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Standard non-target tests for risk assessment of plant protection products are unsuitable for entomopathogenic fungi—a proposal for a new protocol

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Abstract

Purpose The successful implementation of a plant protection product depends on its effectiveness against a target species and its safety for the environment. Risk assessment schemes have therefore been devised to facilitate classification and regulation. These guidelines, however, are directed towards chemical substances and are in many cases less suitable for the assessment of products employing microorganisms.

Methods In this study, we developed a protocol for non-target testing of soil-applied entomopathogenic fungi for the biocontrol of insect pests. Using the predatory mite *Gaeolaelaps* (*Hypoaspis*) *aculeifer* as a non-target model organism, our protocol evaluates the lethal and sublethal effects of the fungus in recommended and ten-fold field concentrations.

Results The proposed protocol considers fungal biology when setting test duration, endpoints, and quality control measures. To assess its practicability, we performed a trial with *Metarhizium brunneum* ART2825 as a representative entomopathogenic fungus. The biocontrol agent was able to infect a susceptible host and reproduce, showing that potential hazards can be detected using our approach. No hazard was detected for the non-target species, with no statistically significant differences in 5-week survival and reproductive output between treated and untreated groups.

Conclusion Based on our results, the protocol is deemed appropriate for the detection of non-target effects. Subject to further validation, our approach could thus provide the basis for standardized protocols for the evaluation of the environmental safety of biocontrol organisms.

Keywords Non-target effect · Biological control agent · Entomopathogenic fungus · Risk assessment

1 Introduction

The environmental safety of chemical and microbial plant protection agents is of great importance (Brühl and Zaller 2019; Scheepmaker et al. 2019). The distinction between products with acceptable and unacceptable risks is the goal of assessment schemes and plays an important role in the registration process of a product (Gwynn 2017). One aspect is the impact on non-target organisms. In the European Union, this assessment follows a tiered approach, where as a first-step standard toxicity tests under laboratory conditions are performed. If the expected exposure of the product is higher than the safe concentration evaluated in the first tier, more complex tests better simulating natural conditions follow as higher tiers (Schäfer et al. 2019). In the past, standardized methods used for registration often did not discriminate between chemical and microbial agents and thus failed to take into account their different modes of action, target specificity, and disparity in environmental behavior, such as persistence and the ability of microorganisms to multiply (Chandler et al. 2008; OECD 2019). As a consequence, data requirements for the registration of microbial products were often not met, complicating and prolonging the process and thereby increasing the cost of the development of new products (Köhl et al. 2019). Distinct

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assessment approaches are thus strongly recommended (Sundh and Goettel 2013). A general guidance protocol to evaluate the environmental safety of microbial control agents, including a chapter on non-target organisms, has been published by the OECD (2014), but detailed guidelines for specific tests are still lacking (Arora et al. 2016; OECD 2016b).

In this paper, we propose a guideline for non-target testing of the soil biota to soil-applied entomopathogenic fungi (EPF) for the biocontrol of insect pests. The goal of this study was to revise the existing protocols for chemical plant protection (Bakker et al. 2003; OECD 2016a) using the predatory mite *Gaeolaelaps* (*Hypoaspis*) *aculeifer* (Laelapidae) to make them appropriate to the assessment of biocontrol agents based on EPF. *G. aculeifer* is classified as a relevant species for ecotoxicological testing (Huguier et al. 2015) and is included in the regulatory scheme of the European Union for non-target testing (EC 2013). This arthropod species is frequently found in arable and grassland soils worldwide (Karg 1993), and its habitat puts it at a high risk of exposure to soil-applied plant protection products. In order to adapt the existing protocols to the specific characteristics of the fungal biology, we addressed (1) meaningful quality controls, (2) the test duration required, and (3) suitable endpoints.

