰ salinity)				
0 (control)	0	10	0	0
$1.3 \pm 0.1 \times 10^6$	$1.6 \pm 0.2 \times 10^6$	10	0	0

[a]See Fig. 2; [b]see Fig. 1C; [c]see Fig. 1B

Larval test

In larval tests, 61 % of larvae exposed to heat-killed conidiospores survived 23 d to metamorphosis. In the experimental bowls 48 %, 68 %, and 46 % survived in the high, medium, and low conidiospore densities, respectively. Thus, there were no significant differences ($p \geq 0.01$) among treatments. In addition, microscopic examination of dead larvae did not reveal any evidence of fungal infection in dead larvae.

Adult test

Conidiospores were found attached to embryos on gravid *Palaemonetes pugio* females. *A* gravid female exposed for 4 h to a conidiospore density of 8.6 X 10^5 ml^{-1}(viable count), harbored 226 ± 64 conidiospores per 200 embryos. Conidiospore-exposed females placed under

flow-through conditions possessed 394 ± 36 and 130 ± 34 conidiospores per 100 eggs after 2 and 6 d, respectively. After 7 d none of the adult shrimp exposed to *Beauveria bassiana* conidiospores in the aquarium water developed infections nor were there any mortalities.

Intrahemocoelic injections of B. *bassiana* conidiospores or blastospores produced high (≥95 %) mortalities (Table B.9.2.2.1-02). Most mortalities occurred 48 h after injection. Microscopic examination of the hemolymph of moribund shrimp revealed that the *B. bassiana* spores had germinated, and began growing within the specimen. Fungal hyphae were observed in numerous tissues and organs, including the hepatopancreas, tail muscle and gills. Heatkilled spores or a sterile filtrate from the spore suspension did not result in mortalities significantly higher than the saline-injection controls.

Table B.9.2.2.1-02 *Palaemonetes pugio*. Mortality of specimens injected with spores of *Beauveria bassiana*. Dosage: 5 X 10^5 spores

Injection	No. tested	Number dead							% Mortality
	Day:	1	2	3	4	5	6	7	
Control (saline)	20	0	0	0	0	0	0	0	0
Heat-killed conidiospores	20	1	0	0	0	1	0	0	10
Heat-killed blastospores	20	1	0	1	0	0	0	0	10
Filtrate from conidiospore suspension	15	0	0	0	0	0	0	0	0
Conidiospores	20	1	15	3	0	0	0	0	95
Blastospores	20	0	17	1	0	0	1	0	95

Conclusion

Adverse effects of *Beauveria bassiana* conidiospores on a nontarget crustacean test species were observed at some individuals during water exposure to developing shrimp embryos. 2 embryos showed strong evidence of fungal infection and a third individual succefully hatched but appeared to be caught in the first larval ecdysis. The remaining 72 embryos exposed to *B. bassiana* conidiospores in Expt 1 successfully hatched and molted without any evidence of fungal infection.

Experimental infections in adults were only observed with intrahemocoelic injections. Apparently, *Palaemonetes pugio* can be infected, albeit infrequently, or under extreme circumstances.

Comments by RMS:

The conidia of *Beauveria bassiana* GHA were not infectious via exposure water only direct injection of blastospores into the adult shrimp haemocoel caused infection. The results can be used in the risk assessment.

A summary of endpoints is given in the table below.

Table B.9.2.2.1-03: Toxicity effects of *Beauveria bassiana* GHA conidiospores to freshwater invertebrates

Test species	*Palaemonetes pugio*
Toxicity embryo test	>1.6 x 10^6 ml^{-1}
Toxicity adult test direct injection	< 5 x 10^5 per test organism

TOXIN/METABOLITE FROM ACTIVE INGREDIENT

Presence of spores in recirculation water in the greenhouse cannot be excluded (especially for non-soil bound use), which in turn can result in exposure of surface water. The metabolites however, will be limited to the host insects and the chance of infected insects being present in the reciculation water in the greenhouses is low. *Beauveria bassiana* does not multiply or show metabolic activity in the absence of hosts insects, and therefore presence of the metabolites in the aquatic environment as result of insect's exposure in the greenhouses is highly unlikely. Nevertheless, all the information available from the peer-reviewed literature will be added in this document.

Reference 8.2.2/03

Reference: Pascale, M, Visconti, A, Pronczuk, M, Wisniewska, H, Chelkowski, J. (2002) Accumulation of fumonisins, beauvericin and fusaproliferin in maize hybrids inoculated under field conditions with Fusarium proliferatum. Mycological Research 106:1026-1030.
Published

Guideline: none

GLP: no

Material and methods:

One hundred and sixty plants of each hybrid were planted in four rows (5 m long), of which 10 plants were inoculated with *Fusarium proliferatum*. The fungal strain (ITEM 1752), used to inoculate maize ears, was isolated in Italy from maize kernels. The strain was previously shown to be pathogenic to maize seedlings (Chelkowski *et al*. 1997) and to produce fumonisins, beauvericin and fusaproliferin on autoclaved maize. Ears from inoculated plants and from control plants (not inoculated plants) were harvested in the third week of October and transported to the laboratory. Disease was scored in 10 inoculated and 10 control ears ; then ears were dried at room temperature. Kernels from

the same ears were separated into two fractions : *Fusarium*-damaged kernels (i.e. visibly mouldy) and healthy-looking kernels.

For the analysis of fumonisins extracts were prepared by shaking 10 g of ground maize in 50 ml of $CH_3CN\}H_2O$ (1:1, v/v) and were, after cleaning, analysed by reverse phase HPLC. Analysis of beauvericin was performed by HPLC diode-array UV detector. The detection limit (signal to noise ratio of 3:1) was 0.01 $\mu g.g^{-1}$.

Fusaproliferin was analysed involving the extraction with methanolwater (containing NaCl) and n-hexane, and determination by HPTLC and/or HPLC. The detection limit (signal to noise ratio of 3:1) was 0.1 $\mu g.g^{-1}$. Beauvericin and fusaproliferin were identified both by retention times and uv spectra.

Results:

The amounts of fumonisin B1 and B2 (FB1 and FB2), beauvericin (BEA) and fusaproliferin (FP) accumulated in maize kernels after inoculation with *F. proliferatum* strain ITEM 1752 were measured. These values represent the weighed averages of the concentrations of FB1, FB2, BEA and FP in *Fusarium*-damaged kernels (FDK) and healthy-looking kernels (HLK) from the same maize samples derived by separate analyses. Fumonisin concentrations were 18-231.9 $\mu g\ g^{-1}$ for FB1 and 0.4-26.5 $\mu g\ g^{-1}$ for FB2, 0.2 to 19.6 $\mu g\ g^{-1}$ for BEA, and 0.3 to 6.4 $\mu g\ g^{-1}$ for FP.

Comments by RMS:

In this study the amount of fungal toxin in maize kernels produced upon infection with *Fusarium proliferatum* strain ITEM 1752 were measured. *F. proliferatum* strain ITEM 1752 is known to produce a.o. beauvericin. From the study it is shown that significant levels can be measured. However, *F. proliferatum* strain ITEM 1752 is a plant pathogen not related to *Beauveria bassiana* and the results of this study can in no way be compared to the species and strain under evaluation. Furthermore, the study was submitted under the section 'effects on aquatic invertebrates' but it does not contain any information on neither exposure nor effects of EPF metabolites in the aquatic environment. The study will not be considered further in the risk assessment.

Reference 8.2.2/04

Report: Genthner, F.J., Cripe, G.M. and Crosby, D.J. (1994) Effect of Beauveria bassiana and Its Toxins on *Mysidopsis bahia* (Mysidacea). *Arch. Environ. Contam. Toxicol.* 26, 90-94

Guidelines: None

GLP: No

Summary

Beauvericin, a cyclic depsipeptide produced by some strains of *B. bassiana*, was evaluated for toxicity to *Americamysis bahia* (formerly *Mysidopsis bahia*). Static acute 96-h tests were conducted with <24-h-old *A. bahia*. A LC_{50} of 0.56 mg/L was estimated. The toxicity of beauvericin persisted in sterile seawater for at least 3, but not, 8 weeks.

Materials and methods

Solutions of beauvericin (Sigma) for the definitive toxicity test were prepared by stirring in seawater at 0.6 or 1.12 mg/L overnight at 4°C to eliminate the potential for breakdown of the test material prior to mysid exposure. Volumes of the suspension were added to exposure vessels to obtain: 0.60, 0.36, 0.22, and 0.13 mg beauvericin per litre. In the toxicity persistence test, beauvericin at 1.12 mg/L was suspended in sterile seawater and incubated at 25°C with stirring to more closely simulate field conditions. This suspension was tested at 1.12, 0.56, and 0.28 mg/L at 0, 2, 3, and 8 weeks.

Ten mysids were added to each exposure vessel. Exposure vessels were covered and incubated at 25 °C with a 14-h light: 10-h dark photoperiod. Each day the mysids were counted, dead animals removed and the remaining mysids fed *Artemia* (48 h post-hydration, /≥30 *Artemia*/mysid/day). Salinity was measured at test initiation. Dissolved oxygen and pH were measured at test initiation and at 96 h. Temperature was recorded continuously.

The trimmed Spearman-Karber method was used to estimate LC_{50} values and confidence limits for the exposures.

Findings

The LC_{50} of beauvericin was estimated to be 0.56 mg/L. Confidence limits could not be calculated because solutions greater than 1.12 mg/L contained undissolved test material. After 2 and 3 weeks of incubation in seawater, the beauvericin retained its toxicity to mysids, exhibiting LC_{50} values of 0.41 and 0.45 mg/L, respectively. After 8 weeks, however, the beauvericin suspension lost all toxicity to mysids, although analysis of beauvericin extracted from the seawater showed no decrease in beauvericin concentration after 8 weeks.

These results were confirmed by additional subsequent exposures.

Conclusions

The LC_{50} of beauvericin was estimated to be 0.56 mg/L for *Americamysis bahia*.

Comments by RMS: The study authors have as well investigated the effects of the fungus to mysids. It was concluded that high mortality is caused at concentrations of ≥ 1.5 x 10^6 conidiospores/ml. The mortality was due to the high density of spores and since the heat-inactivated controls also proved to be lethal. The results as presented under the conclusions can be used in the risk assessment.

B.9.2.2.2 Infectiveness

The conidia of *Beauveria bassiana* GHA were not infectious embryos and larvea of *Palaemonetes pugio* via oral routes. Only direct injection of blastospores into the adult shrimp haemocoel caused infection.

B.9.2.2.3 Pathogenicity

The conidia of *Beauveria bassiana* GHA were not pathogenic to embryos and larvea of *Palaemonetes pugio.*

B.9.2.3 Effects on algae growth (Annex IIM 8.2.3; Annex IIIB 10.2)

ACTIVE INGREDIENT

Reference 8.2.3/01

Report: Forbes T (2015b). *Beauveria bassiana* (PPRI 5339): Freshwater green algae (*Pseudokirchneriella subcapitata*) growth inhibition test. CEM Analytical Services Limited (CEMAS) Imperial House, Oaklands Business Centre, Oaklands Park, Wokingham, RG41 2FD, UK. Study Code: CEMR-6527

Guidelines: Ecological Effects Test Guidelines OCSPP 850.4500 Algal Toxicity

Guidelines for the Testing of Chemicals No. 201, Freshwater Alga and Cyanobacteria, Growth Inhibition Test (adopted 23 March 2006)

GLP: Yes

Deviations: The Tween 80 used in the bioassay was supplied by Sigma Aldrich, Lot Number: MKBQ6327V rather than Acros Organics, Lot Number: A0341586. A new batch of Tween 80 was purchased before starting the biological phase of the study.

Summary

This study was designed to assess the effects of the microbial pest control agent (MPCA) test substance, *Beauveria bassiana* PPRI 5339 to the freshwater green algae *Pseudokirchneriella subcapitata*, under static conditions for 96 hours. Exposure solutions were prepared at a loading rate of 0.1 mg/L, equivalent to 1.91×10^7 CFU/L. Due to its hydrophobic properties test item solutions were prepared with Tween 80 with a final concentration of 0.0001% v/v, except the untreated control. Six replicate vessels were prepared for each treatment or control, inoculated with algae cell to give a starting cell density of 1×10^4 cells/mL.

Exposure of *Pseudokirchneriella subcapitata* to PPRI 5339 at a nominal concentration of 0.1 mg/L equivalent to 1.91×10^7 CFU/L (initial mean measured 3.0×10^7 CFU/L and 1.9×10^5 CFU/L after 96 hours) resulted in no significant inhibition in specific growth rate or yield.

Materials and methods

Start of laboratory phase: 09th February 2015

End of laboratory phase: 22nd February 2015

Test item

Name:	*Beauveria bassiana* PPRI 5339 concentrate
Batch No.:	S826
Physical State:	Light cream powder
Purity:	1.91×10^{11} spores/g
Storage conditions:	Refrigerated

Attenuated test item

Name:	*Beauveria bassiana* PPRI 5339 concentrate – sterile
Batch No.:	S826
Treatment:	*Beauveria bassiana* (PPRI 5339) concentrate autoclaved at 121°C for at least 20 minutes at CEMAS
Storage conditions:	Refrigerated

Treatments

Test concentrations:	Dilution medium, vehicle control (Tween 80 with a final concentration of 0.0001% v/v), attenuated control (0.1 mg sterilised PPRI 5339/L with 0.0001% v/v Tween 80) and nominal test item loading rate of 0.1 mg PPRI 5339/L, equivalent to 1.91×10^7 CFU/L (initial mean measured 3.0×10^7 CFU/L and 1.9×10^5 CFU/L after 96 hours).
Vehicle:	Tween 80 with a final concentration of 0.0001% v/v
Positive control:	None
Analysis of test concentrations:	Yes, samples plated in triplicate on Rose Bengal Agar and number of CFUs counted.

Test organism

Species:	*Pseudokirchneriella subcapitata*, Strain No. CCAP 278/4
Source:	Culture Collection of Algae and Protozoa (CCAP), Oban, PA37 1QA, UK

Test design

Test vessels:	250 mL glass conical flasks, containing 100 mL of test solution, capped with foam bungs
Test medium:	OECD medium
Replication:	Each test group had six replicate test vessels
Starting cell density:	1×10^4 cells/mL
Aeration:	No
Duration:	96 hours

Environmental conditions

Test temperature:	19.3 to 24.1 °C
pH:	Test start: 7.3 – 7.8
	Test end: 7.5 – 8.7
Lighting:	Continuous (mean lux 6633)

A stock solution of PPRI 5339 was prepared by adding 0.5 mL Tween 80 to 50 mg of PPRI 5339. This was mixed together to form a homogenous paste and transferred to 500 mL OECD medium. This was stirred for approximately 20-30 minutes until it formed a homogenous suspension. An aliquot of 1 mL of this stock was added to 1000 mL OECD medium to give a final concentration of 0.1 mg test item/L with 0.0001% v/v Tween 80. The attenuated control was prepared in a similar way however the PPRI 5339 used was sterilised in an autoclave for 20 minutes at 121°C prior to use.

Test solutions were inoculated with a culture of *Pseudokirchneriella subcapitata* to give a starting cell density of 1×10^4 cells/mL. The study design consisted of six replicate test vessels for each test concentration and the dilution medium control. The vehicle control was diluent with Tween 80 added at a rate of 0.001% v/v, the control was untreated diluent only.

The test vessels were transferred to an orbital shaker and were continuously shaken at a nominal 150 revolutions per minute under constant illumination from fluorescent lights. Cell counts were conducted at 24, 48, 72 and 96 hours using a haemocytometer and a microscope.

The concentrations of the test item were determined at the start and end of the exposure period. Triplicate 20 mL samples were taken from the test item vessel and control groups. Samples from 0 hours were refrigerated until they were sent to the analytical laboratory with the 96 hour samples for analysis. Upon receipt at the analytical laboratory samples were thoroughly agitated by hand and then vortex mixed, 1 mL was transferred into 9 mL of sterile phosphate buffered saline (PBS) to produce a 1×10^{-1} dilution. The dilution series was continued, agitating samples in a vortex mixer, until a final dilution of 1×10^{-10} was achieved.

100 µL of each dilution were plated in triplicate on Rose Bengal Agar with cycloheximide. Plates were incubated at 28 ± 2°C for approximately 96 hours. Plates with the highest number of easily distinguishable colonies were selected for enumeration. Counts were made manually on a pressure-sensitive colony counter and the results read directly from the display.

Temperature was continuously monitored in a surrogate vessel. The pH and temperature was additionally recorded at the start and end of the test from each treatment group.

Cell counts were compiled and the growth rate, yield and respective percentage inhibition values were calculated from the cell densities. Comparative analysis was performed (using ToxCalc v.5.023) on both the growth rate and yield after 96 hours to determine if there were any statistical differences between the treatment groups using Dunnett's Test (two-tailed, α = 0.05).

Results and Discussion

Measured test item concentrations ranged from 16.6 % at the start of the test, dropping to 1.0 % at the end (see Table 8.2.3/01-1).

Table 8.2.3/01-1: Analytical results

Treatment	**Mean Spore Count (CFU/L)**	
	0 Hour	**96 hour**
Untreated Control	ND	ND
Tween 80 Control	ND	ND
Attenuated Control	ND	ND
Test Item	3.0×10^6 (15.6%)	1.9×10^5 (1.0%)

ND: not detected

Values in parenthesis = % theoretical nominal 1.91×10^7 CFU/L

The measured *B. bassiana* concentration in the 0.1 mg *B. bassiana* concentrate/L exposure solution was lower than the target dose in fresh and aged samples. The accurate enumeration of microbial substances from aquatic systems is notoriously difficult due to the hydrophobic nature of most spores, their heterogeneous distribution throughout the medium, and the reduced viability of aerobic spores after immersion in water. This would explain the low concentrations of *B. bassiana* that were measured in the aged 0.1 mg *B. bassiana* concentrate/L exposure solution.

Water quality throughout the test was generally within acceptable limits. During the exposure period the pH ranged from 7.3 to 8.7 and temperature ranged from 19.3 to 24.1 °C.

The algae population in the control vessels grew well, resulting in an average cell density of 3.5×10^6 cells/mL after 96 hours. The mean specific rate and yield for each treatment group is presented in the tables below.

Table 8.2.3/01-1: Mean specific growth rate (/day) in each treatment at 24, 48, 72 and 96 hours

Treatment	Mean specific growth rate (/day)					Percent Inhibition 0-96 hour[§] (%)
	0-24 hours	24-48 hours	48-72 hours	72-96 hours	0-96 hour	
Untreated Control	1.43	1.52	1.63	1.27	1.46	-
Tween 80 Control	1.27	1.58	1.72	1.35	1.48	-1.1
Attenuated Control	1.19	1.71	1.76	1.31	1.49	-2.1
Test Item	1.30	1.67	1.81	1.18	1.49	-1.8

[§]: Percent inhibition relative to the control. A positive number represents a decrease and a negative number represents an increase, relative to the control

Table 8.2.3/01-2: Mean yield (×104 cells/mL) in each treatment at 24, 48, 72 and 96 hours

Treatment	Yield ($\times 10^4$ cells/mL)				Percent Inhibition 0-96 hour (%)
	24 hours	48 hours	72 hours	96 hours	
Untreated Control	3.27	18.4	99.9	349	-
Tween 80 Control	2.63	16.5	95.9	370	-6.1
Attenuated Control	2.38	17.3	106	393	-12.8
Test Item	2.71	18.6	118	386	-10.7

[§]: Percent inhibition relative to the control. A positive number represents a decrease and a negative number represents an increase, relative to the control

No significant effect was determined in the Tween 80 control, attenuated control or PRRI 5339 treatment when compared to the untreated control.

Validity Criteria

The mean coefficient of variation (CV) for daily growth rates (0-24, 24-48, 48-72 and 72-96 hours) in the control was 14.8% (< 35 %). The overall (0-96 hours) growth rate CV for the control cultures was 2.3% (< 7%). The biomass in the control cultures increase exponentially by a factor of at least 100. The validity criteria specified in OECD Guideline 201 were satisfied.

Conclusions

Exposure of *Pseudokirchneriella subcapitata* to PPRI 5339 at a nominal concentration of 0.1 mg/L equivalent to 1.91×10^7 CFU/L (initial mean measured 3.0×10^7 CFU/L and 1.9×10^5 CFU/L after 96 hours; geometric mean of 7.38×10^5 CFU/L) resulted in no significant inhibition in specific growth rate or yield.

Comments by RMS: The test was performed according to the US EPA and OECD testing guidelines. The dose level as per OPPTS 885.4300 should be at a "maximum label rate" meaning the amount of active ingredient to be used per land area or applied directly to the surface of a 15 cm column of water. The current calculated PECsw in a water depth of 0.3 m is 5.8×10^5 CFU/L which is equivalent to 1.2×10^6 CFU/L in 0.15 m layer of water. As the geometric mean measured concentration in the test was 7.38×10^5 CFU/L, the RMS considers that the criterion was fulfilled. The test is considered valid and the results can be used in the risk assessment.

TOXIN/METABOLITE FROM ACTIVE INGREDIENT

Presence of spores in recirculation water in the greenhouse cannot be excluded (especially for non-soil bound use), which in turn can result in exposure of surface water. The metabolites however, will be limited to the host insects and the chance of infected insects being present in the reciculation water in the greenhouses is low. *Beauveria bassiana* does not multiply or show metabolic activity in the absence of hosts insects, and therefore accumulation of the metabolites in the aquatic environment as result of insect's exposure in the greenhouses is highly unlikely. Nevertheless, all the information available from the peer-reviewed literature will be added in this document.

B.9.2.4 Effects on plants other than algae (Annex IIM 8.2.4; Annex IIIB 10.2)

No data were submitted. Data are not required as *Beauveria bassiana* strain PPRI 5339 is not a plant pathogen and there are no reported adverse effects on terrestrial plants by any strains (also see B.2.1.6).

B.9.2.5 Summary of the studies on aquatic organisms toxicity, infectiveness and pathogenicity

Table 9.2.5.-01 Summary of the studies on effects on aquatic organisms treated with *Beauveria bassiana*.

Species	Test duration	Dose range	Results/End point	Observations	Reference
TOXICITY					
Inland Silverside	9 days	8.3×10^3 – $1.2 \times$	No dose-	mortality,	Fred J. Genther

Species	Test duration	Dose range	Results/End point	Observations	Reference
Fish (*Menidia beryllina*)		10^7 spores/ml	response effect, larval hatch and thus embryo death was significantly effected at 8.3 10^4 and 8.3 x 10^5 spores/ml	embryo rupture	and Douglas P. Middaugh, 1992
Grass shrimp (*Palaemonetes pugio)*	12 days (embryos) 3 days (larva) 7 days(adult)	10^4 – 10^6 spores/ml	LD50 > 1.6 x 10^6 spores/ml (embryos) LD50 < 5 x10^5 spores/organism	mortality	Genthner, FJ. Et al, 1994b
Fresh green algae (*P. subcapitata*)	96 h	3.0 x 10^7 CFU/L (im)	E_y/E_rC50 > 7.38 x 10^5 CFU/L	Growth rate and yield	Forbes, T. 2015 b
INFECTIVENESS					
Inland Silverside Fish (*Menidia beryllina*)	9 days	8.3 x 10^3 – 1.2 x 10^7 spores/ml	No dose-response effect	Advanced hyphal growth on chorion surface, ruptured embryos	Fred J. Genther and Douglas P. Middaugh, 1992
Grass shrimp *Palaemonetes pugio*	12 days (embryos) 3 days (larva) 7 days(adult)	10^4 – 10^6 spores/ml		Mycelial proliferation throughout the tissue of 2 out of 75 embryos. No infection of larvae. No infection of adults exposed via	Genthner, FJ. Et al, 1994b

Species	Test duration	Dose range	Results/End point	Observations	Reference
				water. Infection of the adults exposed throughintrahemocoelic injections.	
Fresh green algae (*P. subcapitata*)	96 h	3.0 x 10^6 CFU/L (im)	E_y/E_rC50 > 7.38 x 10^5 CFU/L	No infectivity	Forbes, T. 2015 b
PATHOGENICITY					
Inland Silverside Fish (*Menidia beryllina*)	9 days	8.3 x 10^3 – 1.2 x 10^7 spores/ml	No dose-response effect		Fred J. Genther and Douglas P. Middaugh, 1992
Grass shrimp *Palaemonetes pugio*	12 days (embryos) 3 days (larva) 7 days(adult)	10^4 – 10^6 spores/ml		Infection resulted in dead of the 2 embryos and 95% of the adults exposed throughintrahemocoelic injections	Genthner, FJ. Et al, 1994b

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Table 9.2.5.-02. Summary of the studies on toxicity on aquatic organisms treated with metabolite from the active ingredient or the plant protection product.

Species	Test duration	Dose range	Results/Endpoint	Observations	Reference
METABOLITE beauvericin					
Americamysis bahia (formerly *Mysidopsis bahia*)	96-h 2 w 3w	0.13, 0.22, 0.36, 0.60 mg/L	LC50 0.56 mg/L (96 h) LC50 0.41 mg/L (2 w) LC50 0.45 mg/L (3 w)	mortality	Genthner F.J. et al. 1994

B.9.2.6 Risk assessment for aquatic organisms

According to chapter B.8.1.2 the maximum spore density in surface water after a single application would be 4760 CFU/L. Summarising the maximum amount of possible applications in a growth season without taking into account a loss in spore viability, the maximum estimated density would reach 5.8 x 10^5 CFU/L. In the study with *Menidia beryllina,* some responses were observed although these were not dose-response related. The lowest concentration which caused an effect was 8.3 x 10^7 CFU/L which is higher than the current PEC water.

A toxicity value of 1.6 x 10^6 CFU/ml was found for the freshwater shrimp *Palaemonetes pugio,* this is equivalent to 1.6 x 10^9 CFU/L. This value is well above the maximum estimated environmental density in surface water.

For algae, an endpoint of 7.38 x 10^5 CFU/L is available, endpoint close to the maximum PECsw. Considering that the fungus is an enthomopathogenic organism it is not expected that it will have a detrimental effect on algae.

