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Defined technical protocol for test of side effects on invertebrates established

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Technical protocol for tests of direct and indirect side-effects of new BCA formulations on non-target invertebrates

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Introduction

Novel formulations of entomopathogenic fungi may improve the efficacy against target pests. But before taking novel formulations into use it is important to carefully screen them for side-effects on beneficial organisms.

This protocol was established as part of the EU FP7 project INBIOSOIL, under work package 3 - Assessment of the effects of new biocontrol products on the environment and the food chain - for testing direct and indirect side effects of these novel formulations of entomopathogenic fungi applied to the soil (not spraying) on beneficial non-target arthropods. The protocol was developed based on a series of pilot studies, subsequent experimental set-ups and analyses of results aimed at optimising the protocol.

The protocol is intended for use for test of side-effects of formulations of entomopathogenic fungi on invertebrates in the EU FP7 project INBIOSOIL. The protocol has been developed at University of Copenhagen. The cohort rearing described was developed by EWH BioProduction. The protocol may be further adjusted and refined during the project period as novel formulations become available for testing.

For developing the protocol we assessed *Metarhizium brunneum* treatments to a negative control in the direct side effects of longevity, mortality and fungal growth, and indirect side-effects on fecundity and prey preference.

Four non-target beneficial predatory arthropods were selected to represent different levels of contact with soil and different classes of arthropods (insects, mites) and orders of insects (Coleoptera, Diptera, Heteroptera). The non-target test organisms are all widespread and naturally occurring in Europe, in addition all are commercially available.
Fungal concentrations were selected so that they led to an intermediate to low mortality, in all species with significant differences between a negative control and treatment on longevity and mortality. The response level of the test organisms provides a good background for test of side-effects.

The tests of the protocol are arranged to follow a decision tree. Initially maximum exposure by dipping arthropods in a high concentration suspension of the novel formulation is tested against dipping them in a positive control of *M. brunneum* and against the pure formulant or a negative control of water. If no side-effects on any of the arthropods are observed at this stage on longevity, mortality and fungal infestation, no further testing is required. For those formulations where a negative side-effect is observed, direct side effects under more realistic but still high exposure conditions are tested with the formulation in the soil, again assessing longevity, mortality and fungal infestation. If these trials show no significant side-effects compared to a positive control, the formulation has low risk.

Indirect side effects may occur even when no direct side effects are observed. Therefor the protocol includes two tests for indirect side effects. The test for indirect side-effects on fecundity can be done using the same set-up as used in the longevity trial, allowing both tests to be completed in one set-up with limited additional work effort. The final indirect side-effect tests are for predator prey preferences. If preference for treated vs non-treated prey is affected this may have a potential implication for pest control.

Materials

Soil

Soil used in the trials was collected from the UCPH experimental farm area. We used the top 25-30 cm soil from a field (field code: m 29-2 northern part) on the part of the experimental organic farm "Bakkegaarden" in Taastrup, belonging to University of Copenhagen, which has been cropped organically

since 1988. The soil was analyzed in March 2012, where it had a Rt(Ph):6,5 Pt:2,3 Kt:8,1 Mgt:4,3. The soil sample for trials was kept in plastic bags in a cold storage room maintained at 5° C.

The last 5 y crop rotation was:

2008 Oat (*Avena sativa*) with *Lolium spp.*grass
2009 Winter wheat with *Lolium spp.*grass
2010 Barley/lupine (*Lupinus*) with alfalfa (*Medicago sativa*)/*Medicago lupulina*
2011 spring triticale (a crossing of barley and rye) with clover-grass mixture
2012 clover-grass

Fungal material -Culture rearing and conidia suspension preparation

The isolate used in the study was F52 (*Metarhizium brunneum)* received from the fungal collection from the University of Innsbruck, Austria. Conidia for a (stock) isolate should be frozen down at -80°C in a skimmed milk - glycerol mixture. Every 3 month a conidia from this stock should be used to start a "mother"-culture on 4% Sabourad dextrose agar (SDA) (grown at 22°C) to produce conidia for the establishment of cultures for the experiment. These cultures are to be initiated by inoculating a fresh 4% SDA plate with conidia and and to be incubated for 2 weeks at 22°C before they can used in the experiments.