To demonstrate the test setup, we selected as biocontrol model species *Metarhizium brunneum*, one of the most commonly used EPF (Lacey et al. 2015) and known to infect a wide variety of insects and arachnids. Isolates of *M. brunneum* can differ greatly in host specificity (Roberts and St Leger 2004). We selected two isolates: ART2825, a promising biocontrol agent against wireworms (Eckard et al. 2014; Rogge et al. 2017), and the standard isolate BIPESCO 5. The latter is registered in the EU and Switzerland as the active ingredient of plant protection products, such as Met52 granular against *Otiorhynchus* (BLW 2018; BLV 2020).

Non-target tests of EPF have been the topic of several peer-reviewed studies in the past (e.g., Bilgo et al. 2018; Seiedy et al. 2015; Thungrabeab and Tongma 2007). The methods applied in these studies, however, vary considerably, and the reasons for selecting a specific setup are not explained in detail. This makes it difficult to judge and compare their implications and results in the registration process. The newly adapted protocol presented here represents a step towards harmonization of test procedures for EPF, with the goal of facilitating the registration process and removing barriers to the development of new products.

2 Materials and methods

2.1 Protocol revision

Two existing protocols for non-target testing of *G. aculeifer* were reviewed for their compatibility with EPF. An overview of the two test setups and associated questions and concerns (Table 1) provided the basis for the creation of our new protocol.

2.2 Principles of the new test protocol

Our protocol was designed to be applied using fungal conidia, rather than a formulated product, analogous to active substance testing of chemical plant protection products. The protocol is divided into a 2-week exposure period and a 3-week observation period, similar to Bakker et al. (2003). For exposure, fungal conidia are incorporated in the test subjects' (*G. aculeifer*) environment. During the exposure period, mites develop from protonymph to adult. Adult mites are subsequently transferred into an untreated environment for observation, during which three parameters are monitored: weekly mortality, oviposition rate of female mites, and, as an addition to previous protocols, mycosis of cadavers.

For fungal exposure, the recommended field dosage of the biocontrol organism and ten times the field dosage were chosen as treatments as suggested in OECD (2014), rather than an increasing concentration series because the calculation of a dose–response curve was not intended. A negative (no treatment) and a positive control (insecticide treatment) were included in the test, as in previous protocols. Additionally, a susceptible species (larvae of the wireworm *Agriotes obscurus*, Coleoptera: Elateridae) was also tested, using the same exposure method, to confirm the virulence of the biocontrol agent. To calculate the required amount of conidia per experimental unit, the field application rate per area was converted to conidia per gram substrate, based on the suggested incorporation depth of the biocontrol agent in the field and assuming a medium bulk density of the soil of 1500 kg/m^3 (OECD 2014). The experiment was performed twice to increase the robustness of the results. Tests were performed in darkness at 22°C, 70% RH.

2.3 Test subjects

2.3.1 Biocontrol agent

M. brunneum ART2825 was isolated in 2007 from an infected *A. obscurus* larva in the rearing facility at Agroscope in Zurich, Switzerland (Kölliker et al. 2011). Prior to use in the experiment, the fungus was re-isolated from single conidium colonies obtained from *A. obscurus* larvae and maintained on a selective medium (Sabouraud 2% glucose agar (SDA) with the antibiotics cycloheximide (0.05 g/l), streptomycin sulfate (0.6 g/l), tetracycline (0.05 g/l), and the fungicide dodine (50 mg/l); Strasser et al. 1996). Single conidium colonies were propagated on a solid complete medium (Riba and Ravelojoana 1984). Conidia were collected from these plates by adding two tablespoons of quartz sand and gently moving

Table 1 Review of non-target protocols for *G. aculeifer* and their suitability for entomopathogenic fungi