Toxicity of beauvericin to aquatic invertebrates

The lowest toxicity reported for beauvericin to aquatic invertebrates is an LC50 of 0.56 mg/L derived for *Americamysis bahia*. This can be used for the risk assessment. However, as no exposure of the aquatic environment to the metabolites is expected (see above the section on “Toxins/metabolites”), no risk assessment will be conducted.

B.9.3 Effects on bees (Annex IIM 8.3; Annex IIIB 10.3)

B.9.3.1 Toxicity

ACTIVE INGREDIENT

Study 8.3-01

Reference:	Younger C (2012). *Beauveria bassiana* PPRI 5339 spore concentrate. Honey Bee, *Apis mellifera* non-target microbial testing. Stillmeadow Inc., 12852 Park One Drive, Sugar Land TX 77478. Study Code: 16339-12 Unpublished
Guideline:	OCSPP 885. 4380
GLP:	yes
Material and methods:	
Micro-organism	*Beauveria bassiana* PPRI 5339 spore concentrate Batch: S627 C2. Purity: 2.06 × 10^{11} spores/g. Positive control: dimethoate 0.1 mg/L
Test species:	*Apis mellifera*
Number of test animals:	200 per treatment

Treatments:	19.4 g/L which was equivalent to 4.0 × 10^9 CFU/mL
Duration:	10 days
Test conditions:	35±5°C, rel.hum. 30-90%, dark
Deviations from guideline	The provided Certificate of Analysis was not accompanied by a GLP compliance statement. Stability information was not provided to the testing facility. Mixture analysis was not performed. The bees were not dosed at 10× the maximum field application rate (MFAR) because the test substance did not form a homogenous dispersion at 194 g/L. The bees were dosed at the MFAR of 19.4 g/L instead. The protocol deviation had an effect on the outcome of the study. Instead of evaluation of the test substance at 10× MFAR, evaluation was determined at MFAR
Life stage of supplied test bees:	young adults
food	50% sucrose ad libitum
Observations:	pathogenicity, toxicity, mortality, food consumption

Summary

This study was designed to assess the acute oral toxicity potential of the microbial pest control agent (MPCA) test substance, *Beauveria bassiana* PPRI 5339 spore concentrate when administered to the honey bee at the rate of 19.4 g/L which was equivalent to 4.0 × 10^9 CFU/mL. Honey bees, *Apis mellifera*, divided into four groups – Group I – untreated (vehicle control), Group II – inactive *Beauveria bassiana* PPRI 5339 spore concentrate, Group III – *Beauveria bassiana* PPRI 5339 spore concentrate and Group IV – dimethoate (positive control). Each group consisted of 2 cages of 100 bees.

Bees in group III were dosed with the test item at 19.4 g/L. Given that the spore content of the test substance was 2.06 × 10^{11} spores per g the concentration of the test solution was 4.0 × 10^9 CFU/mL. This is 200 × the maximum field application rate (MFAR). All test solutions were dispensed to the bees in glass jars using standard beehive feeders. It was not clearly reported however, it is assumed that the feeding of the test solutions continued during the whole experiment.

The bees were observed daily for pathogenicity, toxicity or mortality. Observations were made until mortality in the control group exceeded 20%. The amount of sucrose solution fed to each group was recorded for each replicate.

Dose verification was performed by diluting 19.4 g of the test material in 1 L of a 50% sucrose solution. This was plated onto Rose Bengal Agar. The plates were incubated for 48 h at 28°C.

Statistical analysis

A one-way parametric analysis of variance (ANOVA) was performed on food consumption and daily mortality.

Results:

Dose verification

The results of the pre-dose enumeration yielded counts of 8.5 x 10^3 CFU/mL whereas the calculated intended dose rate was 4.0 × 10^9 CFU/mL.

toxicity

Results were evaluated by comparing corrected percent mortality between control group and treated group. Mortality observations and percent mortality are presented in Table 9.3.1-01.

Table 9.3.1-01: Percent mortality summary for effect of *Beauveria bassiana* strain PPRI 5339 on honey bees

Day	Mortality (%)				p-value
	Group I Untreated	Group II Inactive	Group III Active	Group IV Dimethoate	
1	0.5 a	0.5 a	0.0 a	1.0 a	0.3813
2	1.0 a	5.0 ab	11.5 b	1.0 a	0.0112
3	2.0 a	51.5 b	69.0 c	1.0 a	<0.0001
4	6.0 a	81.5 b	97.5 c	4.0 a	<0.0001
5	7.5 a	94.5 b	100.0 b	8.5 a	<0.0001
6	10.0 a	100.0 b	100.0 b	19.5 a	<0.0001
7	14.0 a	100.0 b	100.0 b	26.5 c	<0.0001
8	17.5 a	100.0 b	100.0 b	38.5 c	<0.0001
9	17.5 a	100.0 b	100.0 b	43.5 c	<0.0001
10	24.5 a	100.0 b	100.0 b	52.5 c	<0.0001

Different letter within the same row indicate significance at p<0.05

Mortality was 100% for bees fed the inactive test substance and by Day 5 for bees that were fed the active day substance. The study ended on Day 10 when mortality in the control group exceeded 20%.

Food consumption

Comparisons for food consumption could not be made due to the high mortality in the groups.

Conclusion

Given that >50% mortality had occurred in the group treated with the inactive *Beauveria bassiana* by Day 3 and that 100% mortality had occurred by Day 6 this test is not considered to be reliable. Also, the pre-dose enumeration yielded counts of 8.5 x 10^3 CFU/mL. This is far lower than the target dose of 4.0 x 10^9 CFU/mL. It is therefore considered that this study is unacceptable for use in the authorisation process for *Beauveria bassiana* strain PPRI 5339.

Comment by RMS:

Considering the high mortality in the inactive control, the results seen with the active bacteria cannot be attributed to the adverse effects caused by the active microorganism under the field conditions. **The results of this test cannot be used in the risk assessment.**

Study 8.3/02

Reference: Younger C (2012). *Beauveria bassiana* PPRI 5339 spore concentrate. Honey Bee, *Apis mellifera* non-target microbial testing. Stillmeadow Inc., 12852 Park One Drive, Sugar Land TX 77478. Study Code: 16984-12 Unpublished

Guideline: OCSPP 885. 4380

GLP:	yes
Material and methods:	
Micro-organism	*Beauveria bassiana* PPRI 5339 spore concentrate Batch no S627 C2, purity: 2.06×10^{11} spores/g
Test species:	*Apis mellifera*
Number of test animals:	200
Treatments:	toxic ref: Dimethoate 0.1 mg/L
Duration:	16 days
Test conditions:	Temperature 35±5° C· Relative Humidity 30-90% · Dark
Deviations from guideline	Characterization and stability information was not provided to the testing facility.
life stage of bees	young adult
food	50% sucrose ad libitum
Observations:	pathogenicity, toxicity or mortality

Honey bees, *Apis mellifera*, divided into four groups: untreated (vehicle control), inactive *Beauveria bassiana* PPRI 5339 spore concentrate, *Beauveria bassiana* PPRI 5339 spore concentrate and dimethoate (positive control). Each group consisted of 2 cages of 100 bees.

Bees in the group treated with the active spore concentration were dosed with 400 g MCPA/ha. To prepare this 1.57 mg of the test substance was suspended in 1 L of a 50% sucrose solution. Given that the spore content of the test substance was 2.06×10^{11} spores per g the concentration of the test solution would be 3.23×10^{5} CFU/mL. All test solutions were dispensed to the bees in glass jars using standard beehive feeders. Feeding of the test solutions continued during the whole experiment.

The bees were observed daily for pathogenicity, toxicity or mortality. Observations were made until mortality in the control group exceeded 20%.

Dose verification was performed by diluting 1 mL of the test solution in 10 mL with PBS. This was plated onto DRBC agar. The plates were incubated for 72 h at 28°C.

A one-way parametric analysis of variance (ANOVA) with Tukey-Kramer's Multiple Comparisons Test was performed on food consumption and daily mortality.

Results:

This study was designed to assess the acute oral toxicity potential of the microbial pest control agent (MPCA) test substance, *Beauveria bassiana* PPRI 5339 Spore Concentrate, when administered to the honey bee, *Apis mellifera,* at the application rate of 400 g/hectare, which was 10X the maximum field application rate (MFAR) of 40 g/hectare. The pre-dose enumeration analysis of the active and inactive test substance dosing solutions resulted in counts of 3.9×10^{4} Colony Forming Unit (CFU)/mL and 0 CFU/mL, respectively.

Food consumption in the active test substance group was significantly greater than the inactive group. There were no other significant differences among the groups in food consumption.

Results were evaluated by comparing corrected percent mortality between control group and treated group. Mortality observations and percent mortality are presented in Table 9.3.1-02.

Table 9.3.1-02: Daily mean mortality summary for effect of *Beauveria bassiana* strain PPRI 5339 on honey bees

Day	Mortality (%)				p-value
	Vehicle Control	Inactive	Active	Dimethoate	
0 (4 hours)	0.0	0.0	0.0	0.5	0.4789
1	0.0 a	0.0 a	0.0 a	1.0 a	0.4789
2	0.0 a	0.0 a	0.0 a	1.0 a	0.4789
3	0.5 a	0.5 a	1.0 a	1.0 a	0.8766
4	1.0 a	0.5 a	3.0 a	2.0 a	0.5607
5	1.5 a	1.5 a	6.5 a	2.5 a	0.6418
6	1.5 a	1.5 a	6.5 a	3.0 a	0.6369
7	2.5 a	2.0 a	6.5 a	3.5 a	0.7357
8	2.5 a	3.0 a	9.0 a	4.0 a	0.7177
9	2.5 a	2.5 a	10.0 a	5.0 a	0.6086
10	3.0 a	4.0 a	12.5 a	5.0 a	0.4316
11	5.0 a	4.5 a	16.5 a	15.0 a	0.4561
12	8.5 a	12.5 a	17.5 a	23.0 a	0.8116
13	11.5 a	21.0 a	19.0 a	30.0 a	0.8664
14	14.0 a	20.0 a	21.5 a	31.5 a	0.8671
15	19.0 a	22.5 a	24.0 a	42.0 a	0.7468
16	24.0 a	40.0 a	27.0 a	57.5 a	0.7487

Different letter within the same row indicate significance at $p<0.05$

The study ended on Day 16 when the mortality in the control group exceeded 20%. The cumulative mean number of dead honey bees in the control, inactive, active, and positive control groups on Day 16 were respectively, 24.0, 40.0, 27.0 and 57.5 (Table 2). Corrected mortality for the inactive, active, and positive control groups on Day 16 was 21.1%, 3.9% and 44.1%, respectively.

Based on the results of this study, the test substance, *Beauveria bassiana* PPRI 5339 Spore Concentrate, in its active and inactive form was determined to be non-toxic to the honey bee when administered at 400 g/hectare, which was 10x the MFAR of 40 g/hectare. This would be equivalent to a concentration of the test solution of 3.23×10^5 CFU/mL but was actually found to be 3.9×10^4 Colony Forming Unit (CFU)/mL in the dose verification analysis.

Conclusion

The LT_{50} for the test item was determined to be greater than 16 days, and the LD_{50} was determined to be $>3.9 \times 10^4$ CFU/mL. However, mortality in the inactive control was unacceptably high meaning that this study cannot be used in the risk assessment.

Comment by RMS:

Considering the high mortality in the inactive control, the results seen with the active bacteria cannot be attributed to the adverse effects caused by the active microorganism under the field conditions. **The results of this test cannot be used in the risk assessment.**

The maximum field application rate (MFAR) is 2 × 10^7 CFU/mL (i.e. from one application). Therefore *Beauveria bassiana* strain PPRI 5339 was tested at a concentration 512 times lower than the MFAR.

Study 8.3/03

Reference:	Whittaker, M. Effects of *Beauveria bassiana* PPRI 5339 on the honeybee, *Apis mellifera*, in an acute oral and contact toxicity pre-test. APIS, Knaresborough Technology Park, Manse Lane, Knaresborough, North Yorkshire, HG5 8LF. Study No.: APIS-BASF-NGLP-001 Published: No
Guideline:	Modification of OECD 213 and 214, (OECD, 1998)
GLP:	no
Material and methods:	
Micro-organism	*Beauveria bassiana* PPRI 5339 Batch no: S826 Purity: 1 × 10^{11} CFU/g nominal
Test species:	*Apis mellifera*,
Number of test animals:	30
Treatments:	unclear dose rate. toxic ref: Perfekthion (dimethoate) 0.05, 0.10 and 0.15 µg/bee
Duration:	10 days
Test conditions:	The test was conducted in cylindrical Perspex tubes (length 180 mm, internal diameter 45 mm). 25 ± 2°C in the dark
Deviations from guideline	none
Observations:	mortality, signs of *Beauveria bassiana* infection

For the contact test, a 100 µL droplet of *Beauveria bassiana* PPRI 5339 was dosed onto the dorsal surface of the thorax of each bee at the Maximum Hazard Concentration (MHC) of the MPCA. For preparation of this, 0.20 g of MPCA was diluted in 100 g deionised water with a small amount of Tween 80 as adjuvant. Since it is normal practice to apply a 1 µL droplet in contact toxicity tests the test solution was diluted a further 100-fold in order to compensate for the application of a 100 µL droplet.

For the oral test, 200 µL was administered to each cage. This was replaced with untreated 50% sucrose solution after all of the dose had been consumed. The assumption made was that equal feeding between the bees would result in each bee receiving 20 µL.

A single mortality assessment was made 10 days after treatment. The bees in each chamber were classified as alive, affected, moribund or dead.

Results:

The test chamber and feeding tube designswere considered suitable for honey bee studies. Control mortality at 10 days after treatment was acceptable, and the test chamber design was considered fit for purpose.

An application volume of 100 µl per bee in the contact study represented the maximum realistic volume that could be applied before run-off. Control mortality in the contact group was lower than the oral group, suggesting that the higher application volume had no negative effect on bee survival.

Mortality following oral and contact exposure to the MCPA *Beauveria bassiana* PPRI 5339 applied at the MHC

Mortality was 30% and 26.7% for application via the oral and contact routes of exposure, respectively. As mortality was <50% the oral and contact LD_{50} values were greater than the MHC and the LT_{50} was greater than the 10-day study duration.

Conclusions

In a preliminary test, test chamber and feeding tube suitability, control mortality achievable in a 10-day test, feasibility of application of 100 µL as a topical dose and mortality following oral and contact exposure to the microbial pest control agent *Beauveria bassiana* strain PPRI 5339 at the MHC were evaluated.

Everything was acceptable. Also at the MHC *Beauveria bassiana* strain PPRI 5339 exhibited limited infectivity to honey bee. The LT50 was >10 days and the LD_{50} was >MHC.

Comment by RMS:

In this preliminary test a test design was tested for its suitability for honey bee testing with *Beauveria bassiana* strain PPRI 5339 and maybe micro-organisms in general. **The dose is unclear and thus the results cannot be used in the risk assessment.**

Study 8.3/04

Reference:	Whittaker M (2013), Effects of *Beauveria bassiana* PPRI 5339 on the bumblebee, *Bombus terrestris*, in an acute contact toxicity test. APIS, Knaresborough Technology Park, Manse Lane, Knaresborough, North Yorkshire, HG5 8LF. Study No.: APIS-BASF-004 Unpublished
Guideline:	Modification of OECD 214, (OECD, 1998); OPPTS 885.4340 (EPA, 1996); OPPTS 885.4380 (EPA, 1996)
GLP:	yes
Material and methods:	
Micro-organism	*Beauveria bassiana* PPRI 5339 Batch no: S826 Purity: 1×10^{11} CFU/g nominal
Test species:	*Bombus terrestris*
Number of test animals:	30 per treatment
Treatments:	toxic ref: Perfekthion (dimethoate) 0.05, 0.10 and 0.15 µg/bee, water control, adjuvant control, 3.1×10^6 CFU/mL (3.1×10^3 CFU/ µL= 3.1×10^5 CFU/bee).
Duration:	10 days
Test conditions:	temp 22.4 – 27.2°C. humidity 43.7-89.2%. Dark
Deviations from	none

guideline

Observations: mortality, infectivity

Five young worker bumblebees (*Bombus terrestris*) were placed into each of the six test chambers per treatment. The bees were subject to treatment with either a water control solution, Tween 80 adjuvant, inactive *Beauveria bassiana* strain PPRI 5339, active *Beauveria bassiana* strain PPRI 5339 or dimethoate positive control (at low, medium or high concentrations).

The test was conducted in cylindrical Perspex tubes (length 180 mm, internal diameter 45 mm). A single hole (9 mm diameter) had been drilled halfway along the tube. This served as a port for the feeding tubes.

A 100 µL droplet of *Beauveria bassiana* PPRI 5339 was dosed onto the dorsal surface of the thorax of each bee at the Maximum Hazard Concentration (MHC) of the MPCA. For preparation of this, 0.202 g of MPCA was diluted in 100.02 g deionised water with 0.46 g Tween 80 leading to a concentration of 0.00201 g MCPA/mL. Considering the purity of 1×10^{11} CFU/g, the stock solution nominally contained 2.01×10^{8} CFU /mL. Since it is normal practice to apply a 1 µL droplet in contact toxicity tests the test solution was diluted a further 100-fold in order to compensate for the application of a 100 µL droplet. Thus, the concentration in the solution applied to the bees was nominally 2.01×10^{6} CFU /mL (actually 3.1×10^{6} CFU/mL, see below). The inactive MCPA was applied at the same dose as for the active *Beauveria bassiana*.

Dose verification was performed by diluting the spray solution in a logarithmic scale and plating 100 µL from each dilution onto Rose Bengal Agar. The plates were incubated for 96 h.

Mortality assessments were made approximately 4-6 hours after treatment and every 24 hours thereafter, for ten days. At each observation, the bees in each chamber were classified as alive, affected, moribund or dead.

Bees that died during the study were removed from the test chamber and placed in individual Petri dishes. These dishes were placed in an incubator to allow for the growth of any infective organisms. These were subsequently plated onto Rose Bengal Agar to confirm their identity. At the end of the study, three bees from each test chamber were subjected to autopsy. External signs of infection were noted before internal examination of the head and abdomen.

Results

One bee died in the MPCA treatment during the study which showed no overt signs of infections after post-mortem incubation or any sign of infection at autopsy. A further 17 bees were euthanized at study termination and autopsied, none of which showed any signs of infection.

The mortality per treatment group at 10 days after treatment is shown in Table B.9.3.1-03.

Table 9.3.1-03: Mortality of bumble bees (*Bombus terrestris*) by treatment group at 10 days after treatment with *Beauveria bassiana* strain PPRI 5339

Treatment	Observations at 10 days after treatment (30 bees per treatment)			
	Alive	Affected	Moribund	Dead
Water control	28	0	0	2

Adjuvant	27	0	0	3
Inactive MPCA	26	0	0	4
MPCA	29	0	0	1
Toxic Reference (Low)	0	0	0	30
Toxic Reference (Medium)	0	0	0	30
Toxic Reference (High)	0	0	0	30

Dose verification revealed that the concentration of MPCA applied to the bees was 3.1 × 10^6 CFU/mL feeding solution (3.1 x 10^3 CFU/ µL).

For application of *Beauveria bassiana* strain PPRI 5339 at the maximum application rate of 1.0 x 10^{13} CFU/ha (in the minimum application volume of 500 L/ha, corresponding to 2 × 10^{10} CFU/L (= 2 × 10^7 CFU/mL) and assuming that each foraging bee would encounter a 1 µL droplet, then the maximum theoretical amount encountered per bee would be 2 x 10^4 CFU.
Since each bee in this test was treated with a 100 µL droplet, the dose was 3.1 x 10^5 CFU/bee which represents 16 times the maximum field rate. As the test item was applied at a single dose representing the Maximum Hazard Concentration (16 times the maximum field rate), the calculation of an LD_{50} is not appropriate.

Conclusions

At the Maximum Hazard Concentration, *Beauveria bassiana* PPRI 5339 exhibited no infectivity or pathogenicity to bumblebees. The LT_{50} for the test item >10 days. The LD_{50} was >3.1 × 10^5 CFU/bee.

Comment by RMS:

The RMS does not understand why the assumption was made that one bumblebee will come into contact with 1 µL product. Considering the concentration in the formulation of 4 x 10^{12} CFU/L and the application in the field of 100 mL product/100L water, the single maximum field rate will be 4 x 10^9 CFU/L water. This means that the current test was conducted (i.e. 3.1 × 10^6 CFU/mL) at a rate similar to the current maximum single application rate and not at 10-100 times as recommended by the guidelines. No signs of infectivity or pathogenicity were detected upon autopsy and no overt signs of infections after post-mortem incubation. The contact LT_{50} for *Beauveria bassiana* PPRI 5339 at the test rate of 3.1 × 10^6 CFU/mL is >10 days.

Study 8.3/05

Reference: Whittaker M (2013), Effects of *Beauveria bassiana* PPRI 5339 on the bumblebee, *Bombus terrestris*, in an acute oral toxicity test. APIS, Knaresborough Technology Park, Manse Lane, Knaresborough, North Yorkshire, HG5 8LF. Study No.: APIS-BASF-005
unpublished

Guideline: Modification of OECD 213, (OECD, 1998), OPPTS 885.4340 (EPA, 1996), OPPTS 885.4380 (EPA, 1996)

GLP: yes

Material and methods:

Micro-organism	*Beauveria bassiana* PPRI 5339 Batch no: S826 Purity: 1 × 10^{11} CFU/g nominal
Test species:	*Bombus terrestris*
Number of test animals:	30 per treatment
Treatments:	toxic ref: Perfekthion (dimethoate) 0.05, 0.10 and 0.15 µg/bee, water control, adjuvant control, 3.1 × 10^6 CFU/mL (3.1 x 10^3 CFU/ µL= 3.1 x 10^5 CFU/bee).
Duration:	10 days
Test conditions:	temp 25 ± 2°C. humidity 90% first 4-6 hours than 50-70%. Dark
Deviations from guideline	none
Observations:	mortality, infectivity

Five young worker bumblebees (*Bombus terrestris*) were placed into each of the six test chambers per treatment. The bees were subject to treatment with either a sucrose control solution, Tween 80 adjuvant, inactive *Beauveria bassiana* strain PPRI 5339, active *Beauveria bassiana* strain PPRI 5339 or dimethoate positive control (at low, medium or high concentrations).

The test was conducted in cylindrical Perspex tubes (length 180 mm, internal diameter 45 mm). A single hole (9 mm diameter) had been drilled halfway along the tube. This served as a port for the feeding tubes.

The bees were dosed with *Beauveria bassiana* strain PPRI 5339 at the Maximum Hazard Concentration (MHC) of the MPCA. For preparation of this, 0.202 g of MPCA was diluted in 100.02 g 50% sucrose solution prepared in deionised water with 0.46 g Tween 80 leading to a concentration of 0.00201 g/mL. Considering the purity of 1 × 10^{11} CFU/g, this test solution nominally contained 2.01 × 10^8 CFU /mL (actually 2.9 × 10^8 CFU/mL, see below). 500 µL of test solution was provided and this was completely consumed within a 4-6 hour period. It is assumed that the bees shared the dose equally meaning that each bee consumed 100 µL of a 2.9 × 10^8 CFU/mL solution of MCPA. The inactive MCPA was applied at the same dose as for the active *Beauveria bassiana*. After the initial 4-6 h treatment period, bees were fed untreated sucrose solution and pollen *ad libitum*.

Dose verification was performed by diluting the spray solution in a logarithmic scale and plating 100 µL from each dilution onto Rose Bengal Agar. The plates were incubated for 96 h.

Mortality assessments were made approximately 4-6 hours after treatment and every 24 hours thereafter, for ten days. At each observation, the bees in each chamber were classified as alive, affected, moribund or dead.

Bees that died during the study were removed from the test chamber and placed in individual petri dishes. These dishes were placed in an incubator to allow for the growth of any infective organisms. These were subsequently plated onto Rose Bengal Agar to confirm their identity.

At the end of the study, three bees from each test chamber were subjected to autopsy. External signs of infection were noted before internal examination of the head and abdomen.

Results

The study complied with validity criteria. Mortality in the sucrose and adjuvant control groups did not exceed 20%.

The estimated LD_{50} for dimethoate was 0.11 µg/bee, which is within the expected range of 0.10 to 0.35 µg/bee.

Dose verification revealed that the concentration of MPCA in the oral solution was 2.9×10^8 CFU/mL (2.9×10^5 CFU/µL).

For application of *Beauveria bassiana* strain PPRI 5339 at the maximum application rate of 1.0×10^{13} CFU/ha (in the minimum application volume of 500 L/ha, corresponding to 2×10^{10} CFU/L (= 2×10^7 CFU/mL).

The mortality per treatment group at 10 days after treatment is shown in Table 9.3.1-04.

Table 9.3.1-04: Mortality of bumble bees (*Bombus terrestris*) by treatment group at 10 days after treatment with *Beauveria bassiana* strain PPRI 5339

Treatment	Observations at 10 days after treatment (30 bees per treatment)			
	Alive	Affected	Moribund	Dead
Sucrose control	27	0	0	3
Adjuvant	28	0	0	2
Inactive MPCA	28	0	0	2
MPCA	28	0	0	2
Toxic Reference (Low)	0	0	0	30
Toxic Reference (Medium)	0	0	0	30
Toxic Reference (High)	0	0	0	30

Two bees died in the MPCA treatment during the study, neither of which showed overt signs of infections after post-mortem incubation or any sign of infection at autopsy. A further 16 bees were euthanized at study termination, none of which showed any signs of infection.

Since each bee in this test was assumed to have consumed 100 µL, the dose consumed was equivalent to 2.9×10^7 CFU/bee, which represents 15 times the maximum field rate. As the test item was applied at a single dose representing the Maximum Hazard Concentration (15 times the maximum field rate), the calculation of an LD_{50} is not appropriate.

Conclusion:

At the Maximum Hazard Concentration, *Beauveria bassiana* PPRI 5339 exhibited no infectivity or pathogenicity to bumblebees. The LT_{50} for the test item >10 days. The LD_{50} would be $>2.9 \times 10^7$ CFU/bee.