Conidia suspension for the experiments should be prepared as followed: Harvest the conidia by flooding the culture with approx. 20 ml of 0.05% Triton X and agitate it with a sterile Drigalski spatula. Transfer the suspension afterwards to a 50ml vial and centrifuge it for 3 min at 3000 rpm, discard the supernatant, add 20 ml of fresh 0.05% Triton X and repeat the procedure 2 more times. The concentration should be determined by using a haemocytometer (Fuchs-Rosendahl) before the conidia suspension is diluted Spore viability should be examined by placing three droplets of an approx. 1×10^5 conidia/ml suspension on 4% SDA plates followed by incubation for 24 h at 22°C, after which the germination was examined under a compound microscope by observing 100 conidia from each droplet at a magnification of 400.

Arthropod test organisms

Test organisms were selected to represent different orders of beneficial predatory arthropods and species with different degrees of contact with soil, and hence risk of exposure to entomopathogenic fungi.

a. *Gaeolaelaps aculeifer* (Canestrini) (soil dwelling mite) - formerly *Hypoaspis aculeifer*
b. *Aphidoletes aphidomyza* Rondani (pupates in soil)
c. *Atheta coriaria* Kraatz ("soil surface" predator)
d. *Orius majusculus* (Reuter) ("plant surface" predator)

Geolaelaps aculeifer

Geolaelaps aculeifer (Canestrini) (Acari, Mesostigmata, Laelapidae) is a predatory mite of value in biological control of soil living stages of pests. It occurs naturally in Europe and is also commercially available. Both adults and nymphs are polyphagous predators. For biocontrol the species is used in particular against shore flies, thrips, springtails, bulb mites, sciarids, and other species. This mite species stays most of the time within or on the soil searching the upper 20 cm of the soil for prey and only rarely climb onto the plants. They prefer humid conditions. The microhabitat of *G. aculeifer* makes natural exposure to entomopathogenic fungi in the soil very likely.
The species is included in the OECD protocol for tests of soil pesticides (OECD 2008).

Aphidoletes aphidimyza

The larval stages of *Aphidoletes aphidimyza* Rondani (Insecta, Diptera, Cecidomyiidae) are aphid predators of value in biological control. The adult is not predatory but feeds on nectar and/or honeydew. The adult will lay its eggs close to aphid colonies. The species occurs naturally in Europe and is also commercially available. For biocontrol it is used against aphids both in indoor and outdoor crops. Pupation occurs in the soil making the prepupal and pupal stage as well as the emerging adult exposed to entomopathogenic fungi in the soil.

Atheta coriaria

Atheta coriaria Kraatz (Insecta, Coleoptera, Staphylinidae) is a rove beetle of value in biological control of various small soft-bodied preys, in particular fly eggs. It occurs naturally in Europe and is also commercially available. *Atheta coriaria* larvae and adults are found in the soil. Apart from arthropod prey this species can also feed on yeasts and mycelium from other fungi. The microhabitat of *Atheta* makes natural exposure to entomopathogenic fungi in the soil likely. Its biology has been recently described by Wilson (2012).

Orius majusculus

Orius majusculus (Reuter) (Insecta, Hemiptera, Anthocoridae) is a predatory bug of value in biological control of thrips, aphids mites and various other small prey, eggs and larvae. It occurs naturally in Europe and is also commercially available. *O. majusculus* nymphs and adults feed on plant inhabiting prey such as thrips, aphids, mites, lepidopteran eggs, and small larvae. The microhabitat of *O. majusculus* being in the vegetation with only occasional contact with the soil surface makes it less exposed to entomopathogenic fungi in the soil.

Cohort rearing

The cohort rearing of all invertebrates originate from colonies, taken from EWH BioProduction's own mass production. The mass production of EWH BioProduction is regularly renewed with specimens collected from the wild. EWH BioProduction is following the IOBC quality control guidelines for beneficial insects (van Lenteren 2003). Cohort rearing takes place in a climate- controlled cabinet (Panasonic MUC 214). The colonies are maintained at 23 ± 0.5°C temperature, 50-75% relative humidity and L16: D8 light regime.