	(OECD 2016a)	(Bakker et al. 2003)	Concerns identified	Solution proposed
1. Test subjects	*G. aculeifer* adult female mites	*G. aculeifer* protonymphs	Depending on their age, individuals may be more or less sensitive to fungal infection (Maniania and Odulaja 1998; Sedighi et al. 2013).	Observation of full life cycle to recognize sensitive life stages
2. Experimental design	Dose–response test in 5–12 concentrations, calculation of effective concentration (ECx) or no observed effect concentration/lowest observed effect concentration (NOEC/LOEC), alternatively limit test	Limit or dose–response test possible in the setup. For dose–response, 5 concentrations are recommended, calculation of 50% lethal rate (LR50)	Pathogens mostly do not follow dose–response curves, as a single contact event can suffice for infection (Lafferty et al. 2008). Concentration-dependent values, such as NOEC/LOEC, are not necessarily informative.	Development of new classification criteria. For pathogenic effects reduced test setup with two concentrations: the target concentration in the field and an elevated concentration as a safety factor. Dose–response test when indication that toxicity is the main driver of the effect.
3. Endpoints	Reproductive output of *G. aculeifer*	Juvenile mortality after 14 days, number of fertile eggs produced after a 7-day oviposition period on an untreated substrate	After killing their host, EPF produce resting structures or further infective spores for the proliferation to a new host (Roy et al. 2006). The potential increase of the biocontrol agent is not considered.	Incubation of cadavers for observation of mycosis and assessment of potential proliferation on non-target host
4. Information on the test agent	Water solubility of the test chemical		Basic knowledge considering taxonomy, biology, and methods for identification required for all microbial biocontrol agents before risk evaluation (OECD 2014)	Provision of supplementary information on biocontrol agent
5. Reference substance (positive control)	Dimethoate (CAS 60-51-5), Boric acid (CAS 10043-35-3)	Dimethoate	The inanimate substance does not confirm adequate laboratory test conditions for living organisms.	Testing of a susceptible species in addition to positive control to confirm conditions are suitable for the tested EPF
6. Test duration	14 days	34 days	Disease transmission and incubation period may require a longer test duration.	Test for 36 days at 22°C to allow for observation of possible increased mortality and visible mycosis. Reassessment of test duration based on susceptible species in the test
7. Test conditions	Artificial soil substrate; stable water content 40–60% WHC; temperature 20 ± 2°C; gaseous exchange at least twice a week; controlled light:dark cycles with 400–800 lux; test vessels made out of glass or chemically inert materials with tight-fitting cover; food source *Tyrophagus putrescentiae*, small collembolans, enchytraeids or nematodes, ad libitum	LUFA 2.1 soil; stable temperature and soil moisture at 50% WHC 25 ± 2°C; gaseous exchange by passive ventilation through 80-μm gauze; darkness; test vessels glass plates with circular space and plastic cups with humidified plaster as lower surface; food source *Tyrophagus putrescentiae* renewed every 2–4 days	Biotic and abiotic soil factors influence the efficacy of entomopathogenic fungi (Jaronski 2007); choice of substrate should be reviewed.	Use of quartz sand as substrate to reduce the influence of soil factors, improve the homogenous distribution of fungal spores, and facilitate mite retrieval from the substrate
8. Application procedure	Sprayed or mixed into the soil, depending on solubility in deionized water or dry application	As solution in deionized water by mixing through the soil or as spray	Physicochemical properties of fungal spore surfaces range from hydrophilic to hydrophobic (Holder et al. 2007); solutions in water may not be practical.	Application method also suitable for hydrophobic fungal spores through a sand–spore mixture
9. Evaluation	Juveniles collected once after 14 days through heat extraction	Mortality by counting live mites and the number of corpses after 14 days. Fertility by counting juveniles and unhatched eggs after a 7-day oviposition period and 4–6 days of incubation	See point 3. Observation of reproductive ability of fungus in the non-target organism necessary. Generally, fungal pathogens must kill their host to produce new infective spores (Meyling and Hajek 2010). Needs further incubation of cadavers for visible mycosis.	Retrieving live mites from inoculated substrate by flooding containers. Observation of mites on water agar to facilitate detection and incubation of cadavers

Table 1 (continued)