Comment by RMS:

The RMS does not understand why the assumption was made that this test covers 15 times the maximum field rate. Considering the concentration in the formulation of 4×10^{12} CFU/L and the application in the field of 100 mL product/100L water, the single maximum field rate will be 4×10^9 CFU/L water. This means that the current test was conducted (i.e. 2.9×10^8 CFU/mL which is 2.9 ×

10^{11} CFU/L) at a rate 10-100 times as recommended by the guidelines. No signs of infectivity or pathogenicity were detected upon autopsy and no overt signs of infections after post-mortem incubation. The oral LT_{50} for *Beauveria bassiana* PPRI 5339 at the test rate of 2.9×10^8 CFU/mL is >10 days. These results can be used for assessment.

Study 8.3/06

Reference:	Whittaker M (2013), Effects of *Beauveria bassiana* PPRI 5339 on the honeybee, *Apis mellifera*, in an acute contact toxicity test. APIS, Knaresborough Technology Park, Manse Lane, Knaresborough, North Yorkshire, HG5 8LF. Study No.: APIS-BASF-001
Guideline:	Modification of OECD 214, (OECD, 1998), OPPTS 885.4340 (EPA, 1996), OPPTS 885.4380 (EPA, 1996)
GLP:	yes
Material and methods:	
Micro-organism	*Beauveria bassiana* PPRI 5339 Batch no: S826 Purity: 1×10^{11} CFU/g nominal
Test species:	*Apis mellifera*
Number of test animals:	30 per treatment
Treatments:	toxic ref: Perfekthion (dimethoate) 0.05, 0.10 and 0.15 µg/bee, water control, adjuvant control, 3.1×10^6 CFU/mL (3.1×10^3 CFU/ µL= 3.1×10^5 CFU/bee).
Duration:	10 days
Test conditions:	temp 21.7 – 26.3°C. humidity 27.4 – 50.9%, increased for the first 4-6 hours. Dark
Deviations from guideline	The temperature in the growth chamber exceeded 27 °C for extended periods, reaching 31 °C on two occasions.
Observations:	mortality, infectivity

Ten young worker honeybees (*Apis mellifera*) placed into each of the three test chambers per treatment. The bees were subject to treatment with either a water control solution, Tween 80 adjuvant, inactive *Beauveria bassiana* strain PPRI 5339, active *Beauveria bassiana* strain PPRI 5339 or dimethoate positive control (at low, medium or high concentrations).

The test was conducted in cylindrical Perspex tubes (length 180 mm, internal diameter 45 mm). A single hole (9 mm diameter) had been drilled halfway along the tube. This served as a port for the feeding tubes.

A 100 µL droplet of *Beauveria bassiana* PPRI 5339 was dosed onto the dorsal surface of the thorax of each bee at the Maximum Hazard Concentration (MHC) of the MPCA. For preparation of this, 0.20 g of MPCA was diluted in 100 g deionised water with 0.10 g Tween 80 leading to a concentration of 0.002 g MCPA/mL. Considering the purity of 1×10^{11} CFU/g, the stock solution nominally contained 2.0×10^8 CFU /mL. Since it is normal practice to apply a 1 µL droplet in contact toxicity tests the test solution was diluted a further 100-fold in order to compensate for the application of a 100 µL droplet.

Thus, the concentration in the solution applied to the bees was nominally 2.0 × 10^6 CFU /mL (actually 2.9 × 10^6 CFU/mL, see below). The inactive MCPA was applied at the same dose as for the active *Beauveria bassiana*.

Dose verification was performed by diluting the spray solution in a logarithmic scale and plating 100 µL from each dilution onto Rose Bengal Agar. The plates were incubated for 96 h.

Mortality assessments were made approximately 4-6 hours after treatment and every 24 hours thereafter, for ten days. At each observation, the bees in each chamber were classified as alive, affected, moribund or dead.

Bees that died during the study were removed from the test chamber and placed in individual petri dishes. These dishes were placed in an incubator to allow for the growth of any infective organisms. These were subsequently plated onto Rose Bengal Agar to confirm their identity.

At the end of the study, three bees from each test chamber were subjected to autopsy. External signs of infection were noted before internal examination of the head and abdomen.

Results

Dose verification revealed that the concentration of MPCA applied to the bees was 2.9 × 10^6 CFU/mL (2.9 x 10^3 CFU/ µL).

For application of *Beauveria bassiana* strain PPRI 5339 at the maximum application rate of 1.0 x 10^{13}/ha (in the minimum application volume of 500 L ha, corresponding to 2 × 10^{10} CFU/L (= 2 × 10^7 CFU/mL), and assuming that each foraging bee would encounter a 1 µL droplet, then the maximum theoretical amount encountered per bee would be 2 x 10^4 CFU.

The mortality per treatment group at 10 days after treatment is shown in Table 9.3.1-05.

Table 9.3.1-05: Mortality of honey bee (*Apis mellifera*) by treatment group at 10 days after treatment with *Beauveria bassiana* strain PPRI 5339

Treatment	Observations at 10 days after treatment (30 bees per treatment)			
	Alive	Affected	Moribund	Dead
Water control	26	0	0	4
Adjuvant	11	0	0	19
Inactive MPCA	21	0	0	9
MPCA	22	0	0	8
Toxic Reference (Low)	10	0	0	20
Toxic Reference (Medium)	4	0	0	26
Toxic Reference (High)	0	0	0	30

Eight bees died in the MPCA treatment during the study, neither of which showed overt signs of infections after post-mortem incubation. Seven of these bees and two surviving bees, euthanized at study termination, were autopsied, and showed no signs of infection.

Since each bee in this current study was treated with a 100 µL droplet then the dose was 2.9 x 10^5 CFU/bee which represents 15 times the maximum field rate. As the test item was applied at a single

dose representing the Maximum Hazard Concentration (15 times the maximum field rate), the calculation of an LD_{50} is not appropriate.

As mortality at 10 days in the MPCA was only 27% the LD_{50} is greater than the MHC. The LT_{50} is also greater than 10 days.

Conclusions:

At the Maximum Hazard Concentration, *Beauveria bassiana* strain PPRI 5339 exhibited limited infectivity to honey bees. The LT_{50} for the test item >10 days. The LD_{50} was >2.9 × 10^5 CFU/bee.

Comment by RMS:

The RMS does not understand why the assumption was made that this test covers 15 times the maximum field rate. Considering the concentration in the formulation of 4 x 10^{12} CFU/L and the application in the field of 100 mL product/100L water, the single maximum field rate will be 4 x 10^9 CFU/L water. This means that the current test was conducted (i.e. 2.9 × 10^6 CFU/mL which is 2.9 × 10^9 CFU/L) which is similar to the current maximum single field application rate but not at a rate 10-100 times as recommended by the guidelines. At the Maximum Hazard Concentration, *Beauveria bassiana* strain PPRI 5339 exhibited limited infectivity to honey bees. The contact LT_{50} at the test rate of 2.9 × 10^6 CFU/mL>10 days. These results can be used for assessment.

Study 8.3/07

Reference:	Whittaker M (2013), Effects of *Beauveria bassiana* PPRI 5339 on the honeybee, *Apis mellifera*, in an acute oral toxicity test. APIS, Knaresborough Technology Park, Manse Lane, Knaresborough, North Yorkshire, HG5 8LF. Study No.: APIS-BASF-002
Guideline:	Modification of OECD 214, (OECD, 1998), OPPTS 885.4340 (EPA, 1996), OPPTS 885.4380 (EPA, 1996)
GLP:	yes
Material and methods:	
Micro-organism	*Beauveria bassiana* PPRI 5339 Batch no: S826 Purity: 1 × 10^{11} CFU/g nominal
Test species:	*Apis mellifera*
Number of test animals:	30 per treatment
Treatments:	toxic ref: Perfekthion (dimethoate) 0.05, 0.10 and 0.15 µg/bee, sucrose control, adjuvant control, non-infective test item, 2.9 × 10^8 CFU/g
Duration:	10 days
Test conditions:	temp 21.7 – 26.3°C. humidity 27.4 – 50.9%, increased for the first 4-6 hours. Dark
Deviations from guideline	The temperature in the growth chamber exceeded 27 °C for extended periods, reaching 31 °C on two occasions.
Observations:	mortality, infectivity

Ten young worker bees were carefully aspired from the flight tent and placed into each of three test chambers per treatment using a large bore aspirator. The chambers were sealed and a glass feeding tube was inserted. Each tube was filled with the relevant feeding solution using a fine, and was weighed prior to insertion. The bees were dosed with *Beauveria bassiana* PPRI 5339 at the MHC of the MPCA. For preparation of this, 0.20 g of MPCA was diluted in 100 g 50% sucrose solution prepared in deionised water with 0.10 g Tween 80 leading to a concentration of 0.002 g/mL. Considering the purity of 1×10^{11} CFU/g, this test solution nominally contained 2.0×10^{8} CFU /mL (actually 2.9×10^{8} CFU/mL, see below). The inactive MCPA was applied at the same dose as for the active *Beauveria bassiana*. For dosing, 200 µL was administered to each cage. This was replaced with untreated 50% sucrose solution after all of the dose had been consumed. The assumption made was that equal feeding between the bees would result in each bee receiving 20 µL. An attempt was made to increase the relative humidity within the chamber to around 90% for the first 4-6 hours of exposure to give the MPCA optimal conditions for germination. After the initial 4-6 h treatment period, bees were fed untreated sucrose solution and pollen *ad libitum*.

Bees that died during the study were carefully removed from the test chamber and placed in a petri dish. These were placed in the incubator to allow for the growth of any infective organisms, which were subsequently plated on Rose Bengal Agar to check for their identity.

At the end of the study 3 bees from each test item chamber were subjected to autopsy. External signs of infection were noted before internal examination of the head (brain, hypopharangeal glands and cibarium) and abdomen (crop, proventiculus and vetriculus).

The study was conducted as a limit test defined as 10-100 times the maximum field application rate.

Results

Validity criteria

Mortality in the sucrose control was 6 bees out of 30 at 10 days after treatment. This does not exceed the validity threshold of 20%. The estimated LD_{50} for dimethoate was 0.11 µg/bee, which is within the expected range. The study was deemed to be valid.

Mortality

Mortality data are shown in table Table 9.3.1-06.

Table 9.3.1-06: Mortality by treatment group at 10 days after treatment

Treatment	Observations at 10 days after treatment (30 bees per treatment)			
	Alive	**Affected**	**Moribund**	**Dead**
Sucrose control	24	0	0	6
Adjuvant	20	0	0	10
Inactive MPCA	28	0	0	2
MPCA	21	0	0	9
Toxic Reference (Low)	0	0	0	30
Toxic Reference (Medium)	0	0	0	30
Toxic Reference (High)	0	0	0	30

The mean amount of solution consumed after 4 – 6 hours was 0.33 g per cage of 10 bees. If it is assumed that the bees shared the dose equally then each bee consumed 0.033 g.
Dose verification revealed that the concentration of MPCA in the oral solution was 2.9×10^8 CFU/mL.
For application of *Beauveria bassiana* strain PPRI 5339 at the maximum application rate of 1.0 x 10^{13}/ha (in the minimum application volume of 500 L/ha, corresponding to 2×10^{10} CFU/L (= 2×10^7 CFU/mL), and assuming that each foraging bee would consume 20 µL of nectar, the maximum amount consumed per bee would theoretically be 4 x 10^5 CFU.
Since each bee in this study was assumed to have consumed 0.033 g, the dose consumed was equivalent to 9.57 x 10^6 CFU/bee, which represents 24 times the maximum field rate. As the test item was applied at a single dose representing the Maximum Hazard Concentration (24 times the maximum field rate), the calculation of an LD_{50} is not appropriate.
Mortality at 10 days is only 30% and therefore the LD_{50} would be greater than the MHC. Also the LT_{50} is greater than 10 days.

Nine bees died of MPCA treatment during the study, of which five showed overt signs of infection in the form of profuse fluffy white fungal growth over the cadaver post-mortem incubation. This was subsequently identified as *Beauveria bassiana* after culturing. The remaining 4 bees that died during the study showed no signs of infection. Similarly, none of the bees euthanized at study termination showed any signs of infection. It is noted that the observed mortality may have (partly) been caused by the adjuvant, as the adjuvant control had a similar mortality level as the test item, of 33% (10 out of 30 bees).

Conclusion:

At the maximum hazard concentration *Beauveria bassiana* PPRI 5339 showed limited infectivity to honeybees. The oral LT_{50} for the test item at the test rate of 2.9×10^8 CFU/mL was determined to be >10 days and the LD_{50} was determined >2.9 x 10^8 CFU/g.

Comments by RMS:

The RMS does not understand why the assumption was made that one bee will come into contact with 20 µL product. Considering the concentration in the formulation of 4 x 10^{12} CFU/L and the application in the field of 100 mL product/100L water, the single maximum field rate will be 4 x 10^9 CFU/L water. This means that the current test was conducted (i.e. 2.9×10^8 CFU/mL which is 2.9×10^{11} CFU/L) at a rate 10-100 times higher as recommended by the guidelines. The results are used for risk assessment.

A summary of acceptable endpoints from the laboratory studies is given in the table below.

Table B.9.3.1-07: Toxicity effects of Active ingredient to bees

Test species/type of laboratory test	Applied dose CFUs/L	Observations	Test duration
Toxicity			
Bombus	3.1 x 10^9	No signs of infectivity or	10 days

terrestris/contact		pathogenicity were detected upon autopsy and no overt signs of infections after post-mortem incubation	
Bombus terrestris/oral	2.9 x 10^{11}	No signs of infectivity or pathogenicity were detected upon autopsy and no overt signs of infections after post-mortem incubation	10 days
Apis mellifera/contact	2.9 × 10^{9}	At the MHC, *Beauveria bassiana* strain PPRI 5339 exhibited limited infectivity to honey bees	10 days
Apis mellifera/oral	2.9 x 10^{11}	Nine bees out of 30 died of MPCA treatment during the study, of which five showed overt signs of infection in the form of profuse fluffy white fungal growth over the cadaver post-mortem incubation. This was subsequently identified as *Beauveria bassiana* after culturing	10 days

From literature:

MA 8.3/08

Citation: Meikle, WG, Mercadier, G, Holst, N, Girod, V (2008). Impact of two treatments of a formulation of *Beauveria bassiana* (Deuteromycota: Hyphomycetes) conidia on Varroa mites (Acari: Varroidae) and on honeybee (Hymenoptera: Apidae) colony health. *Experimental and Applied Acarology,* 46(1-4): 105-117

Guidelines: None

GLP: No

Abstract

Bee colonies in southern France were treated with conidia (asexual spores) from two strains of *Beauveria bassiana*. One strain was commercial (GHA) and the other had been isolated from Varroa mites in the region. Objectives were to evaluate treatment effect on colony weight, adult bee mass,

capped brood, and on Varroa fall onto sticky boards. Treatments included conidia formulated with either carnauba or candelilla wax powder, candelilla wax powder alone, or control; in two treatment groups, formulation was applied a second time after one week. Treatment did not affect colony health. Colonies treated twice with Bb05002 conidia and carnauba wax powder had significantly higher mite fall compared to colonies treated with blank candelilla wax powder. The proportion of fallen mites that were infected in both conidia treatments was higher than controls for 18 days after the second treatment. The number of fungal propagules on the bees themselves remained elevated for approximately 14 days after the second treatment.

Materials and methods

Four formulations were prepared:

- carnauba wax powder and conidia from *Beauveria bassiana* isolate 05002 (‘‘Bb05002 + carnauba’’);
- carnauba wax powder and conidia from *Beauveria bassiana* isolate GHA (‘‘BbGHA + carnauba’’);
- candelilla wax powder and 05002 conidia (‘‘Bb05002 + candelilla’’);
- and candelilla wax powder alone (‘‘candelilla alone’’).

The per colony dose of Bb05002 + carnauba and BbGHA + carnauba consisted of 1.0 g conidia of the respective isolate mixed with 9.0 g carnauba wax powder and 0.05 g hydrated silica as a flow agent. The per colony dose of Bb05002 + candelilla consisted of 1.0 g conidia of isolate 05002 mixed with 9.0 g candelilla wax powder and 0.05 g hydrated silica. The per colony dose of candelilla alone consisted of 9.0 g candelilla wax powder and 0.05 g silica. The density of colony-forming units (CFU) per g formulation was determined at the time of colony treatment by plating three sub-samples of the formulation diluted in distilled water and Tween 80 onto potato-dextrose agar, and counting the number of colonies 96 h after plating.

Twenty six bee colonies were used for the field experiment. Each colony comprised a 10-frame wooden Langstroth brood box.

Five colonies were selected for each treatment group except the untreated control, which had 6. For each colony treatment, a plastic laboratory wash was filled with a single dose of preparation, the hive lid and super removed, the formulation blown between all brood box frames by squeezing the wash bottle, and the super and lid replaced.

To calculate colony and adult bee weight, hive weight was divided into a ‘‘non-colony’’ part, consisting of the hive pieces, e.g., brood box, lids, super, hive base, and 10 empty frames with foundation comb, and the ‘‘colony’’ part, consisting of the adult bees, brood, honey, pollen and wax (other than foundation comb). Adult bee weight was calculated as the difference between the sum of the weights of all the hive parts and the observed hive weight. The non-colony weight was calculated as the total weight of all the hive parts except brood box frames, plus the weight of 10 empty frames, or approximately 2.87 kg. Colony weight was calculated by subtracting the non-colony component from the total hive weight. The area of sealed brood and sealed honey per frame was estimated from photographs. Brood areas were inspected closely for any signs of fungal infection. Colony entrances were inspected for unusually large numbers of dead bees.

On several occasions throughout May and June, samples of approximately 15 bees were collected from within each hive in order to evaluate the number CFUs on them. Subsamples of the bees were placed in a 50 mL plastic centrifuge tube and vortexed for 3 min. in 10 mL of a 0.1% aqueous solution of Tween 80. Aliquots of 20 and 100 µL of the resulting suspension from each subsample were spread onto each of three Petri dishes containing potato dextrose agar with chloramphenicol (0.4 g/L). The dishes were incubated for at least 14 days at 23°C, and the number of *Beauveria bassiana* CFU were counted in the plates with 20 L of solution; when CFU densities became low, CFU were counted on the 100 µL plates.

Findings

CFU density at time of treatment was 3.70 × 10^{10} CFU/g for the Bb05002 + carnauba formulation, 1.79 × 10^{10} CFU/g for the Bb05002 + candelilla formulation and 1.72 × 10^{10} CFU/g for the BbGHA + carnauba formulation.

No negative effect of application of entomopathogenic fungi on colony health, measured as the colony growth rate, total adult bee weight, surface areas of capped brood, and colony survivorship was observed. Colony growth among all groups was lowest immediately after application, but this was likely to be due to food consumption prior to a nectar flow. Colony growth increased among all groups thereafter. No treatment differences were observed in either total adult weight change or changes in the amounts of sealed brood or honey.

Conclusions

Bee hives were treated with two strains of *Beauveria bassiana* at application rates ranging from 1.72 × 10^{10} CFU/g to 3.70 × 10^{10} CFU/g. No negative effects were observed on colony health, measured as the colony growth rate, total adult bee weight, surface areas of capped brood, and colony survivorship.

Comments by RMS:

From the study no negative effects of *Beauveria bassiana* on bee colonies could be detected.

MA 8.3/09

Citation: Meikle WG, Mercadier G, Holst N, Nansen C & Girod V (2008). Impact of a treatment of *Beauveria bassiana* (Deuteromycota: Hyphomycetes) on honeybee (*Apis mellifera*) colony health and on Varroa destructor mites (Acari : Varroidae). *Adipologie,* 39(2): 247-259

Guidelines: None

GLP: No

Abstract

In 2 experiments bee colonies in southern France were treated with conidia of a *Beauveria bassiana* isolate collected from *Varroa* mites in the region. Objectives were to evaluate treatment effect on colony weight, adult bee mass, and capped brood and honey, and on *Varroa* fall onto sticky boards. Treatments included conidia formulated with either wax powder or wheat flour, flour alone, or control. Treatment did not affect colony health.

Colonies treated with conidia and wax powder had higher mite fall compared to controls while those treated with conidia and wheat flour did not. The proportion fallen, infected mites in both conidia treatments was higher than controls for up to a week. Higher mite fall and infection rates were observed in treated hives in the 2nd, smaller experiment. The relationships between dosage and proportion fallen, infected mites, and between ambient temperature and infection duration,
were examined. Future experiments will explore *Varroa* control using conidia.

Materials and methods

Two field experiments were described in this paper, one of which concentrated on evaluation of any detrimental effects to treated bee colonies. The other evaluated the effects of *Beauveria bassiana* on *Varoa destructor*. The remainder of this summary will focus on the effects of *Beauveria bassiana* on colony health.

Three formulations were prepared prior to treatment:

- wax powder and conidia (“powder + conidia”);
- flour and conidia (“flour + conidia”);
- and flour alone.

The per colony dose of powder + conidia consisted of 1.0 g Bb05002 conidia mixed with 9.0 g Entostator powder, a refined, electrostatically-chargeable carnauba wax powder and 0.05 g hydrated silica. The per colony dose of flour + conidia consisted of 1.0 g Bb05002 coni- dia mixed with 9.0 g commercially-prepared wheat flour and 0.05 g silica prepared on 18 May. The dose per colony of flour alone was 10.0 g wheat flour mixed with 0.05 g silica. The density of colony-forming units (CFU) per g formulation was determined at the time of colony treatment by plating three sub-samples of the formulation diluted in distilled water and Tween 80 onto potato-dextrose agar, and counting the number of colonies 96 h after plating.

The bee colonies were housed in 10-frame, wooden Dadant brood boxes (56 L capacity) with telescoping lids and with screens underneath the frames.

Prior to treatment each hive was weighed. After weighing, each hive was opened, and each hive part (i.e. brood box, lids, colony base, and frames after shaking them free of bees) was weighed using a smaller portable electronic balance. Digital photographs were taken of each side of each. Brood areas were inspected closely for any signs fungal infection. The hive was then reassembled, and one super containing 9 frames with wax foundation was weighed and placed on top of each colony. Hives were then weighed in their entirety once per week thereafter until the end of observations for a total of 6 sampling occasions. The brood box frames were again weighed individually, following the same procedure, and the super was also weighed.

For each colony treatment, a plastic laboratory wash bottle was filled with a single dose of preparation, the hive lid removed, the formulation blown between all the frames in the brood box by squeezing the wash bottle, and the lid replaced.

To calculate colony and adult bee weight, hive weight was divided into a “non-colony” part, consisting of the hive pieces, e.g., brood box, lids, su- per, hive base, and 10 empty frames with foundation comb, and the “colony” part, consisting of the adult bees, brood, honey, pollen and wax (other than foundation comb). Adult bee weight was calculated as the difference between the sum of the weights

of all the hive parts and the observed hive weight. The non-colony weight was calculated as the total weight of all the hive parts except brood box frames, plus the weight of 10 empty frames. The average weight of an empty frame was estimated by weighing 22 frames with only foundation comb. Colony weight was calculated by subtracting the non-colony component from the total hive weight. Colony entrances were inspected for unusually large numbers of dead bees.

Findings

CFU density at time of treatment was 1.96×11^{10} CFU/g for the powder + conidia formulation, and 6.33×10^{9} CFU/g for the flour + conidia formulation.

No negative effect of application of entomopathogenic fungi on colony health, measured as the colony growth rate, total adult bee weight, surface areas of capped brood and honey, and colony survivorship was observed. Colony growth among all groups was lowest immediately after application, but this was likely due to food consumption prior to a nectar flow. Colony growth increased among all groups thereafter. No treatment differences were observed in either total adult weight change or changes in the amounts of sealed brood or honey.

Table 8.3/09-1 shows that there were no significant differences between the control hives and the colonies treated with *Beauveria bassiana*. In fact, the total adult weight was significantly higher in the powder + conidia treatment at both observation periods. This was also the case for the sealed brood area for the first observation period.

Table 8.3/09-1: Effects of *Beauveria bassiana* on various hive parameters

Variable	Treatment	N	23 May		27 June		Daily *r*
			avg[1]	s.d.	avg[1]	s.d.	
Total adult weight	Powder + conidia	5	3.301 ab	0.899	4.773 a	0.954	0.0105
	Flour + conidia	5	2.805 b	0.575	4.245 ab	0.535	0.0118
	Flour alone	4	4.305 a	0.513	4.711 ab	0.866	0.0026
	Control	7	2.161b	0.865	3.242 b	1.083	0.0116
Sealed brood surface area	Powder + conidia	5	4532 a	551	3712 a	732	-0.0057
	Flour + conidia	5	3721 ab	982	3665 a	596	-0.0004
	Flour alone	4	4447 a	548	3790 a	587	-0.0046
	Control	7	2867 b	1105	2812 a	1018	-0.0006
Sealed honey surface area	Powder + conidia	5	4846 a	1800	5489 a	1099	0.0036
	Flour + conidia	5	2836 a	1750	3593 a	1870	0.0068
	Flour alone	4	4535 a	849	6009 a	1545	0.0080
	Control	7	4516 a	1431	5203 a	2524	0.0040
	Flour + conidia	5	2.805 b	0.575	4.245 ab	0.535	0.0118
	Flour alone	4	4.305 a	0.513	4.711 ab	0.866	0.0026

[1] Averages within a variable and within a date followed by different letters are significantly different using Tukey's HSD at $P < 0.05$/

Conclusion

Bee hives were treated with *Beauveria bassiana* at application rates of 1.96×11^{10} and 6.33×10^{9} CFU/g. No negative effects were observed on colony health, measured as the colony growth rate, total adult bee weight, surface areas of capped brood, and colony survivorship.

Comments by RMS:

From the study it is demonstrated that *Beauveria bassiana* has no negative effects on bee colonies.

MA 8.3/10

Citation: Mommaerts, V, Put, K, Smagghe, G (2011) *Bombus terrestris* as pollinator-and-vector to suppress *Botrytis cinerea* in greenhouse strawberry. *Pest Management Science,* 67(9): 1069-1075.

Guidelines: None

GLP: No

Summary

A study was designed to investigate the capacity of *Bombus terrestris* as a pollinator and vector of a biofungicide formulation in greenhouse strawberry flowers. Exposure of bumblebees to the MCA caused no adverse effects on bumblebee workers, with no loss of survival or impairment of flight activity of the workers during the 4 week flowering period.

Materials and Methods

Bombus terrestris bumblebee hives obtained from a mass rearing program at Biobest NV (Westerlo, Belgium) were used for the greenhouse experiment. Each hive contained a queen, her brood and a minimum of 75 workers.