Geolaelaps aculeifer

Berlese technique is used to extract *Geolaelaps* adults from 1 liter production material. The sample is transferred to a funnel with a sieve and placed at a distance of 5 cm under a lamp (150 W) for 4 hours. A coal beaker (100ml volume) is placed under the funnel to collect the falling mites. When approximately 200 adults are extracted, the beaker is removed and all the nymphs are killed. After adding 20 ml moist grove vermiculite and *Tyrophagus putrescentiae* as a food source, the predatory mites are left for egg laying for 48 hours. Then, adults are removed and the beakers are maintained until the new generation of adults appears. *Gaeolaelaps* should be used as newly emerged adults. If mites are shipped this requires shipmentas deuteronymphs 3-4 days before adult.

Aphidoletes aphidimyza

Adults of *Aphidoletes* are left for egg-laying in a cage (40cm x 40cm x 40cm) with a pepper plant, infected with *Mysuz persicae*. After 24 hours adults are removed and a whole pepper plant with eggs is laid down into a tray (10cm x 10cm x 5cm), filled with sand. A fine- mesh hole is incorporated into the lid of the tray for ventilation. The gall midge larvae are daily fed with 10 ml of aphids (*Megoura* sp.). *Aphidoletes* should be used as last instar larvae immediately prior to pupation in the soil. If larvae are shipped this should be done as late instar larvae, 4 days prior to pupation.

Atheta coriaria

500 *Atheta* adults are sucked with a mechanical pooter and released into a small coal beaker (100ml volume), filled with moisten sphagnum (1:10 ration water: sphagnum) and equipped with a net hole. Shell free shrimp food is provided as food. After 24 hours adults are removed and the beakers are maintained until the emergence of a new generation adults. For trials newly emerged adults should be used. If shipped this should be done as late pupae, prior to adult.

Orius majusculus

Adults of *Orius* are left for oviposition in a small cage (40 cm x 40 cm x 40 cm) with a pepper plant and *Ephestia kuhniella* eggs for food. After 24 hours adults are removed and 4-5 pepper leaves are transferred into a tray (30 cm x 20 cm x 10 cm) with water/glycerol agar and 250 ml of buckwheat. The tray is equipped with fine-mesh holes for air flow. *Ephestia kuhniella* eggs are provided as a food source twice per week until the life cycle is completed. Orius are used as adults. If shipped this should be done when cohort nymphs are 2-3 days prior to adults.

Methods for test of side effects

Methods of the protocol are established based on trials with *M. brunneum* and control treatments. For testing side effects of new formulations at least 3 treatments should be used: pure *M. brunneum* as a positive control (the pure pathogen in the formulation), test formulation media alone (the material, chemicals that are added to the "pure" pathogen) and the real formulation (pathogen and formulation material combined, processed). We strongly recommend a control of no treatment, to make sure that the form of application of the different treatments does not influence the tested insects survival rate.

Direct side effects

Direct side effect trials are done as qualitative pathogenicity tests where *M. brunneum* or formulations of *M. brunneum* are tested in concentrations of 10^7 spores per ml. This should be done by a) dipping of adult insects into a *M. brunneum* suspension and b) exposing the adults to a known concentration of *M. brunneum* in soil. $1x10^7$ concentrated conidia suspensions were selected as a concentration which provided a response in all the selected species, at levels from 10 % to 50 % mortality for dipping, while 5×10^6 concentrations were used for exposure to soil. The protocol has been established specifically for inoculation via soil media, hence no spraying tests are included in the protocol.

Dipping –the effect of immersion in a conidial suspension

Dip the test insects (and the mite) in the conidia concentration $1x10^7$ (15-20ml) for 30 seconds, then pour the whole suspension in a Büchner funnel, with filter paper in, and drain the suspension by applying vacuum (Goettel and Inglis, 1997). At least 20 test individuals should be tested in each trial and at least 3 repetitions of trials (i.e. 60 individuals of a species is tested) are strongly recommended to ensure a satisfactory level of precision. As a control treatment (see above), test organisms should be dipped in water with Triton –X only (no conidia).

An initial control should also test the effect of the inoculation technique itself (here: dipping procedure ie. dipping + Büchner funnel) on mortality. Expose a set (20-30) of insects to the dipping procedure and another set transfer only to the containers. Test whether mortality after the dipping procedure is markedly higher than no treatment (just the transfer) for one or more of the test organisms as a criterion to accept the procedure.