	(OECD 2016a)	(Bakker et al. 2003)	Concerns identified	Solution proposed
			Heat extraction retrieves only surviving mites.	
10. Validity of test	Mean adult female mortality, mean number of juveniles per replicate, coefficient of variation	Mortality and fertility	Additional validity criteria needed to estimate the viability and virulence of the test agent	Confirmation of viability by testing spore germination. Examination of virulence by integrating susceptible species in the test

it on the plate. Samples of 5 g of the sand–spore mixture were then diluted in sand at 1:5 and stirred for 1 min to achieve an even distribution of the spores. To determine the concentration of the mixture, 1 g was mixed with 1 ml of 0.1% Tween® 80 and the number of conidial spores counted in the supernatant with a hemocytometer. The required concentrations were achieved by further diluting with sand. For *M. brunneum* ART2825, Rogge et al. (2017) recommended application at a field concentration of 10^{14} conidia/ha. For the approximated incorporation depth of 6 cm, the concentrations in our experiments were thus set to 2.22×10^6 conidia per gram substrate for the recommended concentration (hereafter labeled ART-Field) and 2.22×10^7 conidia per gram substrate for the ten-fold recommended concentration (ART-Field*10). *M. brunneum* BIPESCO 5 conidia (treatment Bip) were produced in the same way, and their concentration was adjusted to match that of ART2825.

Infection of arthropods was identified morphologically based on genus-level characteristics, namely, white mycelia with green conidia. In the case of doubt, conidial morphology was additionally examined microscopically.

2.3.2 Arthropods

G. aculeifer were obtained from EWH BioProduction ApS, Tappernøje, DK, and kept at 20°C in darkness in plastic boxes with plaster of Paris and charcoal mixture at the bottom. Food (*Ephestia kuehniella* eggs) was available ad libitum and renewed twice a week. The culture was synchronized to obtain individuals of similar age (OECD 2016a), and cohorts of 50 adult female mites were put in Petri dishes containing 16 ml of 20% water agar for oviposition. Petri dishes were sealed with Parafilm® to prevent escape. After 2 days, female mites were removed, and the protonymphs developing in the Petri dishes were collected and used for experiments.

A. obscurus larvae used as susceptible species in the experiment originated from the laboratory livestock established according to Kölliker et al. (2009). Prior to experiments, each larva was weighed, and its fitness was assessed based on movement behavior according to van Herk and Vernon (2013). Only larvae showing normal and spontaneous movement (category 0 in van Herk and Vernon 2013) were included in the test. Individuals were randomly assigned to each treatment.

2.4 Test preparation

Prior to the tests, the viability of the EPF was assessed by preparing a conidial suspension with 0.1% Tween® 80 and adjusting it to 1×10^6 spores/ml. Three 50-µl drops of this suspension were then incubated in darkness at 22°C, 70% RH, in Petri dishes on a solid complete medium containing 10 g glucose, 0.36 g KH_2PO_4, 1.78 g Na_2HPO_4, 1 g KCl, 0.6 g $MgSO_4 \cdot 7H_20$, 0.7 g NH_4NO_3, 5 g yeast extract, and 20 g agar in 1000 ml distilled water (Riba and Ravelojoana 1984); 24 h post-inoculation, the germination percentage was calculated by counting 100 spores for each drop at ×40 magnification. Conidia were considered as germinated if the germ tube was at least the length of the spore itself. Only the EPF material with a mean germination rate of ≥95% was considered viable and used for non-target testing.

2.4.1 Exposure period

For exposure, plastic cups (90 cm^3) were filled with 19 g quartz sand and moistened with deionized water (10 % of the total substrate dry weight). Fungal conidia were incorporated in the sand by preparing a sand–spore mixture. One gram of this mixture was then added to the exposure cups and stirred thoroughly for 1 min. For the control, 1 g of untreated sand was added. Sand was chosen as a substrate instead of natural or artificial soil to reduce the impact of soil biotic and abiotic factors on the fungus and to allow the even distribution of conidia. Ten *G. aculeifer* protonymps were carefully placed in each cup using a fine brush. The cups were closed with a perforated lid overlaid with Parafilm® in order to keep moisture levels high but still enable gas exchange, preserve the reopening ability of the cup, and prevent mites from escaping. Cups were randomly distributed and stored in plastic boxes. The temperature and relative humidity were recorded daily and adjusted if deviating. The water content

of the substrate was measured weekly by weighing, and the substrate was rewatered if necessary.