The biofungicide Prestop-Mix, based on *Gliocladium catenulatum* J1446 (now *Clonostachys rosea* f. *catenulata* J1446) [powder formulation containing 10^7 – 10^9 colony-forming units (CFU)/g and developed to be delivered by pollinators], was used in this study as a model product for vectoring by bumblebees to suppress the plant pathogen *B. cinerea*. Before use, the numbers of CFU of *C. rosea* f. *catenulata* J1446 were determined through plating out on potato dextrose agar (PDA) medium.

The experiment was conducted in a greenhouse compartment of 24 × 63 × 3.75 m containing four fine-meshed tents (width 6 m, length 15 m, height 2 m) in Westerlo (Belgium). In each tent, a total of 100 strawberry plants were placed when flower buds appeared.

The flight activity was determined for each hive by counting the numbers of bumblebee workers (foragers) flying in and out over a 30 min period each day at 9 a.m. The foraging intensity of the bumblebee workers was evaluated on a weekly basis over the course of the flowering period. The numbers of bumblebees that were flying in and out were recorded every 10 min over the course of 90 min. In addition, the numbers of dead bumblebee workers present in each tent were recorded on a weekly basis.

Conclusion

Exposure to worker bumblebees to the biofungicide Prestop-Mix caused no adverse effects on bumblebee workers, with no loss of survival or impairment of flight activity of the workers during the 4 week flowering period.

Comments by RMS:

The study was performed with Prestop Mix, a MPCP based on *Clonostachys rosea* f. *catenulata* J1446 (formerly *Gliocladium catenulatum* J1446). This micro-organism is not related to *Beauveria bassiana*, therefore, **the study does not provide information that can be used for further assessment**.

8.3/11

Citation: Ramanaidu, K & Cutler, GC (2013). Different toxic and hormetic responses of *Bombus impatiens* to *Beauveria bassiana*, *Bacillus subtilis* and spirotetramat. *Pest Management Science, (2013) 69(8): 949-954*

Guideline: None

GLP: No

Summary

The effects of a biopesticide formulation of *Beauveria bassiana* on the common eastern bumblebee, *Bombus impatiens*, were evaluated. Microcolonies of bees were exposed to field-rate or lower concentrations, and data were collected over 60 days. Oral or topical application of *Beauveria bassiana* had no adverse effects on bees.

Materials and methods

Formulated *Beauveria bassiana* (Botanigard®ES) was used in experiments. Serial dilutions were prepared in deionized water at concentrations of 2.33×10^9, 4.66×10^9, 1.16×10^{10}, 2.33×10^{10} and 4.66×10^{10} CFU/L. Controls consisted of treatment with water only.

Four 'class A' *Bombus impatiens* colonies were donated by Koppert Canada Ltd (Scarborough, Ontario). Two hives were used in contact exposure experiments, and two were used in oral exposure experiments. Each colony was provided with protein and sugar supplements from Koppert.

Experimental microcolonies were established in 461 mL plastic cups. The bottom of each cup was cut out and covered with netting (6.35 mm mesh) secured with a rubber band. Each cup was placed in a second 473 mL polystyrene container that contained a floral water pick or glass vial feeder stuffed with cotton wool or wicks soaked with a 60:40 honey:water solution. The feeder was placed under but touching the netting, allowing bees to feed on the honey solution *ad libitum*. Honey solutions and feeders were replaced twice per week.

Topical exposure

Four- to seven-day-old bees were used. For each treatment, 50 µL of solution was applied to the dorsal thorax of each bee using a PAX 100-3 automatic microdispensing system. There were at least four replicate microcolonies, each containing three bees for each test concentration or control. As hives only produced 50–70 workers weekly, experiments were staggered approximately 1 week apart. After treatment, microcolonies were held at 25°C under total darkness and approximately 60% relative humidity. When separated from the queen of the parent commercial Koppert colony, one worker in each microcolony became dominant and soon thereafter began ovipositing. The other two workers assisted in brood rearing. As workers were unfertilized, all offspring produced were male drones.

Bees were checked once a day for 60 days. Data were collected on worker bee mortality, drone production, number of days to oviposition and number of days to drone emergence.

Oral exposure

Individual worker bees were selected and randomized among treatments, and microcolonies were established as described in the topical exposure section. *Beauveria bassiana* was administered orally to bees in 60:40 honey:water solution at 1.16×10^{10}, 2.33×10^{10} and 4.66×10^{10} CFU/L, offered in

small glass vials stuffed with cotton. Bees in control microcolonies were given honey water only. Treatment solutions and feeders were replaced twice per week for 30 days. Bees were thereafter given untreated honey:water solution for an additional 30 days. There were five replicate microcolonies in each bioassay.

Data collection, endpoints measured and statistical analysis were conducted as in the topical exposure treatment, except that larval ejection data were also collected.

Findings

Topical exposure

There was no effect on *B. impatiens* worker survival when topically treated with *Beauveria bassiana* (H=0.85; P=0.974). Even at the highest concentration, a median of 3.0 workers remained after 60 days following treatment.

Beauveria bassiana topical treatments had no effect on drone production (F=0.27; P=0.922), and there were no significant treatment – time interaction effects on drone production (F = 1.05; P = 0.346) (Table 8.3/11-1). Topical exposure to *Beauveria bassiana* also had no effect on days to oviposition (F=0.94; P=0.481) or days to drone emergence (F=1.65; P = 0.204).

Table 8.3/11-1. Effects on *Bombus impatiens* microcolonies 60 days after a topical treatment with *Beauveria bassiana*

Treatment concentration (CFU/L)	Mean (± 95% C.I.)		
	Drones produced	Days to oviposition	Days to drone emergence
Control	5.50 (5.88)	6.04 (3.16)	39.50 (2.33)
2.33×10^{9}	5.00 (5.36)	6.00 (0.00)	38.67 (4.57)
4.66×10^{9}	6.25 (3.76)	5.76 (2.32)	36.50 (1.62)
1.16×10^{10}	6.25(7.84)	6.12 (4.24)	44.00 (10.21)
2.33×10^{10}	5.25 (6.15)	4.74 (3.62)	33.67 (2.85)
4.66×10^{10}	5.25 (5.26)	5.24 (4.14)	37.75 (2.58)

Oral exposure

There were no effects on worker *B. impatiens* survival after prolonged (30 days) feeding on diluted honey treated with *Beauveria bassiana* (H = 4.93; P = 0.177).

Beauveria bassiana administered in honey water had no effect on drone production (F = 0.82; P = 0.501), and there was no treatment – time interaction (F = 0.91; P = 0.715) (Table 8.3/11-2). There was also no difference in the number of days to oviposition (F = 0.62; P = 0.613), number of larvae ejected (F = 1.10; P = 0.377) or days to drone emergence (F = 0.31; P = 0.819) with this treatment.

Table 8.3/11-2. Effects on *Bombus impatiens* microcolonies 60 days after an oral treatment with *Beauveria bassiana*

Treatment concentration (CFU/L)	Mean (± 95% C.I.)			
	Drones produced	Days to oviposition	Larval ejection	Days to drone emergence
Control	4.61 (6.31)	6.00 (0.62)	3.80 (3.47)	34.67 (1.73)

1.16×10^{10}	7.82 (2.22)	5.40 (0.78)	3.20 (2.87)	33.00 (1.38)
2.33×10^{10}	5.44 (4.78)	5.20 (1.57)	0.60 (1.18)	33.25 (2.31)
4.66×10^{10}	6.43 (8.17)	6.00 (0.88)	1.80 (2.66)	34.25 (4.48)

Conclusion

Topical application of *Beauveria bassiana* at five concentrations ranging from of 2.33×10^9 to 4.66×10^{10} CFU/L had no adverse effects on bees. Oral application of *Beauveria bassiana* at three concentrations ranging from of 1.16×10^{10} to 4.66×10^{10} CFU/L had no adverse effects on bees.

Comments by RMS:

Topical and oral application of *Beauveria bassiana* at five concentrations ranging from of 2.33×10^9 to 4.66×10^{10} CFU/L did not have any significant effects on bumblebee workers and colonies, not even after prolonged (30 days) exposure via food.

8.3/12

Citation: Mommaerts, V, Sterk, G, Hoffman, L & Smagghe, G (2009). A laboratory valuation to determine the compatibility of microbiological control agents with the pollinator *Bombus terrestris. Pest Management Science,* 65(9): 949-955

Guideline: None

GLP: No

Summary

A study was undertaken to identify any potential adverse side effects of the use of seven microbiological control agents (MCAs) on the bumblebee, *Bombus terrestris* L., in the context of combined use in integrated pest management. This summary will concentrate on effects of Botanigard® (*Beauveria bassiana* GHA). Bumblebee workers were exposed under laboratory conditions to each MCA at its maximum field recommended concentration (MFRC) via three different routes of exposure: dermal contact and orally via either treated sugar water or pollen.

Materials and Methods

All experiments were performed with worker bumblebees obtained from a continuous mass rearing programme and conducted under standardised laboratory conditions of 28 – 30°C, 60 – 65% RH and continuous darkness. The insects were provided *ad libitum* with commercial sugar water and pollen.

Newly emerged workers were collected from the bumblebee colony, and five were placed in artificial plastic nest boxes (15 × 15 × 10 cm). Four artificial nests were exposed for each treatment, and each experiment was repeated twice. Adult workers were exposed to the MCAs at their respective MFRCs via three different routes: via contact by topical application, and orally via treated sugar water and via treated pollen. For each treatment, worker mortality was scored after 72 h and on a weekly basis during a period of 11 weeks.

For the contact treatments, the treatments were prepared in water. Individual bees were topically treated with 50 µL of this aqueous solution on their dorsal thorax with a micropipette. For the oral treatments, bumblebee workers were continuously exposed to 500 mL of sugar water that was dosed with the MCA, or to pollen sprayed until saturation with the MCA in water. The treated sugar water and

pollen were replaced weekly with freshly prepared material. Bees were treated with a concentration of 2.5 x 10^{10} CFU/L.

Results

Lethal effects on worker survival

No lethal effects were exhibited against the workers during the first 72h following treatment, regardless of the different routes of exposure. However, after 11 weeks, worker mortality was recorded in nests that were treated with Botanigard®. 92 ± 3% of the workers were killed following topical treatment. In addition, when the concentration was reduced to 1/2, 1/5 and 1/10 of the MFRC, the mean worker mortality still reached 59 ± 1%, 41 ± 6% and 46 ± 4%, respectively, after 11 weeks. The presence of *Beauveria bassiana* GHA mycelium on the bodies of workers was also confirmed when cadavers were inspected under the microscope.

Sub-lethal effects on reproduction

The production of drones after 11 weeks was not significantly different ($P > 0.05$) from the control nests treated with water. Exposure of worker bumblebees via sugar water treated with Botanigard® for 11 weeks resulted in a reproductive rate that was not significantly different ($P > 0.05$) from that observed in the controls.

Pollen treatment with Botanigard® resulted in significant reduction in the production of males ($P < 0.05$). Although significant, this reduction of 16% is harmless according to the IOBC classification (Class 1).

Sublethal effects on behaviour

After 9 weeks oral exposure to Botanigard® worker mortality was 3%. In addition, the authors examined whether a reduction in foraging by workers may result in a reduced nest reproductive rate, i.e. numbers of drones, through increased larval mortality. None of the MCAs tested at its MFRC caused larval mortality or exerted detrimental effects on the production of drones, with the exception of Botanigard®. Here, significantly ($P < 0.05$) lower numbers of drones were counted after 9 weeks: 13.4 ± 2.9 as opposed to 28.4 ± 2.9 in the control group treated with water. This reduction of 53% corresponds to an IOBC class of moderately harmful (class 3). By comparison, only 5.6±0.1 drones nest−1 was measured in the imidacloprid treatment at 1/10 000 MFRC.

Conclusions

For the insecticidal biopesticide Botanigard® based on *B. bassiana* GHA at 2.5 x 10^{10} CFU/L, severe worker mortality was observed after contact treatment of bumblebee workers, but not via the ingestion of treated sugar water or treated pollen where very low mortality occurred. To explain the high contact toxicity of the fungus *B. bassiana*, it is reported in various publications that environmental conditions such as temperature may play an important role in determining the pathogenicity observed.

In contrast, Al-Mazra'awi *et al*. (see 8.3/13) reported that *B. bassiana* GHA is safe for use in combination with the Eastern North American bumblebee, *B. impatiens*, in a vector system to control pest insects. However, it should be mentioned that in the latter experiments the fungus *B. bassiana* was applied as a dry formulation. Similarly, *B. bassiana* strains isolated from varroa mites have been used in a powder formulation in France to treat honey bee hives against varroasis without any

negative impacts on colony health (see studies 8.3/08 and 8.3/09). In the present tests the bumblebees were treated via contact with a water suspension of the conidia, and this may explain the high worker toxicity observed.

Comments by RMS:

Based on the results of this study high contact toxicity was determined in bumblebee workers. Oral exposure did not show any negative effects on bees.

This outcome is contradictory to the results of the study reported under 8.3/11. As in both studies 8.3/11 and 8.3/12 *B. bassiana* was dosed topically as aqueous solution, the RMS cannot explain this variation in test result. The present authors believe that the toxicity of the fungus *B. bassiana* may differ owing to differences in conidial thermotolerance and the type of formulation and exposure method. Therefore, **it is strongly indicated that the side effects of MCAs under these different conditions should be evaluated before their practical use can be recommended.**

Furthermore the RMS would like to add several peer-reviewed articles that were used at a national level for the registration of a product containing *B. bassiana* strain GHA. According to the information provided in the Vol. B1-B5, B.5.1.1.1, p.83, the sequences of *Beauveria bassiana* strain PPRI 5339, *B. bassiana* isolate GHA, accession number: JN379811.1, and *B. bassiana* isolate ATCC74040, accession number: FJ972972.1 are 98-99% similar to each other. Although the accession number of the tested isolates is not known, the information can give an indication of the associated effects of possible closely related strains. These references can be used in the risk assessment.

8.3/13

Report:	M. S. Al Mazra'awi, J. L. Shipp, A. B. Broadbent, And P. G. Kevan. Dissemination of *Beauveria bassiana* by Honey Bees (Hymenoptera: Apidae) for Control of Tarnished Plant Bug (Hemiptera: Miridae) on Canola. Environ. Entomol. 35(6): 1569-1577 (2006)
Guideline:	No specific guideline was used.
GLP:	No
Abstract:	Large screened cages trials were conducted to assess the potential of honeybees, *Apis mellifera L.*, to vector *Beauveria bassiana* (Balsamo) to canola, *Brassica napus* L., against the tarnished plant bug, *Lygus lineolaris* (Palisot de Beauvois). The bees effectively vectored the inoculum from the hives to the crop. Conidia of *B. bassiana* was recovered from 100, 64-77, 70-82 and 47-83% of bees, flowers, leaves, and *L. lineolaris* samples, respectively, collected on four sampling dates in 2002 and 2003. Mean mortalities of *L. lineolaris* collected from the Beauveria-treated cages in the field were 56 and 48% compared with 9 and 10% in the controls on the first and second sampling dates in 2002, respectively, and 22 and 45% in the treated cages in the field compared with 15 and 22% in the controls on the first and second sampling dates,

respectively, in 2003. These results indicate that bees may provide a new novel means for applying *B. bassiana* to manage *L. lineolaris* on canola. The benefits are better pollination, reduction in pest pressure of *L. lineolaris*, and reduced reliance on insecticides.

RMS addition:

Effects on bees were not an endpoint from this study, but in the discussion, the following is mentioned by the authors of the article:

'Collectively, the data indicated that honey bees were efficient vectors of *B. bassiana* to canola and that the vectored inocula infected the population of *L. lineolaris.* However, for this technology to be practical, *B. bassiana* should not be harmful to honey bees.

Monitoring of the colonies during and after the trials showed no adverse effect on the bees. These observations agreed with previous reports that indicated the safety of *B. bassiana* to colonies of honey bees (Al mazra'awi 2004). Although Vandenberg (1990) reported reduced longevity of honey bees when exposed to *B. bassiana* in cages, this test might be misleading because the exposed workers were isolated from their queen, which rendered them more susceptible than usual to the entomopathogen (Goettel and Jaronski 1997). Exposure of whole hives to *B. bassiana* strain GHA resulted in 1% infection of the workers and no infection of the brood (Goettel and Jaronski 1997). Similar findings were reported by Alves et al. (1996), who showed that both *B. bassiana* and *M. anisopliae* caused high mortality to caged Africanized honey bees maintained at high temperatures, but they did not cause epizootic levels in field hives even when applied at high levels. He concluded that *B. bassiana* and *M. anisopliae* can be used in microbial control programs without any risks to Africanized honey bee colonies (Alves et al. 1996).

8.3/14

Report: William G. Meikle, Guy Mercadier, Niels Holst, Vincent Girod. Impact of two treatments of a formulation of *Beauveria bassiana* (Deuteromycota: Hyphomycetes) conidia on *Varroa* mites (Acari: Varroidae) and on honeybee (Hymenoptera: Apidae) colony health. Exp Appl Acarol (2008) 46:105-117

Guideline: No specific guideline was used.

GLP: No

Abstract: Bee colonies in southern France were treated with conidia (asexual spores) from two strains of *Beauveria bassiana*, an entomopathogenic

fungus. One strain was commercial (GHA) and the other had been isolated from *Varroa* mites in the region (Bb05002). Objectives were to evaluate treatment effect on colony weight, adult bee mass, capped brood, and on *Varroa* fall onto sticky boards. Treatments included conidia formulated with either carnauba or candelilla wax powder, candelilla wax powder alone, or control; in two treatment groups formulation was applied a second time after one week. Treatment did not affect colony health. Colonies treated twice with Bb05002 conidia and carnauba wax powder had significantly higher mite fall compared to colonies treated with blank candelilla wax powder. The proportion of fallen mites that were infected in both conidia treatments was higher than controls for 18 days after the second treatment. The number of fungal propagules on the bees themselves remained elevated for about 14 days after the second treatment. These results were compared to published results from previous experiments with regard to infection duration.

8.3/15

Report: M.S. Al mazra'awi. Impact of the Entomopathogenic Fungus *Beauveria bassiana* on the Honey bees, *Apis mellifera* (Hymenoptera: Apidae). World Journal of Agricultural Sciences 3 (1): 07-11, 2007

Guideline: No specific guideline was used.

GLP: No

Abstract: The impact of entomopathogens on non-target organisms is critical information for the registration of microbial control agents. *Beauveria bassiana* is an excellent potential candidate for biological control of a variety of pests and efforts are being made for its registration in Canada. Four isolates of *B. bassiana* were evaluated to determine their pathogenicity to caged honey bees, *Apis mellifera carnica*. Results showed that three of the tested isolates: GHA, London BB001 and Arkansas ARSEF 3769 caused high mortalities to the caged bees when dusted with a dry formulation at high concentrations (10^8-10^9 CFU g^{-1}). However, exposure of honey bee hives to high inoculum densities of *B. bassiana* resulted in very low mortality that was not different from the control regardless of the isolate. Those results suggest that *B. bassiana* can be applied for pest control in fields where honey bees are used for pollination.

8.3/16

Report: M.S. Al mazra'awi, P. Kevan, L. Shipp. Development of *Beauveria bassiana* dry formulation for vectoring by honey bees *Apis mellifera* (Hymenoptera: Apidae) to the flowers of crops for pest control. Biocontrol Science and Technology, 17:7, 733-741 2007

Guideline: No specific guideline was used.

GLP: No

Abstract: Using bee pollinators as a means for the dissemination of microbial control agents, such as *Beauveria bassiana*, against insect pests of agricultural crops is a novel and interesting approach to biological control. In four laboratory trials, one in Canada and three in Jordan, factors affecting the acquisition of *B. bassiana* by honey bees were evaluated using hive-mounted inoculum dispensers. The numbers of conidia carried by bees emerging from the dispensers differed according to the type of carrier used. Bees that passed through corn flour acquired more inoculum than did those that walked through wheat flour, durum semolina, corn meal, potato starch, potato flakes, oat flour or barley flour. The numbers of conidia acquired by the bees increased with decreasing particle size and moisture content of the carrier, and with increasing density of *B. bassiana* conidia in the formulation. Time required for a bee to pass through the dispenser did not significantly affect the acquisition of conidia. This study indicated that honeybees (*Apis mellifera carnica*) have a great potential for vectoring *B. bassiana* in crop systems. It also opens more avenues for studies on bee delivery of other microbial biological control agents.

Comments by RMS: All four articles above suggest that honeybees are not adversely affected by *B. bassiana* strain GHA under the field conditions. However, this conclusion cannot be applied to the greenhouse uses as the conditions might be more favourable for the Beauveria's growth.

8.3/17

Report: Jean Pierre Kapongo, Les Shipp, Peter Kevan, Bruce Broadbent. Optimal concentration of *Beauveria bassiana* vectored by bumble bees in relation to pest and bee mortality. BioControl (2008) 53:797-812

Guideline: No specific guideline was used.

GLP: No

Abstract: Greenhouse cage trials were conducted to determine the optimal concentration of *Beauveria bassiana* (Balsamo) Vuillemin (Ascomycota: Hypocreales) (BotaniGard 22WP® formulation) as vectored by the bumble bee, *Bombus impatiens* (Cresson) (Hymenoptera: Apidae) pollinator for control of greenhouse whitefly, *Trialeurodes vaporariorum*

(Westwood) (Hemiptera: Aleyrodidae) on greenhouse tomato, tarnished plant bug, *Lygus lineolaris* (Palisot de Beauvois) (Hemiptera: Miridae) and green peach aphid, *Myzus persicae* (Sulzer) (Hemiptera: Aphididae) on greenhouse sweet pepper. Three inoculum concentrations of *B. bassiana*: low, $9x10^9$; middle, 6.24×10^{10}; and high, 2×10^{11} conidia g^{-1} of inoculum and two controls (one with bees and heat-inactivated inoculum, and the other which contained only the host plants and pest species) were tested

in a completely randomized block design. *Beauveria bassiana* killed 18, 54 and 56% of the adult *T. vaporariorum* and 33, 70 and 67% of the adult *L. lineolaris*, respectively, at the low, middle and high concentrations; but no infection from *B. bassiana* occurred in each of the control treatments. Internal infection rates after surface sterilization of the pest insects were 11, 34 and 35% for adult *T. vaporariorum*, 29, 54 and 58% for adult *L. lineolaris*, 22, 34 and 30% for nymphal *M. persicae* and 17, 29 and 32% for nymphal *T. vaporariorum*, respectively, at the low, middle and high concentrations.

Significantly more bumble bees died at the high concentration of *B. bassiana* (42–45%) than at the other concentrations (9–15%) and the controls (5–7%). Spores of *B. bassiana* were collected throughout the plant canopy with the greatest numbers sampled from the top third of the canopy [ca. 1,200 colony forming units (CFU) per cm^{-2}]. The middle concentration was selected as the optimal concentration because it provided the best pest control with the least impact on the bees.

8.3/18

Report: J.P. Kapongo, L. Shipp, P. Kevan, J.C. Stutton. Co-vectoring of *Beauveria bassiana* and *Clonostachys rosea* by bumble bees (*Bombus impatiens*) for control of insect pests and suppression of grey mould in greenhouse tomato and sweet pepper. Biological Control 46 (2008) 508-514

Guideline: No specific guideline was used.

GLP: No

Abstract: Greenhouse cage trials were conducted to assess the effectiveness of bumble bee pollinators for the co-vectoring of two fungi (*Beauveria bassiana* [Balsamo] Vuillemin [BotaniGard 22WP® formulation] and *Clonostachys rosea* Lnk: Fr. [Endofine®]), in greenhouse tomato and sweet pepper for control of insect pests (greenhouse whitefly, *Trialeurodes vaporariorum* Westwood; and tarnished plant bug, *Lygus lineolaris*

[Palisot de Beauvois]), and grey mould (*Botrytis cinerea* Pers: Fr.). Three treatments were evaluated: 6.24 x 10^{10} conidia of *B. bassiana* + 1.38 x 10^{7} conidia of *C. rosea*/g of inoculum vectored by bees (active inoculum); heat-inactivated inoculum vectored by bees; and a control with no inoculum and no bees. In each crop, the treatments were arranged in a completely randomized block design with four replications. When applied in the tomato crop, the active inoculum killed 49% of the *T. vaporariorum* and suppressed grey mould by 57% and 46%, respectively, on the flowers and leaves. In sweet pepper, mortality of *L. lineolaris* was 73% and grey mould was suppressed by 59% and 47%, respectively, on the flowers and leaves in the active inoculum treatment. The incidence of grey mould in sweet pepper and tomatoes treated with the heat-inactivated inoculum or no inoculum (control) was approximately 80% on the flowers and leaves. Thus, the combined inoculum of *B. bassiana* and *C. rosea* can potentially control *T. vaporariorum* and *L. lineolaris*, and suppress grey mould in greenhouse tomatoes and sweet peppers, when vectored simultaneously by bumble bees. Commercial greenhouse trials need to be conducted next to determine control efficacy under large scale production conditions.

Comments by RMS: The second abstract does not report any results on effects on bumble bees.

From the first abstract it can be deduced that when bumblebees were used as vector for *B. bassiana*, inoculum concentrations of 9 x 10^{9}; 6.24 x 10^{10}; and 2 x 10^{11} conidia g^{-1} of inoculum caused 9-15% mortality in the two lowest concentrations and 42-45% mortality in the highest inoculum concentration. The current application rate is 4 x 10^{9} CFU/L and assuming a spray liquid density of 1 L/kg, the application rate will be 4 x 10^{6} CFU/g which is lower than the tested concnetrations in the study above.

B.9.3.2 Infectiveness

In studies 8.3/04, -/05, -/06 and -/07 infectivity was tested by looking at growth of the m.o. on dead bees and autopsy of surviving bees. No signs of infectivity were recorded. However, the RMS is of opinion that the test duration may have been too short in order to capture any infectivity. The guideline recommends a test of at least 30 days.

B.9.3.3 Pathogenicity

In studies 8.3/04, -/05, -/06, and -/07 growth of the m.o. on dead bees and autopsy of surviving bees. No signs of pathogenicity were recorded. However, the RMS is of opinion that the test duration may have been too short in order to capture any pathogenicity. The guideline recommends a test of at least 30 days.