Inoculation via conidia in agricultural soil

To expose both insects and mites to soil with a concentration of 5 x 10^6 conidia/g soil the following procedure is recommended: Sieve soil through a 3 mm mesh and place 200g of this soil into a 10-15 L plastic bag. To produce soil with 5x 10^6 conidia/g soil the according volume of a highly concentrated conidia suspension (>2x10^8 conidia/ml) must be added, by trying to cover most of the soils surface and rough mixing in between. After all the liquid has been applied, close the bag and mix it thoroughly (knead by hand through the plastic bag). Sieve the mixed soil again through a 3 mm mesh, ensuring the highest possible conidia distribution. Transfer 10 mL (~ 11g) of soil into 30 mL medicine cups each. To ensure stable RH during experiments the bases of the cups needs to be covered with water (4-5mL) agar (1.5%).

Add 30 *Geolaelaps aculeifer* newly emerged adults or 10 *Aphidoletes aphidimyza* last instar larvae or 10 *Atheta coriaria* newly emerged adults or 10 *Orius majuscules* newly emerged adults to a single cup. The control group should consists of the same number of insects/mites and the same amount of soil, without conidia but with the same volume of Triton X added.

Close the cups with a perforated lid and incubate the arthropods 3 days at 23°C. Each day carefully turn the cups to ensure movement of the arthropods through the soil. Pour after three days the soil into broad plastic cups and transfer all insects that can be found alive into medicine cups with 1,5% of water agar. Feeding and checking should be done as described for rearing after dipping (above).

For each set-up the relative humidity of the soil needs to be assessed. Fill 5 glass cups (5mL) with the treated soil and 5 glass cups of the untreated control soil, weight them before and after 24 hours in the oven (120°C) and calculate the relative humidity to ensure a stable RH of the used soil over the repetitions.

Rearing of arthropods after exposure

After the treatment of dipping or exposure to soil the arthropods must be transferred singly into 30 ml plastic cups with 5ml of 1.5% water agar in the bottom. Water agar serves to provide a constant humidity. Close the vials with a lid provided with ventilation holes and incubate them at at 23 ± 0.5 °C in 16 h light and 8 h darkness. Provide food on a small spot of the agar surface using a so-called “direct” food deposition. To avoid growth of saprophytic fungi on diet, predators should be transferred to fresh cups with fresh diet every 2nd day or 3rd day.
Use initial set ups to identify optimal experimental conditions and get used to the handling. Factors that might need “fine tuning” include humidity (avoid condensation) and diet (select quality diet, avoid saprophytic fungal growth).

Geolaelaps aculeifer rearing

Geolaelaps aculeifer is fed with *Ephestia kuhniella* eggs. It is also possible to feed them with pollen, but pollen gives some unwanted growth of saprophytic fungi in the rearing cups. After the treatment mites should be kept individually and longevity is recorded daily.

Aphidoletes aphidimyza rearing

Larvae of *Aphidoletes aphidimyza* should be fed with a a diet of aphids (*Megoura viciae*) until ready for pupation. It is also possible to feed *A. aphidomyza* with other species of aphids, such as *M. persicae* or *Rhpalosiphum padi*. For trials with exposure to soil use last instar larvae immediately prior to pupation, so diet in the set-up is not needed. The larvae will pupate in the soil and the number of emerging adults can and should be counted. Some of the emerging adults will be infected by soil contact.. By daily check of cups dead adults should be removed and incubated to record any fungal growth. Cups are checked daily from expected first emergence of adults (typically starting after day 10).

Atheta coriaria rearing

Atheta coriaria should be fed shell free shrimp food using the same brand as used in the cohort rearing.

Orius majusculus rearing

Orius majusculus should be fed *Ephestia kuhniella* eggs, as done in the cohort rearing. As *O. majusculus* tend to spend the major part of its time under the lid where ventilation holes will reduce humidity, this species should be kept in inverted cups for the first 24 h, ensuring that it stays near the water agar (higher humidity) during possible fungal infection.

Recording of mortality and mycosis

Mortality should be recorded daily or every second day. Transfer dead predators to 30 ml plastic cups with 1.5% water agar in order to allow observation of mycosis to occur (fungal sporulation from cadaver). Dead arthropods should not be surface sterilized because especially the mites could be accidentally sterilized totally.