Dimethoate was used as insecticide treatment. It has frequently been included in validation tests (OECD 2016a). In this experiment, a concentration of 3.6 mg a.i./kg dry soil was used, following the protocol of Bakker et al. (2003). A solution was prepared in deionized water in the appropriate water quantity to reach the required moisture content of the sand.

2.4.2 Observation period

After the exposure phase, mites were removed from the substrate by emptying each cup on a 15 × 11-cm dish with high edges. The dish was then flooded with distilled water, and the sand was gently moved. Mites floating on the water surface were transferred using a fine brush to standard Petri dishes (100 × 15 mm) with vents, containing 16 ml of 20% water agar. Petri dishes were sealed with Parafilm® to prevent escape. Any mite not retrieved after the exposure period was recorded as dead, assuming it had decomposed before the assessment. To ensure mating success to allow the assessment of effects of the control agents on the oviposition rate, the sex ratio of the mites was adjusted. In cases where fewer than one male was present per five females, male(s) were added from another replicate of the same treatment, or the experimental unit was discarded when no males were available.

Oviposition was quantified every 3 days for 15 days, starting in week 4 (1 week after transfer into Petri dishes). At each evaluation date, adult mites were moved to a new Petri dish, and the eggs in the used Petri dish were counted.

In the tests involving the susceptible species (larvae of *A. obscurus*), individuals were not transferred to Petri dishes but were shifted to new cups, filled with 20 g of untreated moistened sand. Dead individuals were counted at the end of the exposure period and the end of the observation period (2 and 5 weeks after the beginning of the test). Cadavers were incubated for one further week, and possible signs of mycosis were recorded based on morphological characters (formation of mycelium and spore layers on the outside of the cadavers).

2.5 Statistical analysis

The protocol follows the "proof of hazard" concept (OECD 2006), regarding the test agent as nonhazardous unless there is convincing alternate evidence. It thus tests the hypothesis that there is a difference in mortality/oviposition rates between the treatment and control groups. The statistical software R (version 3.6.1; R Development Core Team 2019) was used for all analyses. Effects of treatments on *G. aculeifer* and *A. obscurus* mortality were tested with linear mixed-effects models fitted by the Laplace approximation using the package "lme4" (version 1.1-23, 2020; Bates et al. (2015)). Boxes and test runs were included as random factors in the analyses. Multiple comparisons of treatment levels were performed using Tukey contrasts in the package "multcomp" (version 1.4-13, 2020, Hothorn et al. 2008). The two species (*G. aculeifer* and *A. obscurus*) were analyzed separately for their mortality after 2 and 5 weeks. The status (alive/dead) of individuals per test vessel was the dependent variable, assumed to follow a binomial distribution. The mean number of eggs laid by female mites per day over the observation period of 3 weeks was tested with a linear model using *t*-tests (Satterthwaite's method). The statistical significance threshold was set to 0.05, compliant with a widely accepted standard in Life Sciences. It remains to be discussed if this threshold is appropriate for the estimation of the risk of environmental hazards. Such discussions, however, are not the topic of this study.

3 Results

3.1 Mortality and mycosis

No elevated mite mortality was detected for ART2825 fungal treatments compared to the control after 2 weeks (ART-Field, $z = 0.204$, $p = 0.999$; ART-Field*10, $z = 1.589$, $p = 0.385$) or after 5 weeks (ART-Field, $z = -0.271$, $p = 0.993$; ART-Field*10, $z = 1.168$, $p = 0.647$) (Fig. 1). Mites treated with *M. brunneum* BIPESCO 5 had a statistically significant higher mortality after the 2-week exposure period ($z = 3.298$, $p = 0.005$), but the same level of mortality as the control group after 5 weeks ($z = 0.121$, $p = 0.999$). No mycosis was visible on any of the mite cadavers for either fungal isolate. The insecticide treatment killed all mites within 2 weeks (Fig. 1a).