B.9.3.4 Risk assessment for bees

A summary of the toxicity data for bees derived from various sources is presented in the table below.

Table B.9.3.4-01.: Summary of bee toxicity data submitted by the applicant

Species	Test type	MCPA	Endpoints	Reference/Source
Bumble bee (*Bombus terrestris*)	10-day contact exposure	*Beauveria bassiana* strain PPRI 5339	LD_{50} >3.1 × 10^9 cfu/L	Whittaker 2013a
	10-day oral exposure		LD_{50} >2.9 × 10^{11} cfu/L	Whittaker 2013b
Honey bee	10-day contact exposure		LD_{50} >2.9 × 10^9 cfu/L	Whittaker 2013c
	10-day oral exposure		LD_{50} > 2.9× 10^{11} cfu/L	Whittaker 2013d
	Field study – whole colony exposure with test substance added directly to the hive	*Beauveria bassiana* isolate 05002	No adverse effects on colony health at treatment rates ranging from 1.72 × 10^{10} cfu/g to 3.70 × 10^{10} cfu/g.	Meikle et al. (2008)
	Field study – whole colony exposure with test substance added directly to the hive	*Beauveria bassiana* isolate 05002	No adverse effects on colony health at treatment rates ranging from 6.33 x 10^9 cfu/g to 1.96 × 10^{10} cfu/g.	
Bumble bee *(Bombus terrestris)*	Oral and topical exposure	*Beauveria bassiana GHA* (Botanigard®)	Oral: no adverse effects at 2.5 × 10^{10} cfu/L Topical: adverse effects at 2.5 × 10^{10} cfu/L	Mommaerts et al. (2011)
Common eastern b umble bee *(Bombus impatiens)*		*Beauveria bassiana* (Botanigard®ES)	Topical and oral application of *Beauveria bassiana* at five concentrations ranging from of 2.33 × 10^9 to 4.66 × 10^{10} CFU/L did not have any significant effects on bumblebee workers and colonies	Ramanaidu & Cutler, (2013)

<u>Within greenhouse</u>

<u>Bumble bees</u>

The concentration of *Beauveria bassiana* strain PPRI 5339 in the spray water after one application in greenhouses is 4 x 10^9 CFUs/L.

In the study by Mommaerts *et al.* 2011, the effects seen on the bumble bees were at a rate similar to that of the current product. Although the effects were seen with a different formulation than the current one, the effects can be indicative of effects when the strain is formulated or just under certain environmental conditions.

Considering the information available, high application number of this entomoptahogenic fungus, and the wide range of growth temperature (i.e. 5 to 35 °C, see Vol. B1-B5) of *B. bassiana*, the RMS is of opinion that a warning sentence should be added on the label of the product (to be determined at the MS level). For the Dutch label the warning sentence will be as follows: "*Warning: This product is harmful for pollinators. Consult your pollinator provider about the use of this product in combination with the use of pollinators to determine an appropriate wait time between product application and introduction of pollinators*". With such a warning sentence, the RMS considers that the risk to pollinating bumblebees in greenhouses is sufficiently addressed. The other MS should consider their national circumstances and the appropriate warning sentences.

Honey bees

All the articles above suggest that honeybees are not adversely affected by *B. bassiana* strain GHA however considering that the product is applied 122 times a year with 3 days interval, and considering no degradation of the product in these 3 days, the exposure concentrations can be 100 times higher and thus a risk to bees cannot be excluded.

Therefore, the RMS concludes that the risk for honeybees as a result of the proposed greenhouse uses is acceptable provided that a warning sentence is placed on the label. For the Dutch label the warning sentence will be as follows: "*Warning: This product is harmful for pollinators. Consult your pollinator provider about the use of this product in combination with the use of pollinators to determine an appropriate wait time between product application and introduction of pollinators*". The RMS considers that the risk to pollinating honey bees in greenhouses is sufficiently addressed. The other MS should consider their national circumstances and the appropriate warning sentences.

B.9.4 Effects on arthropods other than bees (Annex IIM 8.4; Annex IIIB 10.4)

Given the use pattern of the product it was considered that *Orius* and *Aphidius* species would be the most appropriate non-target arthropods to include in the tests given that they are widely used in many glasshouse biocontrol programmes, and as such they serve as useful indicators of non-target effects. *Aphidius rhopalosiphi* is regarded as being a more sensitive indicator species for effects on aphid parasites than the other *Aphidius* species used commercially in glasshouse biocontrol.

Summaries of the studies conducted with *Beauveria bassiana* strain PPRI 5339 are presented below.

B.9.4.1 Toxicity

ACTIVE INGREDIENT

From Literature:

MA 8.4/01

Citation: Devotto, L, Cisternas, E, Gerding, M, Carrillo, R (2007). Response of grassland soil arthropod community to biological and conventional control of a native moth: using *Beauveria bassiana* and lambda-cyhalothrin for *Dalaca pallens* (Lepidoptera : Hepialidae) suppression. *Biocontrol,* 52(4): 507-531
published

Guideline: None

GLP: No

Summary

Two experiments were conducted in winter and spring in Chile to compare non-target effects of *Beauveria bassiana* (Balsamo) Vuillemin and lambda-cyhalothrin on the effects to non-target soil communities. This field study demonstrated that the application of *Beauveria bassiana* at a rate of 10^{12} spores/ha caused no reduction in numbers, diversity or species richness of soil dwelling invertebrate communities. The experiment was validated by including a 'toxic reference' treatment of λ-cyhalothrin for which an adverse impact on invertebrates would be expected. Indeed this was the case for this field evaluation.

Materials and methods

Experiments were set up in winter and spring. For the winter experiment three treatments were prepared: control (0.5 ha); *Beauveria bassiana* QU-B931(1 ha) and λ-cyhalothrin (1 ha). Treatments were 100–150 m apart and surrounded by grassland. *Beauveria bassiana* was treated at the rate of 10^{12} spores/ha.

For the spring experiment, twelve plots (30 × 30 m) were established in a 4 ha field. Four plots were assigned to each treatment at random and four plots remained as control.

No additional plant protection products were applied. All treatments were applied using a horizontal bar mounted on a tractor.

Soil cores were collected from the spring experiment to enumerate the application rate and evaluate spore persistence. A dilution plating method on agar-oat-dodine was used. Spore number in soil was estimated five times in the study: before and 1, 5, 15 and 66 days after spraying. The persistence of spores on leaves was also evaluated.

The abundance and diversity of invertebrates were monitored by extracting soil cores three times in winter (before spraying and 20 and 40 days after spraying) and twice in spring (before spraying and 30 days after spraying). In the winter experiment, 120 cores were taken from every site, and 30 cores were taken from each plot in the spring experiment. In the laboratory, 70% of the cores were put on extraction trays and the remaining 30% were placed in Berlese-Tullgren funnels. Extraction was performed over 96 h. Specimens were examined under a stereoscopic microscope, counted and classified to the lowest possible taxonomic level.

Species richness curves, Shannon index, Hurlbert's PIE and dominance were evaluated. The foliage and soil counts of colony forming units (CFU) were analysed using a one way ANOVA.

Findings

The persistence of spores in the soil cores and on the leaves is presented in the Figure below:

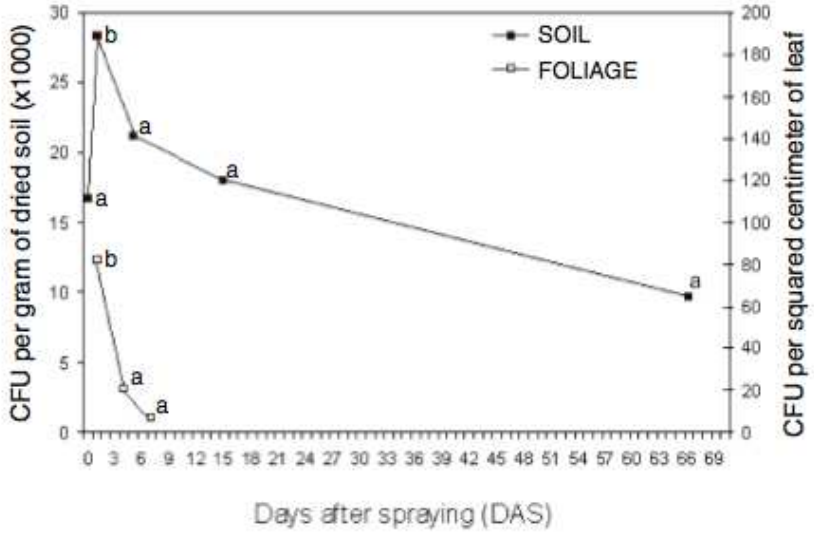


Fig. 8.4/01-1: Estimated numbers of *Beauveria bassiana* spores in soil and pasture foliage. On each curve, means followed by different letters differ according to Fisher's least significant difference test (p < 0.05).

The natural level of *Beauveria bassiana* spores in soil was 1.3 x 10^4 CFU/g of dry soil (Fig. 8.4/01-1). After spraying, the spore number increased by 70%, but they decline dramatically and the second post-spraying estimate did not differ from pre-treatment level (p > 0.05). In the foliage, spore number peaked 82 spores/cm^2 of leaf 1 day after spraying and the spore number by day 7 was close to zero.

A total of 9,555 invertebrates were identified in both experiments. Five taxa accounted for 84% of the total captures: Cantharidae (34%), Acari (30%), Curculionidae (9%), Carabidae (7%) and Araneae (4%). The functional groups represented were herbivores (50%), detritivores (34%), predators (14%) and omnivores (2%).

Diversity of invertebrate communities

In the winter experiment prior to treatment, diversity (estimated using Shannon index) at the control site was higher than both *Beauveria bassiana* and λ-cyhalothrin sites, while no significant differences were observed between these treated sites. Twenty days after a single spraying of *Beauveria bassiana* QU-B931 or λ-cyhalothrin, control and *Beauveria bassiana* sites showed no differences (α = 0.05), while the diversity was significantly lower in the λ-cyhalothrin site. The same result was observed 40 days after spraying.

For the spring experiment, samples taken before spraying indicated that diversity in untreated and treated sites was similar. Thirty days after spraying, estimates of Shannon index showed no differences between control and *Beauveria bassiana* sites, while diversity at the λ-cyhalothrin site was significantly lower than both control and *Beauveria bassiana* sites.

Invertebrate community species richness

In the winter experiment, species richness estimated at n = 495 did not differ between sites before treatment, in spite of marked differences between site abundances (numbers at the *Beauveria bassiana* site were two-fold those at the control site). In both post-spraying sampling dates, species richness at the control site fell slightly out of the confidence interval calculated around the *Beauveria*

bassiana rarefaction curve, while the λ-cyhalothrin rarefaction curve fell out of the lower confidence limit of the *Beauveria bassiana* rarefaction curve. Spring results indicated that the control and λ-cyhalothrin species richness were similar and higher than the *Beauveria bassiana* species richness before treatment, at n = 402. After treatment, the confidence limits of *Beauveria bassiana* and λ-cyhalothrin overlapped and included the control species richness, therefore the treatments did not alter species richness in this season.

Dominance

In the winter experiment, before treatment dominance was similar between sites assigned to *Beauveria bassiana* and λ-cyhalothrin, but it was lower at the control site before treatment applications. Twenty days after spraying, dominance remained high at the λ-cyhalothrin site (where the most dominant species accounted for more than 60% of catches), while dominant species represented just 45 and 39% of catches at the *Beauveria bassiana* and control sites, respectively. At 40 days after treatment, dominance at the control site was slightly higher than dominance at the *Beauveria bassiana* site, but lower than at the λ-cyhalothrin site. In the spring experiment, before spraying, no species accounted for more than 25% of catches, but post-spray samples revealed that dominance was higher at the insecticide site (50%) than the dominance at the *Beauveria bassiana* (27%) and control sites (33%).

Conclusion

Although experiments presented here were not designed to test specific hypotheses on single species, data showed that this strain selected against *D. pallens* did not pose a significant risk to the rest of invertebrate community, at least in the short-term. Corrected mortality of the target pest was 48 and 68% in the *B. bassiana* winter site, 15 and 30 days after spraying, respectively

This field study demonstrated that the application of *Beauveria bassiana* at a rate of 10^{12} spores/ha caused no reduction in numbers, diversity or species richness of soil dwelling invertebrate communities. The experiment was validated by including a 'toxic reference' treatment of λ-cyhalothrin for which an adverse impact on invertebrates would be expected. Indeed this was the case for this field evaluation.

Comments by RMS

No significant effects were detected on invertebrate community on a field scale grassland study. This qualitative information can be used for risk assessment.

MA 8.4/02

Citation: Devotto, L, Cisternas, E, Carrillo, R, Gerding, M (2008). Non-target effects of *Dalaca pallens* Blanchard control examined through principal response curves: a guild approach in Southern Chile. *Chilean Journal of Agricultural Research, (2008) 68 (3): 228-237.*

published

Guideline: None

GLP: No

Summary

A field experiment was conducted to compare the effects of the biological control agent *Beauveria bassiana* (10^{12} spores/ha) and the insecticide λ-cyhalothrin (7.5 g active ingredient/ha) on non-target invertebrate species belonging to different guilds. The soil invertebrates were collected by using pitfall trapping and soil coring before and after spraying the biocontrol agent or the insecticide. Caught individuals were assigned to predator, herbivore or decomposer guilds and then a relatively new multivariate technique called principal response curves (PRC) was adopted to track the potential treatment effects over time in the abundance or activity of the non-target guilds. The products targeting *D. pallens* caused different effects on guilds: λ-cyhalothrin decreased the activity-density of the predator guild but not herbivore or decomposer guilds, while the biological control agent caused no reduction in the activity-density of the three examined guilds. The negative effects of the insecticide were present from 1 to 60 days after treatment.

Materials and methods

The site was located in Chile in a naturalized pasture comprised primarily of *Lolium perenne* L. and *Holcus lanatus* L. A single application of *Beauveria bassiana* strain QU-B931 suspended in water, in a dose equivalent to 10^{12} spores/ha, or the pyrethroid λ-cyhalothrin at 7.5 g a.i./ha was sprayed onto 30 x 30 m open plots established in the pastures. A water treatment was included as a control.

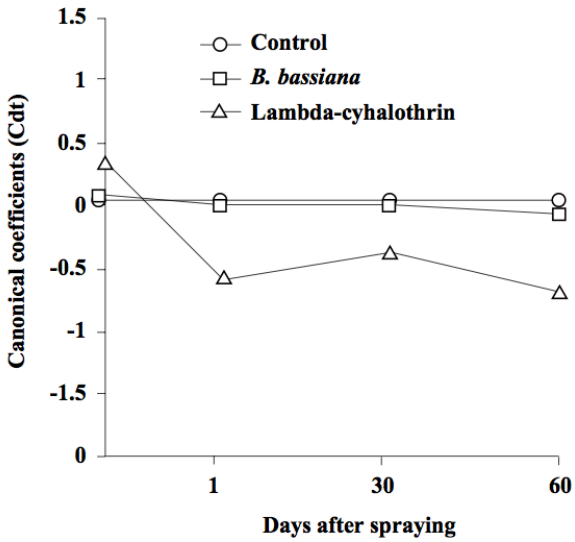
Two techniques were used to sample the invertebrate fauna: pitfall trapping and Tullgren-Berlese funnel extractions of soil cores. Twelve traps were placed in the center of each plot for 4-day periods (one day before spraying and 1, 30, and 60 days after spraying). Thirty soil cores were extracted randomly from each plot, one day before and 30 days after spraying.

A completely randomized design with four replicates was used. Principal response curves (PRC) were used to analyze the time and treatment-dependent multivariate response of selected guilds. The method summarizes all the changes in activity-density of the species belonging to the same assemblage simultaneously, and the principal response, a weighted sum of the abundances of the taxa, is expressed as a canonical coefficient and reflects the behavior of the treated communities relative to the untreated control.

Findings

Over 4111 specimens were identified. The most important taxa were the carabid beetles (42%), oribatid mites (20%), gnaphosid spiders (16%) and curculionid weevils (8%). The remaining taxa accounted for 14%. In terms of ecological functionality, 61% of the invertebrates were predators, 11% herbivores and 28% decomposers.

Fig 8.4/02-1 demonstrates the effects of the different treatments on the predator community. This demonstrates that the response of the communities treated with *Beauveria bassiana* do not differ from the untreated controls. This is not the case for the λ-cyhalothrin. Also, Tables 8.4/02-1 and 8.4/02-2 show significant differences between the *Beauveria bassiana* and λ-cyhalothrin treatments but not between the control and the λ-cyhalothrin.



Weights (b_k)	
2.74	*Ferionomorpha nebroides*
1.79	*Ferionomorpha aerea*
1.35	Lycosidae
0.48	Gnaphosidae
0.27	*Ceroglossus chilensis*
0.27	*Argutoridius chilensis*
0.22	*Allendia chilensis*
0.20	*Trirammatus unistriatus*
0.09	*Parhypates* sp.
0.09	*Calosoma vagans*
0.09	*Metius flavipes*
0.05	*Trechisibus angularis*
0.02	*Mymodromites cynaeus*

Figure 8.4/02-1. Principal response curve (PRC) for the predator guild from pitfall trapping, indicating the effects of a single spraying of *Beauveria bassiana* or lambda-cyhalothrin, compared to the control. Values deviating from the reference value of zero indicate treatment effects. Weights (right) indicate the affinity of the taxon to the PRC trend.

Table 8.4/02-1: Significance level of treatment effects in pitfall traps on the predators according to Monte-Carlo permutation tests (1999 permutations)

Comparisons	**Before treatment**	**Days after treatment**		
		1	**30**	**60**
	P values			
Control vs. *Beauveria bassiana*	>0.16	>0.88	>0.62	>0.34
Control vs. lambda-cyhalothrin	>0.10	0.02	0.02	0.05
Beauveria bassiana vs. lambda-cyhalothrin	>0.76	0.02	0.02	0.10

Table 8.4/02-2: Significance level of treatment effects in the soil cores on the predator and non-target herbivore guilds 30 days after spraying according to Monte-Carlo permutation tests (1999 permutations)

Comparisons	**Predators**	**Herbivores**
	P values	
Control vs. *Beauveria bassiana*	0.77	0.79
Control vs. lambda-cyhalothrin	0.02	0.27
Beauveria bassiana vs. lambda-cyhalothrin	0.02	0.80

Conclusions

A field trial was conducted in Chile to compare the effects of *Beauveria bassiana* and λ-cyhalothrin on non-target arthropod communities. λ-cyhalothrin significantly reduced the predator guild compared to the *Beauveria bassiana* and control treatments for 30 days after treatments were applied. This clearly demonstrates that no unacceptable effects occurred on non-target arthropods when an application of 10^{12} spores *Beauveria bassiana*/ha were applied.

Comments by RMS:

No unacceptable effects occurred on non-target arthropods communities with an application of 10^{12} spores *Beauveria bassiana*/ha. This qualitative information can be used for risk assessment.

MA 8.4/03

Citation: Steinwender, BM, Krenn, HW, Wegensteiner, R (2010). Different effects of the insectpathogenic fungus *Beauveria bassiana* (Deuteromycota) on the bark beetle *Ips sexdentatus* (Coleoptera: Curculionidae) and on its predator Thanasimus formicarius (Coleoptera: Cleridae). *Journal of Plant Diseases and Protection,* 117(1): 33-38 published

Guideline: None

GLP: No

Abstract

In our study, *Beauveria bassiana* was tested on the bark beetle *Ips sexdentatus* (Coleoptera: Curculionidae) and its predator *Thanasimus formicarius* (Coleoptera: Cleridae). Infection experiments were conducted in the lab at 20°C and long day conditions. *B. bassiana*, isolated from a different bark beetle species *Ips typographus*, was grown on malt extract agar. *I. sexdentatus* was collected from infested pine log sections and *T. formicarius* from pheromone baited traps. To check for insect pathogens that would influence the infection experiments significantly, a pathogen analysis was conducted. Insect pathogens were found in different frequencies (multiple infections were also noted): the ascomycete *Metschnikowia cf. typographi* (29.8%), the protozoan *Gregarina cf. typographi* (36.0%) as well nematodes in the hindgut (38.5%) and nematodes in the haemolymph (16.0%). Adult *I. sexdentatus* were inoculated with two different concentrations of conidia suspension (1×10^6, 1×10^7 conidiospores ml^{-1}) and dry conidia, stripped off from infected bark beetle cadavers. *T. formicarius* was inoculated with conidia suspension (1×10^7 conidiospores ml^{-1}), dry conidia and a very high amount of dry conidia. The experiments showed that *B. bassiana* killed up to 93.7% of the inoculated *I. sexdentatus* within 8 days, but no *T. formicarius* at these doses and only seven out of 15, inoculated with the highest amount of conidiospores.

Materials and methods

Three treatment regimes were used. One of these involved 30 beetles being treated with dry conidia of *Beauveria bassiana* on the last distal sternites, called ‘normal’. The other treatment, which was considered to be ‘extreme’ involved covering the whole ventral side of the body of 15 beetles with dry conidia. The concentration of colony forming units in the dry conidia was not cited. The third treatment

involved dipping the beetles for 3 seconds in a conidia suspension which had a concentration of 1 × 10^7 spores/mL.

The beetles were maintained individually at 20 ± 1°C and were fed with one untreated *Ips sexdantus* per day.

Findings

Together 75 *T. formicarius* were tested with conidia suspension, dry conidia "normal" or dry conidia "extreme". Further 30 beetles were used for the untreated control group. None of the *T. formicarius* (n = 30) that were inoculated with the conidia suspension died because of *B. bassiana*.

Their mean life span was 157 days. The 30 beetles inoculated with the "normal" dose of dry conidia showed a mean life span of 151 days, with no deaths caused by *B. bassiana*. From 15 *T. formicarius* that were inoculated with the "extreme" dose of *B. bassiana* eventually 7 died. The first died after 24 days, the last after 48 days. The last *T. formicarius* of this group died after 108 days, but not because of *B. bassiana*. The mean life span was 52 days.

Mortality and feeding assessments of the treated beetles are given in the table below.

Table 8.4/03-1: Mean life span (MLS), mortality and feeding behaviour of *Thanasimus formicarius* after inoculation by *Beauveria bassiana*

Treatment	**No. of beetles**	**MLS (days) [S.D.] [c]**	**No. of dead beetles**	**Mean *I. typographus* consumed/day [S.D.] [c]**
DC 'extreme'	15	52 [± 24][a]	7	0.56 [± 0.14][a]
DC 'normal'	30	151 [± 68][b]	0	0.61 [± 0.17][a]
1 x 10^7 conidospores/mL	30	157 [± 85][b]	0	0.62 [± 0.11][a]
Control (untreated)	30	188 [± 78][b]	0	0.73 [± 0.15][b]

[c] Values with different letters represent significant difference (P<0.05)

After the inoculation of 30 beetles with a "normal" amount of dry conidia and the inoculation of 30 beetles with the conidia suspension the beetles were observed during 30 days and there were no dead. Following this test 15 beetles were inoculated with the "extreme" amount of conidia, in order to check whether it is at least possible to infect *T. formicarius* with a high dosage of this strain.

The seven dead beetles proofed that it is possible, but only with a very high amount of conidia. It is remarkable that the first *T. formicarius* did not die before day 24 and that the last beetle that was killed by *B. bassiana* died on day 48.

There was a significant difference (P < 0.05) between the mean life span of the "extreme" inoculated group and all other groups.

Conclusions

The results demonstrate that only the 'extreme' dry conidia treatment resulted in significant adverse effects on adult *T. formicarius.* However, no information was provided on conidia concentrations in this

treatment. As it is indicated 'extreme' compared to the conidia suspension of 1 x 10^7 spores/mL used, it is assumed that the *Beauveria bassiana* was applied at >1 × 10^7 spores/mL.

Comments by RMS:

At concentrations up to and including 1 × 10^7 spores/mL there were no negative effects on the non-target arthropod *T. formicarius,* predator of the target *I. sexdentatus.* At unknown concentration larger than >1 × 10^7 spores/mL almost 50% of the exposed *T. formicarius* was killed over an extended time period. Dead beetles were not searched for signs of infection.

A summary of endpoints is given in the table below.

Table B.9.4.1.-01: Toxicity effects of Active ingredient to arthropods

Grassland arthropod community	Field	*Beauveria bassiana*	No reduction in numbers, diversity or species richness of soil dwelling invertebrate communities at 10^{12} spores/ha	Devotto *et al.* (2007)/LIT
			No unacceptable effects occurred on non-target arthropods communities with an application of 10^{12} spores *Beauveria bassiana*/ha.	Devotto *et al.* (2008)/LIT
Thanasimus formicarius	Laboratory	*Beauveria bassiana*	50% effect >1 x 10^7 spores/mL	Steinwender *et al.* (2010)/LIT

PLANT PROTECTION PRODUCT

Reference: MP 10.4.1/01
Whittaker, M (2014). Effects of Broadband (BAS 480 00 I) on the parasitic wasp, in a glass plate contact toxicity test. APIS, Knaresborough Technology Park, Manse Lane, Knaresborough, North Yorkshire, HG5 8LF, UK. Study No. APIS-BASF-012.

Guideline: Modification of IOBC/OILB guidelines on the evaluation of the side effects of plant protection products on non-target arthropods

GLP: yes

Material and methods:

Test substance: Broadband OD (containing *Beauveria bassiana* strain PPRI 5339) Batch P1370

Test species: *Aphidius rhopalosiphi* 'Mummies' (parasitised aphids)

Number of test animals: 4 x 10

Treatments: Nominal: 4 × 10^9 CFU/mL; Actual: 7.91 × 10^9 CFU/mL, toxic reference Perfekthion, Water control, Non-infective MPCP (Broadband deactivated by

	autoclaving)
Duration:	10 days
Test conditions:	temp 22.2"C - 24.8"C; rel. hum.12.4% - 92.8%; light intensity 900lux; 16:8 hours
Deviations from guideline	none
Observations:	Adult survival and fecundity

The study was based on a modification of IOBC/OILB guidelines on the evaluation of the side-effects of plant protection products on non-target arthropods, as the original guideline is not wholly suitable for microbial substances.