Two factors should be recorded a) mortality; i.e the day of death of an individual and b) mycosis (yes or no).

Indirect side effects

Indirect effects should be assessed by testing effects of *M. brunneum* and its formulations on non-target invertebrate fitness parameters of fecundity and prey preference.

Fecundity

Fecundity should be assest after exposure to the soil. Exposure and rearing conditions should be identically to the description for direct side effects, with modifications as described below. This allows the fecundity test to be integrated with the direct side effect. This is especially relevant in cases where low mortality occurs.
In fecundity trials, provide oviposition material. Record oviposition for sufficient time to clearly identify differences in fecundities, if any, or till the adult dies. This depends on the species oviposition period (see below for details).

We strongly suggest preliminary tests that help one to identify the time of key events, and allow to later reduce the number of observations needed on each individual.

Geolaelaps aculeifer fecundity trial

Place 20 mites in a 30ml 1.5% water agar medicine cup covered with soil for 3 days, and turn the cup once daily as described for test for direct side effects in soil. Then, transfer 1 female and 1 male mite to a new 1.5% water agar medicine cup with few *Ephestia kuhniella* eggs provided. After 48 hours, move the female, without the male, to a new 1.5% water agar medicine cup and count the eggs laid. Continue egg counting every 48 hours until day 10. Hereafter, keep moving each mite to a new 1.5% water agar medicine cup every 48 hours with *Ephestia kuhniella* eggs. Stop the experiment after 24 days . Incubate any dead to check for *Metarhizium* mycosis.

Aphidoletes aphidimyza fecundity trial

Place a female and a male, alive but emerged from treated soil, into a 30 ml medicine cup with 1.5% water agar in the base covered with filter paper, and with a barley leaf infested with 5 adult aphids, *Rhopalosiphum padi,* inserted into the agar. Provide a strip of filter paper dipped in a 10% honey solution, and ensure by ventilation holes that RH inside the oviposition cage is near 70-75%, as *A. aphidimyza* is very sensitive to both high and low humidity. Move adults to new oviposition cup every 48 hours. Count the eggs laid till day 8. Incubate dead *A. aphidimyza* adults on water agar for analysis of *Metarhizium* mycosis after 24 days.

Atheta coriaria fecundity trial

Separate *A. coriaria* by sex and keep them singly in water agar cups (as described above – do not forget to change cup/ food every 2^{nd}/3^{rd} day). The sex of live *A. coriaria* is difficult to determine - only by visual inspection of the 8^{th} abdominal sternite under a stereomicroscope. To stop movements of *A. coriaria* expose the adults to CO_2 that has a narcotic effect on insects. The sexed adults are to be kept separated for 7-10 days. Afterwards one male and one female should be put together in a cup with soil (treated/ untreated) and exposed to it for 2 days (during this time mating will/ can take place). Remove the adults after the 2 days the cups with soil and again keep them singly in fresh water agar cups, a small amount of dried sphagnum and are provide shell free shrimp food.
Appearing larvae are quite mobile and therefore can escape “normal” lids - therefore cups that could bear larvae must be secured with fine meshed gaze to make sure no larvae will escape. After 48 hours adults are moved to a new cup. The old cup is saved in an incubator at 23 ^{o}C to check for fecundity- eggs are difficult to see, but after 6 days first instar larvae can be observed and the number noted down.
By the end of the experiment, freeze down the adult A. *coriaria* to determine the sex again to be sure that one male and one female were put together in the soil cup (checking of “couples” that showed to produce offspring is of course not necessary).

Longevity of adult *A. coriaria* can be up to 60 d, but it is suggested to end the experiment after 25 days.