For the susceptible insect species, *A. obscurus* mortality in the EPF treatment was not higher than control mortality after 2 weeks when exposed to the recommended field concentration of isolate ART2825 ($z = 0.846$, $p = 0.670$). However, after 5 weeks, mortality in EPF-treated *A. obscurus* was significantly higher than in the control group ($z = 4.084$, $p < 0.001$). For the ten-fold field concentration, the treatment effect was already statistically significant after 2 weeks ($z = 2.348$, $p = 0.048$) and more strongly so after week 5 ($z = 4.940$, $p < 0.001$). Visible signs of mycosis were present on 93 and 83% of cadavers from the ART-Field group in the two replicates and on 100% of cadavers from the ART-Field*10 group at the end of the experiment (Table 2).

3.2 Reproduction

During the observation period, the mean number of eggs laid per female mite per day over all groups was 0.89 ± 0.42. The number of eggs laid per day slightly increased towards the end of the experiment: from 0.88 ± 0.68 eggs per day on the first count (days 24–26) to 1.08 ± 0.67 eggs on the last count (days 33–

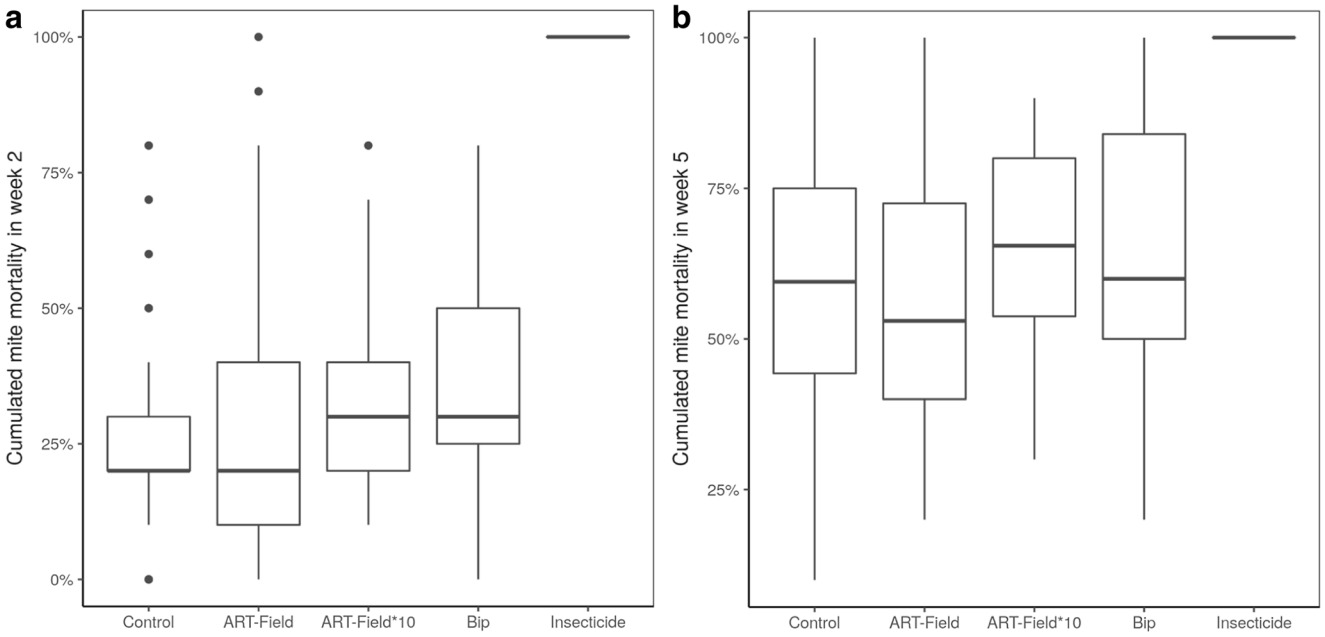

Fig. 1 Cumulative mortality of *G. aculeifer* mites ***a*** after 2 weeks (the end of the exposure period) and ***b*** after 5 weeks (the end of the observation period). Control, negative control (no biocontrol treatment); ART-Field, *M. brunneum* ART2825 conidia applied at recommended field concentration; ART-Field*10, *M. brunneum* ART2825 conidia applied at ten-fold recommended field concentration; Bip, fungal reference strain *M. brunneum* BIPESCO 5 conidia applied at recommended field concentration; insecticide, dimethoate. For each treatment, $n = 20$, 10 mites per test unit. Boxplot middle line indicates the median, lower line 25% quantile, and upper line 75% quantile