Test arenas

Test arenas consisted of square stainless steel frames of internal diameter 10 x 10 x2 cm (H x W x D). Fine slits laser-cut into three of the four sides allowed for ventilation whilst preventing the escape of test organisms. A single hole (1 cm diameter) in the fourth side allowed for the introduction of wasps and was sealed with a cotton plug soaked in 1:3 honey:water solution during the initial 48 hour contact phase. Arenas were sealed on either side with a glass plate, held in place across each axis with a pair of rubber bands.

Test organisms

The parasitic wasp *Aphidius rhopolosiphi* was obtained as synchronised mummies. Mummies were kept under experimental conditions and the adults used within 48 hours of emergence.

For the fecundity assessments, mixed-age *Rhopolosiphum podi* were used. Aphids were inoculated onto young cereal plants.

The glass plates were sprayed using an Iwata Revolution SAR single action airbrush with the Maximum Hazard Concentration (MCP) of the MPCA. For this, 5 mL of MPCA was diluted in 100 mL deionised water. This represented a concentration of 2 x 10^8 CFU/mL, which is 10 times higher than the maximum intended application rate.

After 48 hours, 15 surviving females from the water control, NI MPCP and MPCP groups were removed and confined individually over pots of aphid-infested cereal plants using clear ventilated bags. After 24 hours the wasps were removed and confined individually in Petri dishes with a 1:3 honey:water solution provided *ad libitum*.

Mortality assessments were made approximately 2 hours after treatment and every 24 hours thereafter, for fourteen days. At each observation, the wasps in each arena were classified as alive, affected, moribund or dead.

The aphids were allowed to develop on the plants for a further 10-12 days, after which the number of parasitized aphids that had developed through to pupae was assessed.

Dose verification was performed by diluting the spray solution in a logarithmic scale and plating 100 µL from each dilution onto Rose Bengal Agar. The plates were incubated for 96 h.

Results

Validity criteria

The 48 hour mortality in the water control group was zero, which does not exceed the validity threshold of 13%. The 48 hour mortality in the toxic reference treatment was 100%, which meets the minimum criterion of 75%. All wasps in the control treatments in the fecundity assessment produced a minimum of 5 mummies by study termination, with no zero values.

As all the validity criteria were met the study was deemed to be valid.

mortality

The mortality per treatment group at 10 days after treatment, together with fecundity data are shown in Table B.9.4.1-01.

Table B.9.4.1-01: Mortality and fecundity data for *Aphidius rhopalosiphi* by treatment group at 10 days after treatment with Broadband

Treatment	Number of dead wasps	% mortality	Number of mummies
Water control	0	0	18.2
Inactive MPCA	1	2.5	17.3
MPCA	1	2.5	16.1
Toxic Reference	40	100	-

Dose verification revealed that the concentration of MPCP applied to the glass plates was 3.7×10^8 CFU/mL. As there was 2.5% mortality at this concentration the 10-day LD_{50} >3.7×10^8 CFU/mL. Also, as there was no significant effect on fecundity the NOER = 3.7×10^8 CFU/mL.

Fecundity

The full fecundity data are presented in table B.9.4.1-02 . Mean fecundity at study termination was 18.2 ± 1.4 mummies in the water control and 16.1 ± 1.1 in the MPCP treatment. The difference was not significant (p = 0.266, ns).

Table B.9.4.1-02: raw data of fecundity test

Treatment	No. of parasitized aphids by wasp No.														
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Water	18	21	15	26	10	14	17	22	24	19	18	21	11	10	27
NI MPCP	20	10	16	22	15	16	24	12	21	19	17	25	14	13	15
MPCP	14	21	18	14	23	20	13	9	16	11	16	21	20	9	17

Conclusions

The LT for the test item was determined to be > 10 days, and the 10-day 50% effect level was > 3.7×10^8 CFU/mL. There was no significant difference between control and treatment mortality on any day following exposure to the MPCP (p > 0.3 in all cases), and no significant difference in fecundity (p = 0.266)

Comments by RMS:

The study is considered well performed and the results that no significant effect on mortality and fecundity of *Aphidius rhopalosiphi* occurs can be used for further assessment.

Reference:	MP 10.4.1/02 Whittaker, M (2014). Effects of Broadband (BAS 480 00 I) on the predatory bug, Orius laevigatus, in a glass plate contact toxicity test. APIS, Knaresborough Technology Park, Manse Lane, Knaresborough, North Yorkshire, HG5 8LF, UK. Study No. APIS-BASF-013.
Guideline:	Modification of IOBC/OILB guidelines on the evaluation of the side effects of plant protection products on non-target arthropods
GLP:	yes
Material and methods:	
Test substance:	Broadband OD (containing *Beauveria bassiana* strain PPRI 5339) Batch P1370
Test species:	*Orius laevigatus* Synchronised cohort of 2nd instar nymphs
Number of test animals:	8 x 10
Treatments:	Nominal: 4×10^9 CFU/mL; Actual: 7.91×10^9 CFU/mL, toxic reference Perfekthion, Water control, Non-infective MPCP (Broadband deactivated by autoclaving)
Duration:	10 days
Test conditions:	temp 23.1"C - 24.6"C; rel. hum.61.5% - 92.8%; light intensity 900lux; 16:8 hours
Deviations from guideline	none
Observations:	Adult survival and fecundity

Second instar nymphs of *Orius laevigatus* were held in groups of ten in test arenas consisting of treated glass plates held apart by a shallow untreated metal frame of 10 × 10 × 2 cm (H × W × D). The insects were subject to treatment with either inactive *Beauveria bassiana* strain PPRI 5339 (NI MPCP), active *Beauveria bassiana* strain PPRI 5339 or dimethoate positive control. There were eight replicate arenas per treatment.

The glass plates were sprayed using an Iwata Revolution SAR single action airbrush with the Maximum Hazard Concentration (MCP) of the MPCA. For this, 5 mL of MPCA was diluted in 100 mL deionised water. This represented a concentration of 2×10^8 CFU/mL, which is 100 times higher than the maximum intended application rate.

Second instar nymphs of *Orius laevigatus* were introduced into the arenas once spray deposits had dried. Exposure lasted for 10 days, by which time at least 80% of the bugs in the water control had reached adulthood. Mortality was assessed at this point, and after a further four days surviving females were transferred individually to approximately 3 cm leaf discs of cow pea (*Vigna sinensis*) kept on moist cotton wool in ventilated plastic petri dishes.

After two days the number of eggs was counted and the oviposition substrate replaced. The old substrate was retained for five days and hatching success assessed. The substrate was replaced again after another two days, and the number of eggs counted.

Dose verification was performed by diluting the spray solution in a logarithmic scale and plating 100 µL from each dilution onto Rose Bengal Agar. The plates were incubated for 96 h.

Results

Validity criteria

The mortality in the water control group was four out of 80, which does not exceed the validity threshold of 25%. The mortality in the toxic reference treatment was 100%, which was higher than the guideline estimate of 40%. Nonetheless the test organisms were shown to be susceptible to the toxic reference product, and the result is considered acceptable. In the water control, mean oviposition in the fecundity assessment was above the threshold of 2 eggs per female per day (2.78 eggs per female per day) with no zero values, and egg hatch was 81%, above the 70% threshold.

As all the validity criteria were met the study was deemed to be valid.

mortality

The mortality per treatment group at 10 days after treatment, together with fecundity data are shown in Table 9.4.1-03.

Table 9.4.1-03: Mortality and fecundity data for *Orius laevigatus* by treatment group at 10 days after treatment with Broadband

Treatment	Number of dead insects	% mortality	Mean number of eggs/female/day
Water control	4	5	2.78
Inactive MPCA	4	5	
MPCA	5	6.25	2.73
Toxic Reference	80	100	-

There was no significant difference between the water control and the MCPC treatment in terms of fecundity (p = 0.831, ns) or egg hatching (p = 0.925 n.s.).

Dose verification revealed that the concentration of MPCP applied to the glass plates was 4.1×10^8 CFU/mL. As there was 2.5% mortality at this concentration the 10-day 50% effect level $>4.1 \times 10^8$ CFU/mL. Also, there was no significant effect on fecundity the NOER $> 4.1 \times 10^8$ CFU/mL.

Fecundity

Mean fecundity at study termination was 2.78 eggs per female per day in the water control and 2.73 eggs per female per day in the MPCP treatment (table 9.4.1-03). The difference was not significant (p = 0.831, ns). Egg hatching was 81% in the water control and 87% in the MPCP treatment. The difference was not significant (p = 0.925, ns).

Conclusions

The LT for the test item was determined to be > 10 days, and the 10-day LD_{50} was > 4.1 × 10^8 CFU/mL. There was no significant difference between control and treatment mortality at 10 days after treatment, and no significant difference in fecundity.

Comments by RMS

The study is considered well performed and the results that no significant effect on mortality and fecundity of *Orius laevigatus* occurs can be used for further assessment.

A summary of endpoints is given in the table below.

Table B.9.4.1.-04: Toxic effects of plant protection product to athropods other than bees

Test species	*Aphidius rhopalosiphi*
Toxicity of plant protection product	LT_{50} >10 days; <<25% effect on survival and fecundity
Test species	*Orius laevigatus*
Toxicity of plant protection product	LT_{50} >10 days; <<25% effect on survival and fecundity

Additional information from published literature

According to a review by Zimmerman:

It is well known that *Beauveria bassiana* has a wide host range, occurring on several hundred arthropod species; however, host specificity is really a strain-specific trait. For example, *Beauveria bassiana* isolates from the lady beetle, *Olla v-nigrum*, were pathogenic to adult *O. v-nigrum* but not to adults of the Asian lady beetle, *Harmonia axyridis* (Cottrell & Shapiro-Ilan 2003). The GHA strain of *Beauveria bassiana* was not significantly pathogenic to either *O. v-nigrum* or *H. axyridis*. In contrast, *B. brongniartii* has a much narrower host range being mostly restricted to members of the coleopteran family Scarabaeidae.

Generally, there is a difference between the physiological host range and the ecological host range (Hajek & Butler 2000). The physiological host range demonstrates the range of insect species that can be infected in the laboratory, while the ecological host range demonstrates which insects can be infected in nature or under field conditions. Non-target insects which are infected under laboratory conditions may not necessarily be infected in nature. This topic was also discussed in detail by Hajek and Goettel (2000) and Jaronski *et al*. (2003).

One of the first comprehensive reports was given by Goettel *et al* (1990), who listed the effects of *Beauveria bassiana* on nontarget invertebrates, such as bees and other pollinators, silkworms, predators and parasitoids. Further, general information is mentioned by Goettel *et al*. (1997, 2001) and Vestergaard *et al*. (2003). The last authors conclude that despite the wide host range of *Beauveria bassiana*, evidence to date suggests that this fungus can be used with minimal impact on non-target organisms, especially when isolate selection and spacio-temporal factors are taken into consideration. *Beauveria brongniartii* has a narrower host range, mainly including Scarabaeidae, and occurs worldwide in soil habitat. Laboratory bioassays demonstrated that it was possible to infect

collembolans, cicindellid and carabid beetles under stress conditions, while honey bees and earthworms were not affected. Data from field investigations did not reveal any indication of possible adverse effects on vertebrates, honeybees, beneficial insects, earthworms and plants (Vestergaard *et al*. 2003).

RMS remarks:

The studies by Goettel (1990, 1997), Hajek and Goettel (2000) and Jaronski *et al*. (2003) are reviews from which endpoints cannot be used as such. Therefore they are considered as additional information.

B.9.4.2 Infectiveness

No infectivity was tested.

B.9.4.3 Pathogenicity

No pathogenicity was observed.

B.9.4.4 Summary and risk assessment for non-target arthropod species other than bees

Orius and *Aphidius* species are among the most important components of glasshouse IPM programmes, and are the mainstay of most biological control programmes for the control of thrips and aphids in protected salad crops. Specifically, *Orius laevigatus* was chosen as a test species because it is a member of an important group of leaf-dwelling predators with a pan-European distribution, and *Aphidius rhopalosiphi* because it is a member of an important group of aphid parasites, has a wide geographic range, and has been found to be more sensitive and more reliable in toxicity tests than other *Aphidius* species.

A summary of the toxicity data for non-target arthropods derived from various sources is presented in the table below.

Table 9.4.4-1: Summary of non-target arthropod toxicity data

Species	Test type	Test substance	Endpoints and observations	Reference/Source
Aphidius rhopalosiphi	Tier I	Broadband (BAS 480 00 I)	LT > 10 days, 10-day 50% effect > 3.7×10^8 cfu/mL for mortality and fecundity	Whittaker (2014b)
Orius laevigatus			LT > 10 days, 10-day 50% effect > 4.1×10^8 cfu/mL for mortality and fecundity	Whittaker (2014c)
Grassland arthropod community	Field	*Beauveria bassiana*	No reduction in numbers, diversity or species richness of soil dwelling invertebrate communities at 10^{12}	Devotto *et al.* (2007)

			spores/ha	
			No unacceptable effects occurred on non-target arthropods communities with an application of 10^{12} spores *Beauveria bassiana*/ha.	Devotto *et al*. (2008)
Thanasimus formicarius	Laboratory	*Beauveria bassiana*	50% effect >1 × 10^7 spores/mL	Steinwender *et al.* (2010)

LIT = Literature review conducted in 2014

Within greenhouse

The highest concentration of *Beauveria bassiana* strain PPRI 5339 that would be used in greenhouses after one application is 4 x 10^9 spores/L. According to the environmental fate section, the viable *B. bassiana* conidia were almost completely inactivated by exposure to 60 minutes of direct sunlight or 20 seconds of UV light at a wavelength of 302 nm. Therefore it can be assumed that under greenhouse conditions where the light intensity is should mimick the sunlight, the conidia of the fungi will not survive on the plants between the 122 applications. However, survival of spores in the nectar of plants which can be a food source of adult parasitic beneficials cannot be excluded. For the predatory natural enemies feeding on the prey, it is considered that the exposure will be dependent on the number of prey available which have the pathogenic fungus, thus for these types of natural enemies the exposure of the EPF to light is not considered relevant and thus the exposure is assumed throught the maximum total application rate of 4 x 10^{11} CFU/L.

In the field studies on grassland arthropod community there were no effects on soil dwelling invertebrates at application rates of 10^{12} spores/ha. In the second study, at unknown concentration larger than >1 × 10^7 spores/mL almost 50% of the exposed *T. formicarius* was killed over an extended time period. Considering that these concentrations were 10-1000 times lower than the current maximum application rate in the greenhouses of 10^{15} CFUs/ha or 4 x 10^{11} CFU/L, and that this is an EPF with a possible broad host range (see Vol. B1-B5, B.2.1.3), the RMS is of opinion that the a warning sentence should be placed on the label in order to protect the natural enemies used in IPM. The warning sentence should read: "*Warning: This product is harmful for natural enemies. Consult your experts (the supplier of natural enemies, the producer of this product, your advisor) about the use of this product in combination with natural enemies*". The MSs might decide if to apply this warning sentence at the national level.

B.9.5 Effects on earthworms (Annex IIM 8.5; Annex IIIB 10.5)

B.9.5.1 Toxicity

ACTIVE INGREDIENT

MA 8.5/01

Citation: Shapiro-Ilan, D.I. and Brown, I. (2013) Earthworms as phoretic hosts for *Steinernema*

carpocapsae and *Beauveria bassiana*: Implications for enhanced biological control. Biological Control 66:41–48

Guideline: None

GLP: No

Summary

The authors hypothesized that biocontrol efficacy of entomopathogenic fungi could be enhanced in the presence of earthworms based on increased nematode dispersal through the soil. The effects of *Lumbricus terrestris* on dispersal of the entomopathogenic fungus *Beauveria bassiana* in soil columns was determined. *B. bassiana* dispersal was enhanced by earthworm presence. The fungus was carried through soil by the earthworm; conidia recovered from the earthworm casts remained viable and was pathogenic to *Galleria mellonella*.

Materials and methods

Soil columns were made of four sections of polystyrene pipe (4.6 cm diameter with 5.0 cm height) were used. The columns were filled with approximately 454 g of autoclaved loamy sand soil with a final moisture level at field capacity (16 %).

Treatments included *B. bassiana* with and without *L. terrestris*. A control with earthworms only was also included. Approximately, 3.6×10^8 conidia (2.7×10^7 cm^2; equivalent to 2.7×10^{15} CFU/ha) were added to fungus-treated columns in 5 ml tap water. The controls only received 5 mL water. After 4 h, two earthworms were added to the appropriate columns and all columns were covered. The columns were incubated at 23–25 °C, and to maintain soil moisture levels, they were placed in large plastic bags (containing moist paper towels and separated by replicate). There were eight columns for each treatment and control. One half of the columns (four replicates) were assessed 7 d post treatment, and the other half of the columns (four replicates) were assessed 14 d post-treatment. Both the 7 and 14 d experiments were repeated once in time (two trials each).

A bioassay was used to estimate dispersal of *B. bassiana* within the columns. On the appropriate assessment date (7 or 14 d), columns were dismantled and soil from each section was transferred to a 150 mm Petri dish. Each dish was baited with 10 last instar *G. mellonella*. *G. mellonella* was chosen for the bioassay because it has previously been demonstrated to be highly susceptible to the fungus, and may be used to indicate relative population levels of *B. bassiana*. Petri dishes were stored at 25 °C in plastic boxes (by replicate) and the boxes were placed in plasticbags containing moist paper towels. The number of *B. bassiana* infected insects 14 d after exposure was recorded.

In addition to evaluating the distribution of *B. bassiana* within the columns, *L. terrestris* recovered from columns were assessed for evidence that *B. bassiana* conidia were transported via earthworm casts, and if the recovered conidia remained viable and pathogenic. Earthworms were initially rinsed with approximately 2 mL water, then surface sterilized in 70% ethanol and subjected to an additional rinse in sterile water. The earthworms were then placed in 100 mm Petri dishes lined with filter paper and allowed to cast for 48 h. Three last instar *G. mellonella* were added to one half of the Petri dishes containing earthworm casts and assessed for *B. bassiana* infection as described above. The other half of the casts were removed from Petri dishes and streaked onto selective media using a sterile curved glass rod. Presence of viable conidia on the selective media plates was indicated by germination and

formation of *B. bassiana* colonies.

In the assay described above, conidia recovered from *L. terrestris* casts were found to be viable on selective media but pathogenicity to *G. mellonella* was not detected. An additional experiment was conducted to determine if higher rates of application would reveal pathogenic conidia in the earthworm casts. The experiment was conducted in 473 mL plastic cups. The cups were filled with approximately 250 g dry autoclaved soil (the same as described above). *B. bassiana* conidia were added in 5 mL of tap water at a rate of 4.6×10^9 per cup (2.77×10^8 per mL; 1.84×10^{10} CFU/kg dry soil). A control with no conidia (water only) was included. The soil was mixed thoroughly and three *L. terrestris* were added to each cup. After 5 d, earthworms were removed from the cups, allowed to cast, and the pathogenicity of recovered conidia was determined as described above. There were four replicates for the treatment and control, and the experiment was repeated once in time.

ANOVA and the LSMEANS test were used to determine effects of earthworm presence on *B. bassiana* dispersal (based on infection of *G. mellonella* in the various column sections). Data from repeated experiments (trials) were pooled and each trial was considered as a block effect. Percentage data (*G. mellonella* infection) were arcsine transformed prior to analysis. Growth of viable conidia on selective media plates was analyzed by the Krusksal–Wallis test (the number of plates showing growth was compared between the earthworm treatment with *B. bassiana* and the control without *B. bassiana*).

Findings

In the experiment in which columns were dismantled 7 d post-treatment, evidence for enhanced dispersal of *B. bassiana* in the presence of earthworms was observed. Higher levels of *B. bassiana* infection in *G. mellonella* were observed in treatments with earthworms than without earthworms in sections 1, 2 and 4. Relative to the control, the *B. bassiana* treatment without earthworms only caused higher infection in section 1, whereas the *B. bassiana* treatment with earthworms caused higher infection than control in all sections except number 3.

Evidence for enhanced dispersal of *B. bassiana* in the presence of earthworms was also observed in the experiment in which columns were dismantled 14 d post-treatment. (Higher levels of *B. bassiana* infection in *G. mellonella* were observed in treatments with earthworms than without earthworms in sections 2, 3, and 4. Relative to the control, the *B. bassiana* treatment without earthworms only caused higher infection in section 1, whereas the *B. bassiana* treatment with earthworms caused higher infection than the control in all sections.

Evidence that conidia were carried through soil via earthworm casts, and the conidia remained viable and pathogenic, was also observed. When earthworm casts from columns with *B. bassiana* were plated onto selective media, 50% showed formation of *B. bassiana* colonies, which was statistically greater than control plates. All of the *B. bassiana* positive plates except one were from the 14-d experiment. None of the earthworm casts extracted from columns with or without *B. bassiana* exhibited pathogenicity to *G. mellonella*. However, when the assay was conducted in soil cups with an increased rate of *B. bassiana*, earthworm casts from the *B. bassiana* treatment caused *G. mellonella* infection in one half of the replicates (in both trials).

In this study, no apparent harm was inflicted upon the earthworms from the entomopathogens. No

earthworm acute mortality was noted, and no observations of changed behavior in terms of reduced mobility or feeding based on cast levels were documented.

Conclusions

B. bassiana was neither infective nor pathogenic to earthworms at an application rate of 2.7× 10^{15} CFU/ha.

Comments by RMS: The authors demonstrated that the B. bassiana GHA dispersal was enhanced by the earthworm *Lumbricus terrestris* presence with viabile conidia being recovered from earthworms casts. Exposure of earthworms to 2.17 x 10^{15} CFU/ha and 2.77 x 10^{16} CFU/ha for 7 and 14 d, and 5 d, respectively did not in mortality of the earthworms. The authors state that there were no behavioural changes in terms of reduced mobility or feeding based on casts levels. The results can be used in the risk assessment.

MA 8.5/02

Citation: Nuutinen V. et al. (1991) The effects of four entomopathogenic fungi and an entomoparasitic nematode on the hatching of earthworm (Aporrectodea caliginosa) cocoons in laboratory. Journal of invertebrate pathology 58:147-149

Guideline: None

GLP: No

Comment by RMS: The applicant did not provide the a study summary. The following short summary is prepared by the RMS. Cocoons of the earthworm *Aporrectodea caliginosa* were exposed to Beauveria bassiana (Bb66) isolated from the Finnish agricultural soils. Suspensions of 2.5 x 10^{7} spores/ml were prepared and 4 ml of this solution was sprayed onto the soil surface containing earthworm's cocoons. The earthworms were covered with an additional soil layer. The tests were run for 48 days when the esrthworms. Unhatched cocoons, and cocoons shells from the soils were determined. The hatching rate in the control was lower than that in the cocoons exposed to the fungi which make the interpretation of the results difficult. The fungi did not appear to infect the cocoons. In the light of the results obtained with the control, the RMS belives that the results cannot be used in the risk assessment.

Table B.9.5.1.a: Toxicity effects of Active ingredient to earthworm

Test species	*Lumbricus terrestris*
Toxicity 5 d LD50	> 2.77 x 10^{16} CFU/ha

PLANT PROTECTION PRODUCT

No studies with the product were submitted. In the Netherlands, the management practice in the greenhouses includes regular sterilisation of the soil, which prevents the formation of a natural soil organism community and therefore, a study with the product is not considered necessary. The MS should asses if this management practice is applicable at their national level.

Effects on other soil invertebrates

From a review by Zimmerman (2007):

Beauveria bassiana and *B. brongniartii* are commonly found in soil. They generally have a broad host range and are often used for biocontrol of soil dwelling pests. Therefore, possible interactions or effects on other non-target soil inhabiting invertebrates should be noticed.

Other information from open literature:

In 1964, Samšiňák found that the mites *Tyrophagus putrescentiae* and *Acarus siro* are not susceptible to *Beauveria bassiana*. *Tyrophagus putrescentiae* feeds on insects and also on dead, *Beauveria*-infected larvae. This mite species is also able to transmit spores of *Beauveria bassiana* from fungus-infected larvae of *Galleria mellonella* to healthy ones. *Beauveria bassiana* was also found in high frequency on a great number of the collembolan *Onychiurus subtenuis* present in soil litter (Visser *et al*. 1987). The authors did not report any indication that *Beauveria bassiana* affected the collembolan. The collembolan *Folsomia candida* was not susceptible to *Beauveria bassiana*. *F. candida* consumed and inactivated the insect pathogen without suffering mortality, reproductive disturbance or any other harmful effects. Conidia ingested by Collembola were partially inactivated in the gut of these insects. *Beauveria bassiana* conidia recovered from the gut and feces of *F. candida* had lost from 30 to 50% viability. (Broza *et al*. 2001). Pathogenicity tests of *Beauveria bassiana* (3 strains) and *Beauveria brongniartii* (3 strains) were conducted against adults of *Folsomia fimetaria*, *Hypogastrura assimilis* and *Proisotoma minuta* (Dromph & Vestergaard 2002) by dipping the collembolans in 1 x 10^7 conidia/mL suspension. None of the fungal isolates increased mortality over the controls. In a test of the attractiveness of the fungi for the three collembolan species, *B. brongniartii* was found to be more attractive than baker's yeast. For *H. assimilis B. bassiana* was less attractive than baker's yeast. Mites were also observed feeding on *B. brongniartii* killed white grubs of *Melolontha* spp. without any sign of infection (Zimmermann, unpubl.).

Dispersal of entomopathogenic fungi by Collembolans has been demonstrated by several authors (Samšiňákova & Samšiňák 1970; Zimmermann & Bode 1983; Dromph 2001, 2003). For example, *Beauveria bassiana* is distributed both in a horizontal and vertical direction by the mite *Sancassania phyllognathi* which is resistant to fungal infection (Samšiňákova & Samšiňák 1970). The transmission of spores of *Beauveria bassiana* and *B. brongniartii* to a susceptible host, *Tenebrio molitor*, by the collembolans *F. fimetaria*, *H. assimilis* and *P. minuta* was also demonstrated (Dromph 2003).

These findings show that there are no or very low detrimental effects on the tested soil-dwelling collembolans and mites. In contrast, collembolans can act as vectors of *Beauveria* spp. and thus may play an important role for the dispersal and transmission of these fungi in soil.

B.9.5.2 Infectiveness

From the submitted information on collembolans it is demonstrated that *Beauveria bassiana* is not infective to earthworms and soil mites.