Orius majusculus fecundity trial

Expose pairs of one newly emerged female and one male *O. majusculus* together for 3 days in treated soil. First, to ensure mating, the pair is kept together in an empty cup until mating is observed. This will normally happen within few minutes (15-30 min). After 48h exposure to treated soil, females are placed in 30 ml medicine cups with 1.5% water agar and provided with *Ephestia kuhniella* eggs including a 2 cm piece of green bean as oviposition material. Organic beans are used. It is strongly recommended to wash with soap to ensure any agrochemicals are removed. Females are moved to a new cup every 2nd day and eggs are counted. The eggs are counted till day 12 and the female is left in the same medicine cup until day 24 to check for *Metarhizium* -infected *Orius.*

Preference

Use the same setup for *Geolaelaps* adults, *Atheta* adults, and *Orius* adults. In all three cases treat *Ephestia kuhniella* eggs with *M. brunneum* spores 24 hours before use. Place infested and healthy *Ephestia kuhniella* eggs at opposite sides of the same Petri-dish. Place adult arthropod in the middle of the Petri-dish when released.

For the for *Aphidoletes* use a no-choice experimental procedure and 3^{rd} instar *Rhpalosiphum padi*, either healthy or exposed for 24 h to *M. brunneum* spores, are provided as prey.

All experiments are conducted at 23 ^{o}C cabinet. For the short duration trials use light, , for the 24 h trials us a L:D of 16:8.

Geolaelaps- preference

The preference trial with *Geolaelaps* is a choice experiment between healthy *Ephestia kuhniella* eggs and eggs with *M. brunneum* spores. Newly emerged females are starved for 24 hours in 1.5% water agar medicine cups.

To expose the *Ephestia kuhniella* eggs to *M. brunneum* spores, initially place eggs placed in an empty Petri-dish. With an inoculating loop the spores from an *M brunneum* plate are shaved off and transferred to the Petri dish with the *Ephestia kuhniella* eggs. After the lid of the Petri-dish is placed covering all eggs, the Petri dish is shaken to make sure all *Ephestia kuhniella* eggs are infested. The Petri-dish is placed at 23°C for 24 hours before use.

For the preference trial use a 9 cm diameter Petri-dish covered with 20ml water agar and with infected and healthy *Ephestia kuhniella* eggs. 10 healthy *Ephestia kuhniella* eggs and 10 infested *Ephestia kuhniella* eggs are placed on opposite sides of the Petri dish, ensuring that all eggs are equidistantly removed from Petri dish centre (2 cm distance). Use a fine brush to move an egg to the 20ml water agar Petri dish. Before releasing the insect, make sure no eggs have been punctuated. Use a stereomicroscope at a magnification of x 20-x100.

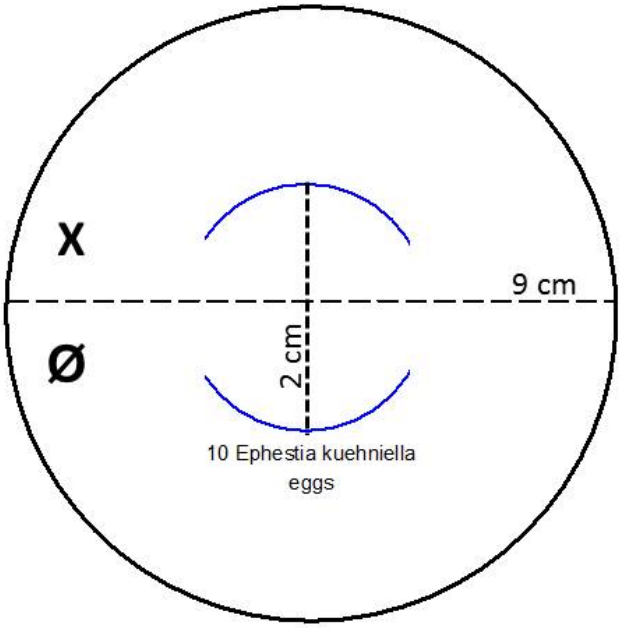


Figure 1 Position off eggs for choice experiment. 10 eggs are placed equidistantly on each side of the release point. Ø = control, X = treated eggs

One female is released in the centre of the Petri dish, and to avoid any effect of Petri dish orientation the orientation of dishes must be randomised (Figure 3) The Petri dishes are kept at 23°C with light and the number of eggs eaten are recorded after 1.5h and 3h.

Figure 2. Ensure randomisation of Petri dish orientation to eliminate any side effects from for example differences in light intensity in the climate cabinet used.

Atheta- preference

For *A. coriara* preference use a 24 h starved adult. The same prey and set up is used as described for *Geolaelaps*-preference trial. Also in this case predation is assessed after 1.5h and 3h.

Orius- preference

For *O. majusculus* preference use a 24 h starved adult female . The same prey and set up is used as described for *Geolaelaps*-preference trial. Predation is assessed after 1.5h and 3h.

Aphidoletes- preference

For the *A. aphidimyza* preference trials, use L4 larvae. In this case use a no-choice set-up as live aphids are used. This predator is an aphid specialist so the prey for the preference trial was healthy *Rhopalosiphum padi* or aphids which had been exposed to *M. brunneum* spores.

To expose *R. padi* to spores, they are initially placed in an empty Petri dish. With an inoculating loop spores from an *M. brunneum* culture plate are collected and transferred to the Petri dish with the aphids. When the aphids are brought into contact with *M. brunneum*, the dish is closed and next shaken carefully to make sure that all aphids are covered with spores. Seal the Petri-dish with parafilm and placed in an incubator at 23^{o}C for 24 hours before use. Only use aphids still alive, as spores may have killed some aphids already.

Conduct the experiment in 30 ml plastic cups with the bottom covered with 1.5 % water agar. Place 10 aphids, from the same treatment, in each water agar medicine cup with one larva. Do the experiment at 23^{o}C. Record preyed aphids after 1.5 h, 3 h and if few aphids are consumed again after 24 hours.

Data analysis

Longevity

Lethal time **LT**, the time from inoculation to death, can be analysed with a nonparametric analysis for survival distributions, the LIFETEST-Procedure (SAS Institute, 2009), which yields survival distribution plots and product-limit survival estimates (Kaplan and Meier, 1958). Tests for homogeneity of survival curves over treatments include the log-rank test, which is most sensitive to differences late in the survival curves, and the modified Wilcoxon test, which is most sensitive to differences early in the curves. Data were adjusted for right-censored observations to correct for any lost specimens. Note: Average survival time AST, is an alternative way to calculate, however this will not alter results and interpretation.

Fecundity

The fecundities of females on the different treatments are compared, and the development in fecundity is analysed using repeated measurements analysis (Proc Glimmix, SAS Inst. 2009). Pairwise comparisons of means (t-test, $P < 0.05$), are used to compare individual treatments.