B.9.5.3 Pathogenicity

From the submitted information on collembolans it is demonstrated that *Beauveria bassiana* is not pathogenic to earthworms and soil mites.

B.9.5.4 Summary and risk assessment for earthworms

According to the OECD 67 (2012) document (ENV/JM/MONO(2012)1)and also EFSA (2014)[3] on the environmental safety evaluation of microbial biocontrol agents, exposure of soil organisms is not considered relevant and therefore no risk assessment is required.

The study by Shapiro Ilan D and Brown I. (2013) demonstrated that no mortality or behavioural effects on earthworms occurred at application rates 10 times higher than the current maximum application rate in the greenhouse. The earthworms served as phoretic hosts for the fungi and contributed to their dispersal in the soil.

For collembolan and mite species the information provided demonstrates that these are not affected by *Beauveria bassiana.* They use the fungus as food source and act as conveyor and thus may play an important role for the dispersal and transmission of these fungi in soil.

B.9.6 Effects on non-target soil micro-organisms (Annex IIB 8.6; Annex IIIB 10.6)

MA 8.6/13

Report: Hirsch, J, Galidevara, S, Strohmeier, S, Devi, KU, Reineke, A (2013). Effects on Diversity of Soil Fungal Community and Fate of an Artificially Applied *Beauveria bassiana* Strain Assessed Through 454 Pyrosequencing. *Microbial Ecology, 66(3): 608-620*

Guidelines: None

GLP: No

Abstract The entomopathogenic fungus *Beauveria bassiana* is widely used as a biological control agent (BCA) for insect pest control, with fungal propagules being either incorporated into the potting media or soil or sprayed directly onto the foliage or soil. To gain a better understanding of entomopathogenic fungal ecology when applied as a BCA to the soil environment, a case study using tag encoded 454 pyrosequencing of fungal ITS sequences was performed to assess the fate and potential effect of an artificially applied *B. bassiana* strain on the diversity of soil fungal communities in an agricultural field in India. Results show that the overall fungal diversity was not influenced by application of *B. bassiana* during the 7 weeks of investigation. Strain-specific microsatellite markers indicated both an establishment of the applied *B. bassiana* strain in the treated plot and its spread to the neighboring non-treated control plot. These results might be important for proper risk assessment of entomopathogenic fungi-based BCAs.

[3] *EFSA Guidance Document on clustering and ranking of emissions of active substances of plant protection products and transformation products of these active substances from protected crops (greenhouses and crops grown under cover) to relevant environmental compartments. EFSA Journal 2014;12(3):3615, 43 pp., doi:10.2903/j.efsa.2014.3615*

Materials and methods

Experiments were conducted from October to December in a cultivated agricultural with a standing crop of 4-week-old chili plants. Chili was chosen as a crop in this study, as it is frequently attacked by insect pests such as *Spodoptera litura* and *Helicoverpa armigera* (Lepidoptera: Noctuidae) that are, at the same time, potential targets for the applied *Beauveria bassiana* isolate. No naturally occurring entomopathogenic fungal epizootics have been documented in the selected field over the last 15 years and the last artificial introduction of *Beauveria* sp. was made 14 years previously. In addition, no insecticides or fungicides had been applied in the field over the previous 2 years. Two 50 m^2 plots were prepared. One of them was treated (T) once with *Beauveria bassiana* strain ITCC 4688 and the other was left as an untreated control (C). There was no replication. Before the application of *Beauveria bassiana*, soil cores (approximately 4 × 4 × 15 cm depth) were collected in order to determine pre-treatment levels of *Beauveria bassiana*.

For application of the fungus to the treated (T) plot, 200 g of rice containing sporulated *Beauveria bassiana* strain ITCC 4688 was suspended in 30 L water with 2 mL Tween 80 to give a final concentration of ~10^9 conidia per mL. The whole suspension was dispensed manually onto the soil and plants in the (T) plot, resulting in a concentration of approximately 3×10^{13} conidia/50 m^2.

For assessment of effects of this *Beauveria bassiana* strain on indigenous soil fungal community structure, soil samples were collected in each plot as described above at weekly intervals for a duration of 7 weeks.

In the laboratory the soil samples were homogenized and genomic DNA was extracted. In total, 92 fungal PCR products, tag- encoded according to sampling date and plot, were pooled at equimolar concentrations and 454 pyrosequencing was performed commercially on a Roche (454) FLX Genome Sequencer.

Findings

Prior to the application of *Beauveria bassiana*, no significant differences in the diversity (Shannon index) of fungal communities between control and treatment plots were evident.

After application, a remarkable increase in the number of sequence reads homologous to *Beauveria bassiana* and *C. bassiana,* a teleomorph *B.bassiana,* were observed in samples from both the control and treatment plots, with the highest number of *Beauveria bassiana*.

During the 7 week investigation, no effect of *Beauveria bassiana*, applied at a concentration of 3×10^{13} conidia per 50 m^2, was evident on the diversity of indigenous fungal communities.

Conclusion

At an application rate of *Beauveria bassiana* at 3×10^{13} conidia/50 m^2 no adverse effect on indigenous fungal communities was observed.

Comment by RMS

The study provides information on the effects of application of *Beauveria bassiana* on soil microbial community. No adverse effects occurred. This information will be used for further assessment.

Table 9.6-1: Summary of soil micro-organisms toxicity data

Species	Test type	Test substance	Endpoints	Reference/Source
Soil micro-organisms	Field study	*Beauveria bassiana*	No adverse effects at 3×10^{13} conidia/50 m^2	Hirsch *et al*. (2013)

Other information from open literature:

Investigations on the natural prevalence of *Beauveria bassiana* have shown that this fungus widely occurs in the soil as well as on insects in the aerial environment. This means that there is a long lasting evolutionary coexistence with other microorganisms that includes different forms of interactions (Zimmermann, 2007).

From the viewpoint of safety, the main concern is that microorganisms applied for biological control could potentially pre-empt or displace other non-target microorganisms. After application on plants or in the soil, biocontrol agents should be able to survive and maintain themselves for biocontrol activity, but they should not interfere with the resident microbiota. In a risk analysis case study using *Fusarium* species, the effect of antagonistic *Fusarium oxysporum* to control *Fusarium* wilts on the resident soil microbiota revealed that the introduction of wild-type and genetically manipulated antagonistic strains of *Fusarium oxysporum*, released alone or in mixture, did not interfere with the microbial equilibrium of a natural soil (Gullino *et al*. 1995). Similar results were also obtained by Wang *et al*. (2004) (Doc M-MA 8.6/03) in *Beauveria bassiana* and Enkerli *et al*. (2004) (Doc M-MA 8.6/04) in *B. brongniartii*. Wang et al. (2004) monitored the fate of inundatively applied strains of *Beauveria bassiana* against *Dendrolimus punctatus* in southwest China. During one year, the indigenous and exotic strains were reisolated, but the indigenous strains predominated in the local environment, indicating that they were not displaced by the exotic ones. Enkerli *et al*. (2004) studied the behaviour of introduced *B. brongniartii* strains for control of *M. melolontha* grubs in Switzerland. The results suggested that applied and indigenous strains of *B. brongniartii* could coexist in the same habitat.

Furthermore, in *Beauveria bassiana*, a genetic exchange between indigenous and introduced strains in the field is unlikely due to the large number of vegetative compatibility groups (Castrillo *et al*. 2004) (Doc M-MA 8.6/05). Strains of *B. bassiana* released in the field as mycoinsectides showed a high level of genetic diversity as evidenced by resolution of the 34 strains into 32 separate genotypes and into 23 VCGs (n.b. vegetative compatibility group). The large number of VCGs observed and the low frequency of in vivo recombination limited to vegetatively compatible strains indicate that this self/non-self-recognition system may be an effective barrier preventing genetic exchange between dissimilar strains in the field. The paper does indicate that it is possible that heterokaryon may be formed during cross incubation of two vegetative strains, which is completely natural for an anamorphic fungus such as *Beauveria bassiana.* However recombination in fungi, particularly in species that are not pathogenic to mammals, is not a significant issue compared with the potential recombination in bacterial species.

There are several reports on interactions of *Beauveria* spp. with hyperparasitic, antagonistic and especially, phytopathogenic fungi. A hyperparasitic fungus attacking *Beauveria bassiana* and *B. brongniartii* is the ascomycete *Syspastospora parasitica*, formerly known as *Melanospora parasitica*

(Markova 1991; Posada *et al*. 2004) forming a tritrophic association. Lingg and Donaldson (1981) investigated the survival of conidia of *Beauveria bassiana* under various conditions (temperature and relative humidity in sterile and nonsterile soil. In nonsterile soil amended with carbon sources, nitrogen sources or combinations of both, the conidia recovery was reduced compared to the same situations in sterile soil. The reduced survival in nonsterile soil was possibly related to the presence of *Penicillium urticae* in the soil, which produced a water-soluble inhibitor of *Beauveria bassiana*. No conidia were recovered from sterile soil that was amended with neopeptone and *Penicillium urticae* filtrate.

There are also various interactions with *Clonostachys* spp. and *Trichoderma* spp. which may suppress or overgrow *Beauveria bassiana in vitro* (Krauss *et al*. 2004; Zimmermann unpublished). There has been increased interest to test and use *Beauveria bassiana* also against plant pathogens (Ownley *et al.* 2004). According to the experiments, *Beauveria bassiana* isolate 11-98 could reduce *Rhizoctonia sola*ni damping-off of tomato in greenhouse tests, and also protect cotton against a seedling disease complex in some sites. Laine and Nuorteva (1970) had previously observed that *Beauveria bassiana* and *B. brongniartii* (*B. tenella*) had a strong antagonistic effect on the plant root pathogenic fungus *Fomes* (*Heterobasidium*) *annosus*. *Beauveria bassiana* had also an antagonistic activity against *Ophiostoma ulmi* (*Ceratocystis ulmi*) (Gemma *et al*. 1984) another plant pathogen.

B.9.6.1 Summary and risk assessment for soil micro organisms

According to the OECD 67 (2012) document (ENV/JM/MONO(2012)1)and also EFSA (2014)[4] on the environmental safety evaluation of microbial biocontrol agents, exposure of soil organisms is not considered relevant for glasshouse use. In the Netherlands, the management practice in the greenhouses includes regular sterilisation of the soil, which prevents the formation of a natural soil organism community. The MS should asses if this management practice is applicable at their national level. Exposure of natural soils is not expected.

For the surrounding of a glasshouse a conservative approach assuming that 0.1% of the product would drift from the greenhouse during a spraying event, the maximum external exposure to soil would be 1×10^{10} cfu/ha. Assuming a soil bulk density of 1.5 g/cm^3 and an incorporation depth of 5 cm, the maximum concentration would be 1.33×10^7 cfu/kg soil.

The field study conducted in India at concentration of 3×10^{13} conidia/m^2, which is far in excess of the maximum predicted concentration following application of BAS 480 00 I, demonstrated no adverse effects on microbial populations. It can therefore be concluded that the application of BAS 480 00 I according to the proposed use pattern would have no adverse effects on soil micro-organisms present in soil external to the treated greenhouses.

[4] *EFSA Guidance Document on clustering and ranking of emissions of active substances of plant protection products and transformation products of these active substances from protected crops (greenhouses and crops grown under cover) to relevant environmental compartments. EFSA Journal 2014;12(3):3615, 43 pp., doi:10.2903/j.efsa.2014.3615*

B.9.7 Effects on terrestrial plants (Annex IIB 8.6; Annex IIIB 10.7)

In summarising the past literature, Müller-Kögler (1967) concluded that side-effects or any phytopathogenic activity on plants are not known. *Beauveria bassiana* has been reported to be an endophyte of certain plants, especially corn (Bing & Lewis 1992). Studies have demonstrated that the fungus, applied to whorl-stage corn by foliar application or injection, colonised, translocated in corn plants possibly along with plant photosynthates. Although *B. bassiana* was isolated from many of the soil samples collected from the field plots before fungal application, the

conidia in the soil did not appear to invade many plants, because none of the plants sampled ahead of fungal application were colonized by *B. bassiana,* and by the end of experimentation, only a few uninjected plants were colonized by the fungus.

In the greenhouse, *Beauveria bassiana* was applied as a liquid seed treatment to Bt transgenic corn hybrids and their near isolines (2 x 10^{10} conidia/mL), and no significant differences in seed germination or presence of root pathogens were observed. The same lines of corn were used in field experiments with treatments of seeds soaked in a suspension of *B. bassiana*, a foliar application of a granular formulation of *B. bassiana*, and corresponding untreated checks. Plants were sampled throughout the growing season and evaluated for growth of individual plant components, including sheaths, leaves, stem, husk, ear, plant leaf-to-stem ratio and overall plant growth. There were no significant differences in overall plant growth between the *B. bassiana* treatments or in the growth of each plant component. The results of this study indicate that *B. bassiana* readily forms an endophytic relationship with transgenic and non-transgenic corn and causes no plant pathology (Lewis et al. 2001).

B.9.8 Additional studies (Annex IIM 8.7; Annex IIIB 10.7)

Effects of *Beauveria bassiana* on additional taxa to those covered in previous sections were also described in a review by Zimmermann (2007) from the public information as presented below:

<u>Amphibia</u>

A fungal suspension of *Beauveria bassiana* was fed to the leopard frog, *Rana pipiens* via gastric incubation. The dosage was 9.8 x 10^8 conidia. No mortality or fungus recovery was recorded in any of the tissues other than the stomach where recoveries are expected because of the oral intake. The viscera were free of any fungal elements. Viability of spores was established in fecal washings of pellets indicating that the spores are inactivated in the gut, but remain viable. (Donovan-Peluso *et al*., 1980).

<u>Reptiles</u>

A fungus attributed to be *Beauveria bassiana* was observed to cause infections in a captive American alligator (Fromtling *et al*. 1979). The reptiles were in captivity and under temperature stress which may explain their susceptibility to the fungus.

Remark by RMS: in this study it is only suspected the intestines of the alligators showed infection by *Beauveria bassiana.* The applied methods do not demonstrate this to any extend.

When a tortoise was kept at 22°C and injected with 0.5 mL of 10^6 spores of *Beauveria bassiana* into the lung, no mortality was observed, while a second contaminated tortoise died when kept only at 16°C (Müller-Kögler 1967). The authors' conclusion was that nothing can be predicted as to the

pathogenicity of these fungi for cold-blooded vertebrates. Perhaps stress conditions may promote fungal infections in these animals.

B.9.9 References relies on

Report: *Beauveria bassiana* strain PPRI 5339, DOCUMENT M-MA, Section 9, literature search. Biosphere Biopesticide Consulting – September 2014

Guidelines: European Food Safety Authority; Submission of scientific peer-reviewed open literature for the approval of pesticide active substances under Regulation (EC) No 1107/2009 (OJ L 309, 24.11.2009, p. 1-50). EFSA Journal 2011;9(2):2092. [49 pp.]. doi:10.2903/j.efsa.2011.2092

GLP: no

A literature search was carried out by the notifier following the EFSA guideline "Submission of scientific peer-reviewed open literature for the approval of pesticide active substances under Regulation (EC) No 1107/2009"[5] (M document, section 9).

Selection of databases

Search engines used were Thomson Innovation (which covers Web of Science), TOXLINE and Pubmed. A justification for the chosen search engines was provided and were agreed with by the RMS. The search was carried out in June 2014 and covered the 10 year period prior to the search date.

Table 9.9-1: Reporting of the search process for scientific peer-reviewed open literature

Thomson Innovation	TOXLINE	PubMed
Justification for choosing the source: A bibliographic database covering scientific literature from the other Thomson Reuters products: Web of Science, Current Contents Connect, Conference Proceedings and Inspec. **Web of Science:** The Web of Science provides access to current and retrospective multidisciplinary information from more than 10,400 of the most prestigious, high impact research journals in the world in the sciences, social sciences and arts and	**Justification for choosing the source:** The TOXLINE database is the National Library of Medicine's (NLM) bibliographic database for toxicology, a varied science encompassing many disciplines. TOXLINE records provide bibliographic information covering the biochemical, pharmacological, physiological, and toxicological effects of drugs and other chemicals. It contains over 4 million bibliographic citations, most with abstracts and/or indexing terms and CAS Registry Numbers. TOXLINE covers much of the	**Justification for choosing the source:** PubMed comprises more than 23 million citations for biomedical literature from MEDLINE, life science journals, and online books. Approximately 25,000 records are included in the PubMed journal lists which are updated daily and include all MEDLINE® titles as well as OLDMEDLINE and other non-MEDLINE titles in PubMed. The PubMed journal lists cover the entire span of MEDLINE, not just currently indexed journals.

[5] *European Food Safety Authority; Submission of scientific peer-reviewed open literature for the approval of pesticide active substances under Regulation (EC) No 1107/2009 (OJ L 309, 24.11.2009, p. 1-50). EFSA Journal 2011;9(2):2092. [49 pp.]. doi:10.2903/j.efsa.2011.2092. Available online: www.efsa.europa.eu*

humanities – with coverage back to 1900 (sciences), 1956 (social sciences) and 1975 (arts & humanities).	standard journal literature in toxicology, complemented with references from an assortment of specialized journals and other sources		
Date of search: 25 June 2014	**Date of search:** 25 June 2014	**Date of search:** 25 June 2014	
Date span of the search: 2004 - present	**Date span of the search:** 2004 - present	**Date span of the search:** 2004 - present	
Date of the latest database update included in the search: Thomson Innovation is updated weekly	**Date of the latest database update included in the search:** TOXLINE is updated at least weekly	**Date of the latest database update included in the search:** PubMed is updated daily	
Search strategy used for this data requirement			
(Beauveria bassiana) OR (Tenellin) OR (Vivotoxins) OR (Bassianolide) OR (Bassianin) OR (Beauvericin)			
Total number of summary records retrieved: 2229	**Total number of summary records retrieved:** 272	**Total number of summary records retrieved:** 881	
Total number of summary records retrieved after removing duplicates:			***n* = 2494**

Search strategy

For all databases, a single concept search strategy was adopted, i.e. using terms for *Beauveria bassiana* and its metabolites only (e.g. synonyms, IUPAC name and CAS number).

Criteria for relevance

Relevance criteria were created to determine if a literature paper was:

- relevant without restriction;
- relevance is unclear;
- clearly irrelevant.

This was achieved by comparing the paper with discriminating relevance criteria. Two tiers of relevance criteria were applied as follows:

- key qualifying criteria;
- assessment level qualifying criteria.

Key qualifying criteria identify individual fundamental aspects that, if on their own, are deviated from or details of such are omitted, it can be concluded with acceptable certainty that the paper is identified as being “clearly irrelevant”. Therefore, identifying a deviation from a key qualifying criteria is sufficient for the assessment to stop.

If all key qualifying criteria were met then the relevance assessment continued to the second tier where the literature was compared to “assessment level qualifying criteria”.

Assessment level qualifying criteria are based on a number of scored secondary criteria. These criteria consider the relevance and robustness of the methods used in key areas of the study reported in the literature. Each criterion was scored either “0” meaning unacceptable deviation or “1” meaning that literature detail was acceptable for relevance scoring. The assumption was that if the majority of these secondary criteria were met then the literature can be classified as being “relevant without restriction”, if the score was lower, literature was classified as either “relevance is unclear” (e.g. ~50% criteria met) or “clearly irrelevant” (e.g. <50% criteria met). The criteria bands depend on the number of criteria defined as being assessment level and are shown in the Table 9.9-2 to Table 9.9-4. The process is transparent and removes bias.

Table 9.9-2: Ecotoxicology relevance criteria – laboratory studies (including semi-field)

Key qualifying criteria	
1.	Well-defined test material (
2.	Effects are directly related to the test material in question
3.	Applicable test species
4.	Control health/mortality reported
5.	Relevant endpoint data
Assessment level qualifying criteria (*each conforming point will score 1, each non-conforming point will receive 0*)	
1.	Where appropriate, test organisms have not been previously exposed to the test material of other contaminants
2.	Appropriate test material dosing or application into the system based on the physicochemical characteristics of the test material
3.	Experimental conditions similar to guidelines where appropriate and of appropriate length
4.	Dependent on the endpoint, sufficient doses/ concentrations tested, sufficient number of organisms used and replication for statistical robustness
5.	Information regarding the stability of test material in the system
6.	Where individual data not available evidence that any statistical method used to derive the endpoint was acceptable and appropriate

[a] For trade name products details may be verified independently

[b] Where results rely on measured results evidence that a suitable analytical method was used is required

Table 9.9-3: Ecotoxicology relevance criteria – field studies

Key qualifying criteria	
1.	Well-defined test material
2.	Relevant geoclimatic conditions
3.	Relevant species, community and/or function studied
4.	Suitable control included in study design
5.	Relevant endpoint data
Assessment level qualifying criteria (*each conforming point will score 1, each non-conforming point will receive 0*)	
1.	Appropriate test material dosing or application into the system

2.	Experimental conditions similar to guidelines where appropriate and of appropriate length.
3.	Meteorological information provided
4.	Where individual data not available, evidence that any statistical method used to derive the endpoint was acceptable and appropriate
5.	Suitable analytical method used and reported or referenced (where relevant)
6.	Minimum detectable difference considered (or independently calculable)

[a] For trade name products details may be verified independently

Table 9.9-4: Ecotoxicology relevance criteria – monitoring studies

Key qualifying criteria[a]	
1.	Relevant geoclimatic conditions
2.	Pesticide usage data available for catchment (e.g. crops)
3.	Suitable analytical method used and reported or referenced
4.	Relevant endpoint studied, effects are demonstrably related to the test material in question (interfering stressors)

[a] Due to the nature and wide protocols used in monitoring studies, no assessment level qualifying criteria are assignable. Literature conforming to all key qualifying criteria are considered relevant without restriction.

Results

The summary results of the literature search are presented in Tables 9.9-5.

Table 9.9-5: An overview of the results of the literature search for *Beauveria bassiana* and its major metabolites

Data requirements	**Number of references**
Total number of summary records retrieved after all searches of peer-reviewed literature (excluding duplicates)	2494
Number of summary records excluded from search after first tier assessment (*rapid assessment*) of relevance [a]	2428
Total number of full-text documents assessed in detail (*detailed assessment*)	80
Number of studies excluded from further consideration after second tier assessment of relevance	40
Number of studies not excluded for relevance after second tier assessment (i.e. relevant studies and studies of unclear relevance)[b]	40

[a] Most duplicates excluded by the search engine

[b] For all these studies a final and detailed assessment for relevance was made alongside the assessment of methodological quality of the studies based on the full text

During the initial rapid assessment, the study titles were scanned to identify whether the studies were relevant to the areas of ecotoxicology, environmental fate or human health. Any studies that were not falling within these areas were excluded. Tables in the report lists the titles of all relevant or unclear studies resulting from this initial rapid assessment ordered by data requirement, and by author according to the requirements of the guidance.

After evaluation of the full text of the papers identified as relevant or of unclear relevance, they were further classified as suitable for inclusion or non-relevant for this dossier.

All the papers considered suitable for inclusion in the Volume B 9 are listed in tables, ordered by data requirement and by author.