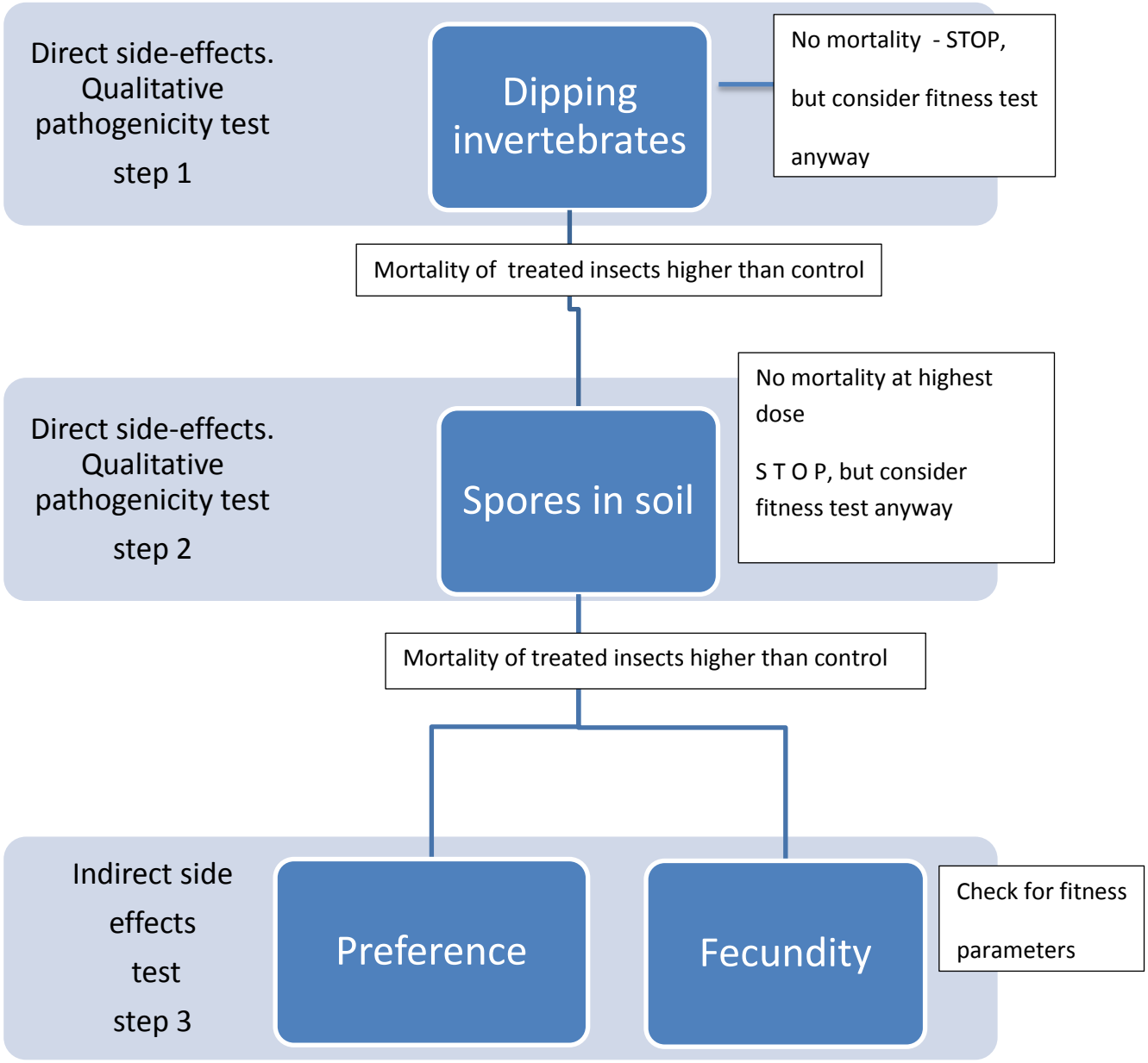
Preference

Preference should be analysed by calculating Manlys Alpha index of prey preference (Manly et al. 1972). Prior to these analyses preference data need to be corrected with Abbotts mortality factor (Abbott, 1925) for mortality in controls. The index is calculated by the following formula, which takes food depletion into account:

$$\alpha_i = \frac{\ln((n_{i0} - r_i)/n_{i0})}{\sum_{j=1}^{m} \ln((n_{j0} - r_j)/n_{j0})}, i = 1, \ldots, m \qquad (1)$$

Where α_i is the preference for prey type 1; ni_0 and nj_0 are the number of prey type 1 (control) and prey type 2 (treated), respectively, present at the beginning of an experiment; and r_i and r_j are the number of prey type 1 and prey type 2, respectively, remaining after experimentation. Thus, the value of α_i will fall between 0 and 1, with values larger than 0.5 indicating a preference. Manlys alpha are compared between prey types using a two-tailed paired *t*-test. In addition to the calculation of preference index, transformed mortality data are analysed by ANOVA to test for differences in the number of prey killed in the choice and no-choice experiments.

Figure 3. Decision tree for a given formulation



Recommended literature

Abott W.S. 1925. A method of computing the effectiveness of an insecticide. Journal of Economic Entomology, 18, 265-267.

Dromph, K.M., Vestergaard, S., 2002. Pathogenicity and attractiveness of entomopathogenic hyphomycete fungi to collembolans. Appl. Soil Ecol. 21, 197–210.

Wilson, ERE 2012. Life cycle of the rove beetle, *Atheta coriaria* (kraatz) (coleoptera: staphylinidae) and suitability as a biological control agent against the fungus gnat, *Bradysia* sp. nr. *Coprophila* (lintner). Phd thesis. Manhattan, Texas, USA.

Goettel, M. S., & Inglis, G. D. 1997. Fungi: hyphomycetes. *Manual of techniques in insect pathology*, *5*, 3.

Manly, B.F.J., 1972. A model for certain types of selection experiments. Biometrics 30, 281–294.

OECD Guidelines for the Testing of Chemicals Test No. 226: Predatory mite (*Hypoaspis* (Geolaelaps) *aculeifer*) reproduction test in soil, adopted guideline October 3, 2008.

van Lenteren JC, ed. 2003. *Quality Control and Production of Biological Control Agents: Theory and Testing Procedures*. Wallingford, UK: CABI Publ. 327 pp.