Table 9.9-6: Report of all relevant studies and studies of unclear relevance (ordered by data requirement)

Data requirements	Author(s)	Year	Title	Source
CA 8.1	Devotto, L, Cisternas, E, Carrillo, R, Gerding, M	2008	Non-target effects of *Dalaca pallens* Blanchard control examined through principal response curves: a guild approach in Southern Chile	Chilean Journal of Agricultural Research, (2008) 68 (3): 228-237
CA 8.1	Haas-Costa, J, Alves, LFA, Daros, AA	2010	Safety of *Beauveria bassiana* (Bals.) Vuill. to *Gallus domesticus* L.	Brazilian Archives of Biology and Technology, (2010) 53(2): 465-471
CA 8.3	Meikle, WG, Mercadier, G, Holst, N, Girod, V	2008	Impact of two treatments of a formulation of *Beauveria bassiana* (Deuteromycota: Hyphomycetes) conidia on *Varroa* mites (Acari: Varroidae) and on honeybee (Hymenoptera: Apidae) colony health	Experimental and Applied Acarology, (2008) 46(1-4): 105-117
CA 8.3	Meikle, WG, Mercadier, G, Holst, N, Nansen, C, Girod, V	2008	Impact of a treatment of *Beauveria bassiana* (Deuteromycota : Hyphomycetes) on honeybee (*Apis mellifera*) colony health and on *Varroa destructor* mites (Acari : Varroidae)	Adipologie, (2008) 39(2): 247-259
CA 8.3	Mommaerts, V, Put, K, Smagghe, G	2011	*Bombus terrestris* as pollinator-and-vector to suppress *Botrytis cinerea* in greenhouse strawberry	Pest Management Science, (2011) 67(9): 1069-1075
CA 8.3	Mommaerts, V, Sterk, G, Hoffmann, L, Smagghe, G	2009	A laboratory evaluation to determine the compatibility of microbiological control agents with the pollinator *Bombus terrestris*	Pest Management Science, (2009) 65(9): 949-955
CA 8.3	Ramanaidu, K , Cutler, GC	2013	Different toxic and hormetic responses of *Bombus impatiens* to *Beauveria bassiana*, *Bacillus subtilis* and spirotetramat	Pest Management Science, (2013) 69(8): 949-954

Data requirements	Author(s)	Year	Title	Source
CA 8.4	Castrillo, LA, Griggs, MH, Liu, HP, Bauer, LS, Vandenberg, JD	2010	Assessing deposition and persistence of *Beauveria bassiana* GHA (Ascomycota: Hypocreales) applied for control of the emerald ash borer, *Agrilus planipennis* (Coleoptera: Buprestidae), in a commercial tree nursery	Biological Control, (2010) 54 (1): 61-67
CA 8.4	Devotto, L, Cisternas, E, Gerding, M, Carrillo, R	2007	Response of grassland soil arthropod community to biological and conventional control of a native moth: using *Beauveria bassiana* and lambda-cyhalothrin for *Dalaca pallens* (Lepidoptera : Hepialidae) suppression	Biocontrol, (2007) 52(4): 507-531
CA 8.4	Kirkland, BH, Eisa, A, Keyhani, NO	2005	Oxalic acid as a fungal acaracidal virulence factorin	Journal of Medical Entomology, (2005) 42(3): 346-351
CA 8.4	Leland, JE, Behle, RW	2005	Coating *Beauveria bassiana* with lignin for protection from solar radiation and effects on pathogenicity to *Lygus lineolaris* (Heteroptera : Miridae)	Biocontrol Science and Technology, (2005) 15(3): 309-320
CA 8.4	Quesada-Moraga, E, Vey, A	2004	Bassiacridin, a protein toxic for locusts secreted by the entomopathogenic fungus *Beauveria bassiana*	Mycological Research, (2004) 108(4): 441-452
CA 8.4	Steinwender, BM, Krenn, HW, Wegensteiner, R	2010	Different effects of the insectpathogenic fungus *Beauveria bassiana* (Deuteromycota) on the bark beetle *Ips sexdentatus* (Coleoptera: Curculionidae) and on its predator *Thanasimus formicarius* (Coleoptera: Cleridae)	Journal of Plant Diseases and Protection, (2010) 117(1): 33-38
CA 8.4	Rehner, SA, Buckley, E	2005	A Beauveria phylogeny inferred from nuclear ITS and EF1-alpha sequences: evidence for cryptic diversification and links to *Cordyceps teleomorphs*	Mycologia, (2005) 97(1): 84-98

Data requirements	Author(s)	Year	Title	Source
CA 8.5	Shapiro-Ilan, DI, Brown, I	2013	Earthworms as phoretic hosts for *Steinernema carpocapsae* and *Beauveria bassiana*: Implications for enhanced biological control	Biological Control, (2013) 66(1): 41-
CA 8.6	Castrillo, LA, Griggs, MH, Vandenberg, JD	2004	Vegetative compatibility groups in indigenous and mass-released strains of the entomopathogenic fungus *Beauveria bassiana:* likelihood of recombination in the field	Journal of Invertebrate Pathology (2004) 86(1-2)
CA 8.6	Krauss, U, Hidalgo, E, Arroyo C, Piper, SR	2004	Interaction between the entomopathogens *Beauveria bassiana*, *Metarhizium anisopliae* and *Paecilomyces fumosoroseus* and the mycoparasites *Clonostachys* spp., *Trichoderma harzianum* and *Lecanicillium lecanii*	Biocontrol Science and Technology (2004) 14(4): 331-346
CA 8.6	Posada, F, Vega, FE, Rehner SA, Blackwell M, Weber D, Suh SO, Humber RA	2004	Syspastospora parasitica, a mycoparasite of the fungus *Beauveria bassiana* attacking the Colorado potato beetle *Leptinotarsa decemlineata*: a tritrophic association	Journal of Insect Science (2004) 4(24).
CA 8.6	Wang, C, Fan, M, Li, Z, Butt, TM	2004	Molecular monitoring and evaluation of the application of the insect-pathogenic fungus *Beauveria bassiana* in southeast China	Journal of Applied Microbiology (2004), 96(4): 861-870

Table 9.9-7: Report the studies excluded from the risk assessment after second tier assessment

Author(s)	Year	Title	Source	Reason(s) for not including the study in the dossier
Abe, M, Ikegami, T	2005	Susceptibility of five species of thrips to different strains of the entomopathogenic fungus, *Beauveria bassiana*	Applied Entomology and Zoology, (2005) 40(4): 667-674	Reasons, key qualifying criteria 3 and 5, Table 9.9-2

Author(s)	**Year**	**Title**	**Source**	**Reason(s) for not including the study in the dossier**
Acevedo, JPM, Samuels, RI, Machado, IR, Dolinski, C	2007	Interactions between isolates of the entomopathogenic fungus *Metarhizium anisopliae* and the entomopathogenic nematode *Heterorhabditis bacteriophora* JPM4 during infection of the sugar cane borer *Diatraea saccharalis* (Lepidoptera : Pyralidae)	Journal of Invertebrate Pathology, (2007) 96(2): 187-192	Reasons, key qualifying criteria 2, 3 and 5, Table 9.9-2
Ahmed AA, bd-Elhady HK	2013	Efficacy of two fungus-based biopesticide against the honeybee ectoparasitic mite, *Varroa destructor*	Pak J Biol Sci, (2013) 16(16): 819 -825	Reasons, key qualifying criteria 1 and 2, Table 9.9-2
Bidochka, MJ | Clark, DC, Lewis, MW, Keyhani, NO	2010	Could insect phagocytic avoidance by entomogenous fungi have evolved *via* selection against soil amoeboid predators?	Microbiology – SGM (2010) 156(7): 2164-2171	Reasons, key qualifying criteria 3 and 5, Table 9.9-2 not met
Du, W, Zhou, J, Jiang, P, Yang, T, Bu, YQ, Liu, CH, Dai, CC	2013	Effects of Beauveria bassiana and acephate on enzyme activities and microbial diversity in paddy soil	Plant Soil and Environment (2013), 59(12): 562-567	Reasons, key qualifying criteria 2 and 5, Table 9.9-2 not met
Geisseler, D, Horwath, WR, Joergensen, RG, Ludwig, B	2010	Pathways of nitrogen utilization by soil organisms – a review	Soil Biology & Chemistry, (2010) 42(12): 2058-2067	Reasons, study is a review and was only used to obtain potential relevant studies within the literature as per EFSA guidance (EFSA Journal 2011;9(2):2092).
Kryukov, VY, Kryukova, NA, Glupov, VV	2013	Susceptibility of *Galleria mellonella* larvae to anamorphic entomopathogenic ascomycetes under envenomation and parasitization by *Habrobracon hebetor*	Russian Journal of Ecology, (2013) 44 (1): 89-92	Reasons, key qualifying criteria 2, Table 9.9-2 not met
Meikle, WG, Mercadier, G, Guermache, F, Bon, MC	2012	Pseudomonas contamination of a fungus-based biopesticide: Implications for honey bee (Hymenoptera: Apidae) health and *Varroa* mite (Acari: Varroidae) control	Biological Control, (2012) 60(3): 312-320	Reasons, key qualifying criteria 1 and 5, Table 9.9-3 not met

Author(s)	Year	Title	Source	Reason(s) for not including the study in the dossier
Ormond, EL, Thomas, APM, Pell, JK, Freeman, SN, Roy, HE	2011	Avoidance of a generalist entomopathogenic fungus by the ladybird, *Coccinella septempunctata*	FEMS Microbiology Ecology, (2011) 77 (2): 229-237	Reasons, key qualifying criteria 1 and 5, Table 9.9-2 not met
Reber, A , Chapuisat, M	2012	No Evidence for Immune Priming in Ants Exposed to a Fungal Pathogen	Plos One, (2012) 7(4): e35372	Reasons, key qualifying criteria 3, 4 and 5, Table 9.9-2 not met
Seger C, Sturm S, Langle T, Wimmer W, Stuppner H, Strasser H;	2005	Development of a sensitive high-performance liquid chromatography-diode array detection assay for the detection and quantification of the *Beauveria* metabolite oosporein from submerged culture broth and bio-control formulations	Journal of Agricultural Food Chemistry, (2005) 53(5): 1364-1369	Reasons, key qualifying criteria 4, Table 9.9-4 not met
Shipp, L, Kapongo, JP, Park, HH, Kevan, P	2012	Effect of bee-vectored *Beauveria bassiana* on greenhouse beneficials under greenhouse cage conditions	Biological Control, (2012) 63(2): 135-142	Reasons, key qualifying criteria 5, Table 9.9-2 not met
Zimmermann, G	2007	Review on safety of the entomopathogenic fungi *Beauveria bassiana* and *Beauveria brongniartii*	Biocontrol Science and Technology, (2007) 17(5-6): 553-596	Reasons, study is a review and was only used to obtain potential relevant studies within the literature as per EFSA guidance (EFSA Journal 2011;9(2):2092).

Comments by RMS:

The literature search provided is in line with the requirements of the guidance. There is a general agreement to include the literature research at the beginning of the references relied for each aspect of the DAR.

The studies by Castrillo et al. (2010), Kirkland et al. (2006), Leland J.E. and Behle R.W (2005), Quesada-Moraga E. and Vey A (2004), Rehner S.A. and Buckley E. (2005), are included in the Table 9.9-6, however the applicant did not submit these studies. The applicant is requested to clarify why these studies are not part of the data package.

The studies by Castrillo et al. (2004), Krauss et al. (2004 were used as a reference in the risk assessment, however no further summary was provided by the applicant.

The study by Posada F. and Vega F.E. 2005 was as well submitted for section 2 as MA 2.1.2/08. The study by Wang et al (2004) was as well submitted for section 2 as MA 2.2.3/05.

In regards to the data presented in Table 9.9-7, it is not completely clear to RMS why these studies were not included in the risk assessment. The applicant set the relevance criteria (see above), however RMS is of opinion that relevance and reliability where at times mixed. According to the EFSA supporting publication 2013: EN-511, the relevance criteria to be considered are as follows: the test material should be given (e.g. the name/code of the active substance or metabolite or plant protection products) and the test should be related to the data requirements for ecotoxicology (IIA 8 and IIIA 10). A criterion such as effects related to the test material in question is not considered a relevance criterion. This can be determined during the evaluation of the study and not already in this step. Further, the applicant might consider that the effects are not directly related to the test material however, the information contained in a study can be used as supporting information. The same applies to the criteria defined by the applicant as “relevant endpoint data”.

Criteria such as suitable analytical methods and suitable controls are, in the view of RMS, reliability criteria and not relevance.

In the view of these, the applicant is requested to provide a better explanation on why the studies were excluded from the data package.

Furthermore, RMS requested the full study summary of the study presented below; however during the update of the dossier the applicant removed this reference. The RMS is of opinion that this study should be included in the current dossier.

Middaugh, DP, Genthner, FJ	1994	Infectivity and teratogenicity of *Beauveria bassiana* in *Menidia beryllina* embryos. Archives of Environmental Contamination and Toxicology, 27, 95-102 Non GLP Published

Reference list

Data point	Author(s)	Year	Title Company Report No. Source (where different from company) GLP or GEP status Published or not	Vertebrate study Y/N	Data protection claimed Y/N	Justification if data protection is claimed	Owner
Annex II Data and Information							
MA 8.1/0,	Althouse, CM, Petersen, BE, McEwen, LC	1997	Effects on young American kestrels (Falco sparverius) exposed to Beauveria bassiana bioinsecticide. Bulletin of Environmental Contamination and	Y	N	N/A	Public

Data point	Author(s)	Year	Title Company Report No. Source (where different from company) GLP or GEP status Published or not	Vertebrate study Y/N	Data protection claimed Y/N	Justification if data protection is claimed	Owner
			Toxicology 59:507-512. Non GLP Published				
MA 8.1/02	Johnson, DL, Smits, JE, Jaronski ST, Weaver, DK	2002	Assessment of health and growth of ring-necked pheasants following consumption of infected insects or conidia of entomopathogenic fungi, Metarhizium anisopliae var. acridum and Beauveria bassiana, from Madagascar and North America. Journal of Toxicology and Environmental Health, Part A 65:2145-2162. Non GLP Published	Y	N	N/A	Public
MA 8.1/03	Haas-Costa, J, Alves, L.F.A & Daros, A.A.	2010	Safety of Beauveria bassiana (Bals.) Vuill. to *Gallus domesticus* L.Not GLP. Brazilian Archives of Biology and Technology, (2010) 53(2): 465-471 Non GLP Published	Y	N	N/A	Public
MA 8.1/04	Johnson, DL, Smits, JE, Jaronski ST, Weaver, DK	2002	Assessment of health and growth of ring-necked pheasants following consumption of infected insects or conidia of entomopathogenic fungi, Metarhizium anisopliae var.	Y	N	N/A	Public

Data point	Author(s)	Year	Title Company Report No. Source (where different from company) GLP or GEP status Published or not	Vertebrate study Y/N	Data protection claimed Y/N	Justification if data protection is claimed	Owner
			acridum and Beauveria bassiana, from Madagascar and North America. Journal of Toxicology and Environmental Health, Part A 65:2145-2162. Non GLP Published				
K MA 8.2.1/01 And K MP 10.2.01	[redacted]	2015	Beauveria bassiana (PPRI 5339): Toxicity, infectivity and pathogenicity to rainbow trout (O. mykiss) during a 28 day test, CEMS 6525 GLP: Yes Published: No	Y	Y	N/A	BASF
MA 8.2.1/02	Genthner, FJ, Middaugh, DP	1992	Effects of *Beauveria bassiana* on embryos of the inland silverside fish (*Menidia beryllina*). Applied and Environmental Microbiology 58:2840-2845. Non GLP Published	N	N	N/A	Public
K MA 8.2.2./01 And K MP 10.2.02	Forbes T	2015	Beauveria bassiana (PPRI 5339): Toxicity, infectivity and pathogenicity to Daphnia magna (Strauss) during a 28 day test, CEMS 6526 GLP: Yes Published: No GLP: Yes Published: No	N	Y	N/A	BASF
MA 8.2.2/02	Genthner, FJ, Foss, SS, Fisher, WS	1994b	Testing of the insect pest control fungus Beauveria bassiana in grass shrimp	N	N	N/A	Public

Data point	Author(s)	Year	Title Company Report No. Source (where different from company) GLP or GEP status Published or not	Vertebrate study Y/N	Data protection claimed Y/N	Justification if data protection is claimed	Owner
			Palaemonetes pugio. Diseases of Aquatic Organisms 20:49-57. Non GLP Published				
MA 8.2.2/03	Pascale, M, Visconti, A, Pronczuk, M, Wisniewska, H, Chelkowski, J	2002	Accumulation of fumonisins, beauvericin and fusaproliferin in maize hybrids inoculated under field conditions with Fusarium proliferatum. Mycological Research 106:1026-1030. Non GLP Published	N	N	N/A	Public
MA 8.2.2/04	Genthner, FJ, Cripe, GM, Crosby, DJ	1994a	Effect of Beauveria bassiana and its toxins on Mysidopsis bahia (mysidacea). Archives of Environmental Contamination and Toxicology 26:90-94. Non GLP Published	N	N	N/A	Public
K MA 8.2.3/01 And K_MP 10.2.03	Forbes T	2015	Beauveria bassiana (PPRI 5339): freshwater green algae (P. subcapitata) growth inhibition assay, CEMS 6527 GLP: Yes Published: No	N	Y	N/A	BASF
MA 8.3/01	Younger C	2012	*Beauveria bassiana* PPRI 5339 spore	N	Y	N/A	BASF

Data point	Author(s)	Year	Title Company Report No. Source (where different from company) GLP or GEP status Published or not	Vertebrate study Y/N	Data protection claimed Y/N	Justification if data protection is claimed	Owner
			concentrate. Honey Bee, *Apis mellifera* non-target microbial testing. Stillmeadow Inc., 12852 Park One Drive, Sugar Land TX 77478 Study Code: 16339-12 Report Date: GLP: Yes Published: No				
MA 8.3/02	Younger C	2012	*Beauveria bassiana* PPRI 5339 spore concentrate. Honey Bee, *Apis mellifera* non-target microbial testing. Stillmeadow Inc., 12852 Park One Drive, Sugar Land TX 77478 Study Code: 16984-12 Report Date: GLP: Yes Published: No	N	Y	N/A	BASF
MA 8.3/03	Whittaker M	2014	Effects of *Beauveria bassiana* PPRI 5339 on the honeybee, *Apis mellifera*, in an acute oral and contact toxicity pre-test. APIS, Knaresborough Technology Park, Manse Lane, Knaresborough, North Yorkshire, HG5 8LF. Study No.: Study No.: APIS-BASF-NGLP-001 GLP: No Published: No	N	Y	N/A	BASF

Data point	Author(s)	Year	Title Company Report No. Source (where different from company) GLP or GEP status Published or not	Vertebrate study Y/N	Data protection claimed Y/N	Justification if data protection is claimed	Owner
MA 8.3/04	Whittaker M	2013	Effects of *Beauveria bassiana* PPRI 5339 on the bumblebee, *Bombus terrestris*, in an acute contact toxicity test. APIS, Knaresborough Technology Park, Manse Lane, Knaresborough, North Yorkshire, HG5 8LF. Study No.: APIS-BASF-004 GLP: Yes Published: No	N	Y	N/A	BASF
MA 8.3/05	Whittaker M	2013	Effects of *Beauveria bassiana* PPRI 5339 on the bumblebee, *Bombus terrestris*, in an acute toxicity test. APIS, Knaresborough Technology Park, Manse Lane, Knaresborough, North Yorkshire, HG5 8LF. Study No.: APIS-BASF-005 GLP: Yes Published: No	N	Y	N/A	BASF
MA 8.3/06	Whittaker M	2013	Effects of *Beauveria bassiana* PPRI 5339 on the honeybee, *Apis mellifera*, in an acute contact toxicity test. APIS, Knaresborough Technology Park, Manse Lane, Knaresborough, North Yorkshire, HG5 8LF. Study No.:	N	Y	N/A	BASF

Data point	Author(s)	Year	Title Company Report No. Source (where different from company) GLP or GEP status Published or not	Vertebrate study Y/N	Data protection claimed Y/N	Justification if data protection is claimed	Owner
			APIS-BASF-001 GLP: Yes Published: No				
MA 8.3/07	Whittaker M	2013	Effects of *Beauveria bassiana* PPRI 5339 on the honeybee, *Apis mellifera*, in an acute oral toxicity test. APIS, Knaresborough Technology Park, Manse Lane, Knaresborough, North Yorkshire, HG5 8LF. Study No.: APIS-BASF-002 GLP: Yes Published: No	N	Y	N/A	BASF
MA 8.3/08 And 8.3/14	Meikle, WG, Mercadier, G, Holst, N, Girod, V	2008	Impact of two treatments of a formulation of *Beauveria bassiana* (Deuteromycota: Hyphomycetes) conidia on Varroa mites (Acari: Varroidae) and on honeybee (Hymenoptera: Apidae) colony health. *Experimental and Applied Acarology, (2008) 46(1-4): 105-117* GLP: No Published: Yes	N	N	N/A	Public
MA 8.3/09	Meikle, WG, Mercadier, G, Holst, N, Girod, V	2008	Impact of a treatment of *Beauveria bassiana* (Deuteromycota: Hyphomycetes) on honeybee (*Apis mellifera*) colony health and on Varroa destructor mites	N	N	N/A	Public

Data point	Author(s)	Year	Title Company Report No. Source (where different from company) GLP or GEP status Published or not	Vertebrate study Y/N	Data protection claimed Y/N	Justification if data protection is claimed	Owner
			(Acari : Varroidae). *Adipologie, 39(2): 247-259* GLP: No Published: Yes				
MA 8.3/10	Mommaerts, V, Put, K, Smagghe, G	2011	*Bombus terrestris* as pollinator-and-vector to suppress *Botrytis cinerea* in greenhouse strawberry. *Pest Management Science, 67(9): 1069-1075.* GLP: No Published: Yes	N	N	N/A	Public
MA 8.3/11	Ramanaidu, K & Cutler, GC	2013	Different toxic and hormetic responses of *Bombus impatiens* to *Beauveria bassiana*, *Bacillus subtilis* and spirotetramat. *Pest Management Science, (2013) 69(8): 949-954.* GLP: No	N	N	N/A	Public
MA 8.3/12	Mommaerts, V, Sterk, G, Hoffman, L & Smagghe, G	2011	A laboratory valuation to determine the compatibility of microbiological control agents with the pollinator *Bombus terrestris. Pest Management Science, 65(9): 949-955* GLP: No Published: Yes	N	N	N/A	Public
MA 8.3/13	M. S. Al Mazra'awi, J. L. Shipp, A. B. Broadbent,	2006	Dissemination of *Beauveria bassiana* by Honey Bees (Hymenoptera:	N	N	-	Public

Data point	Author(s)	Year	Title Company Report No. Source (where different from company) GLP or GEP status Published or not	Vertebrate study Y/N	Data protection claimed Y/N	Justification if data protection is claimed	Owner
	And P. G. Kevan		Apidae) for Control of Tarnished Plant Bug (Hemiptera: Miridae) on Canola Environ. Entomol. 35(6): 1569-1577				
MA 8.3/14	William G. Meikle, Guy Mercadier, Niels Holst, Vincent Girod	2008	Impact of two treatments of a formulation of *Beauveria bassiana* (Deuteromycota: Hyphomycetes) conidia on *Varroa* mites (Acari: Varroidae) and on honeybee (Hymenoptera: Apidae) colony health Exp Appl Acarol (2008) 46:105-117	N	N	-	Public
MA 8.3/15	M.S. Al mazra'awi	2007	Impact of the Entomopathogenic Fungus *Beauveria bassiana* on the Honey bees, *Apis mellifera* (Hymenoptera: Apidae). World Journal of Agricultural Sciences 3 (1): 07-11	N	N	-	Public
MA 8.3/16	M.S. Al mazra'awi, P. Kevan, L. Shipp	2007	Development of *Beauveria bassiana* dry formulation for vectoring by honey bees *Apis mellifera* (Hymenoptera: Apidae) to the flowers of crops for pest control. Biocontrol Science and Technology, 17:7, 733-741	N	N	-	Public
MA 8.3/17	Jean Pierre Kapongo,	2008	Optimal concentration of	N	N	-	Public

Data point	Author(s)	Year	Title Company Report No. Source (where different from company) GLP or GEP status Published or not	Vertebrate study Y/N	Data protection claimed Y/N	Justification if data protection is claimed	Owner
	Les Shipp, Peter Kevan, Bruce Broadbent		*Beauveria bassiana* vectored by bumble bees in relation to pest and bee mortality. BioControl 53:797-812				
M.A 8.3/18	J.P. Kapongo, L. Shipp, P. Kevan, J.C. Stutton	2008	Co-vectoring of *Beauveria bassiana* and *Clonostachys rosea* by bumble bees (*Bombus impatiens*) for control of insect pests and suppression of grey mould in greenhouse tomato and sweet pepper. Biological Control 46 508-514	N	N	-	Public
MA 8.4/01	Devotto, L, Cisternas, E, Gerding, M, Carrillo, R	2007	Response of grassland soil arthropod community to biological and conventional control of a native moth: using *Beauveria bassiana* and lambda-cyhalothrin for *Dalaca pallens* (Lepidoptera : Hepialidae) suppression. *Biocontrol, 52(4): 507-531* GLP: No Published: Yes	N	N	N/A	Public
MA 8.4/02	Devotto, L, Cisternas, E, Carrillo, R, Gerding, M	2008	Non-target effects of *Dalaca pallens* Blanchard control examined through principal response curves: a guild approach in Southern Chile. *Chilean Journal of*	N	N	N/A	Public

Data point	Author(s)	Year	Title Company Report No. Source (where different from company) GLP or GEP status Published or not	Vertebrate study Y/N	Data protection claimed Y/N	Justification if data protection is claimed	Owner
			Agricultural Research, (2008) 68 (3): 228-237.				
MA 8.4/03	Steinwender, BM, Krenn, HW, Wegensteiner, R	2010	Different effects of the insectpathogenic fungus *Beauveria bassiana* (Deuteromycota) on the bark beetle *Ips sexdentatus* (Coleoptera: Curculionidae) and on its predator Thanasimus formicarius (Coleoptera: Cleridae). *Journal of Plant Diseases and Protection, 117(1): 33-38* Non GLP Published	N	N	N/A	Public
MA 8.5/01	Shapiro-Ilan, DI, Brown, I	2013	Earthworms as phoretic hosts for Steinernema carpocapsae and Beauveria bassiana: Implications for enhanced biological control. Biological Control, (2013) 66(1): 41 Non GLP Published	N	N	N/A	Public
MA 8.5/02	Nuutinen V et al	1990	The effects of four entomopathogenic fungi and an entomoparasitic nematode on the hatching of earthworm (Aporrectodea caliginosa) cocoons in laboratory GLP: no Published: yes	N	N	N/A	Public
MA 8.6/01	Hirsch, J, Galidevara, S, Strohmeier, S,	2013	Effects on Diversity of Soil Fungal Community and Fate	N	N	N/A	Public

Data point	Author(s)	Year	Title Company Report No. Source (where different from company) GLP or GEP status Published or not	Vertebrate study Y/N	Data protection claimed Y/N	Justification if data protection is claimed	Owner
	Devi, KU, Reineke, A		of an Artificially Applied *Beauveria bassiana* Strain Assessed Through 454 Pyrosequencing. *Microbial Ecology, 66(3): 608-620* GLP: No Published: Yes				
Annex III Data and Information							
MA 8.2.1/01 And MP 10.2.01	[redacted]	2015	Beauveria bassiana (PPRI 5339): Toxicity, infectivity and pathogenicity to rainbow trout (O. mykiss) during a 28 day test, CEMS 6525 GLP: Yes Published: No	Y	Y	N/A	BASF
MA 8.2.2./01 And MP 10.2.02	Forbes T	2015	Beauveria bassiana (PPRI 5339): Toxicity, infectivity and pathogenicity to Daphnia magna (Strauss) during a 28 day test, CEMS 6526 GLP: Yes Published: No GLP: Yes Published: No	N	Y	N/A	BASF
MA 8.2.3/01 And MP 10.2.03	Forbes T	2015	Beauveria bassiana (PPRI 5339): freshwater green algae (P. subcapitata) growth inhibition assay, CEMS 6527 GLP: Yes Published: No	N	Y	N/A	BASF
MP 10.4.1/01	Whittaker M	2014	Effects of Broadband (BAS 480 00 I) on the parasitic wasp, *Aphidius rhopalosiphi*, in a glass plate contact toxicity test. APIS, Knaresborough Technology Park,	N	Y	N/A	BASF

Data point	Author(s)	Year	Title Company Report No. Source (where different from company) GLP or GEP status Published or not	Vertebrate study Y/N	Data protection claimed Y/N	Justification if data protection is claimed	Owner
			Manse Lane, Knaresborough, North Yorkshire, HG5 8LF, UK. Study No. APIS-BASF-012 GLP: Yes Published: No				
MP 10.4.1/02	Whittaker M	2014	Whittaker, M (2014). Effects of Broadband (BAS 480 00 I) on the predatory bug, *Orius laevigatus*, in a glass plate contact toxicity test. APIS, Knaresborough Technology Park, Manse Lane, Knaresborough, North Yorkshire, HG5 8LF, UK. Study No. APIS-BASF-013. GLP: Yes Published: No	N	Y	N/A	BASF