36). Fungal treatment had no significant effect on the number of eggs laid per female per day during the course of the 3-week observation period (ART-Field, $t = 0.97$, $p = 0.334$; ART-Field*10, $t = 0.448$, $p = 0.654$; Bip, $t = 1.146$, $p = 0.254$; Fig. 2)

4 Discussion

The reduction of environmental risk is one of the main justifications for prioritizing biological control over chemical plant protection products. The limitations in host range and species specificity of biocontrol agents suggest low risk for non-target

Table 2 Percentage mortality and percentage of larvae showing signs of mycosis in *Agriotes* larvae exposed to *M. brunneum* ART2825 conidia in two concentrations and untreated controls for two replicates ($n = 20$ in each case). ART-Field, conidia applied at the recommended field concentration; ART-Field*10, conidia applied at tenfold the recommended concentration

Treatment	Week 2	Week 5
Control		
Mortality	0%/0%	0%/0%
Mycosis	0%/0%	0%/0%
ART-Field		
Mortality	15%/5%	75%/30%
Mycosis	10%/5%	70%/25%
ART-Field*10		
Mortality	40%/10%	90%/70%
Mycosis	10%/10%	90%/70%

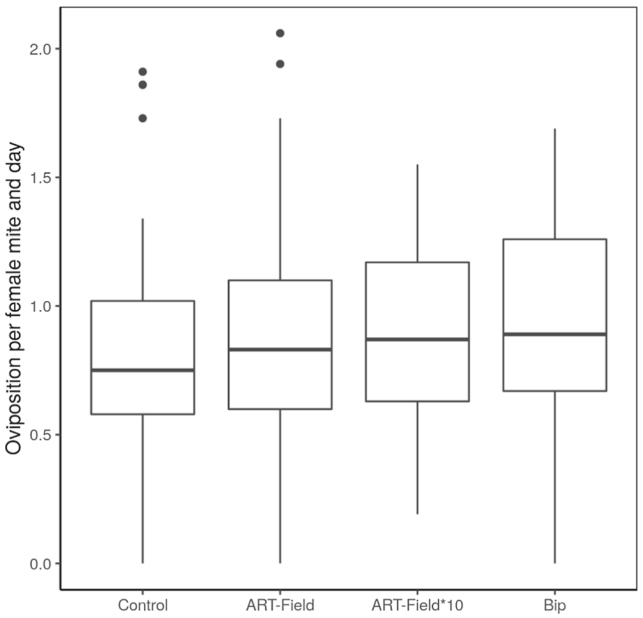

Fig. 2 Oviposition rates per female mite per day. Oviposition was recorded every third day over a 3-week period ($n = 20$). Control, negative control (no biocontrol treatment); ART-Field, *M. brunneum* ART2825 conidia applied at recommended field concentration; ART-Field*10, *M. brunneum* ART2825 conidia applied at ten-fold recommended field concentration; Bip, fungal reference strain *M. brunneum* BIPESCO 5 conidia applied at recommended field concentration. Boxplot middle line indicates the median, lower line 25% quantile, and upper line 75% quantile

organisms (Lacey et al. 2015). For EPF, the need to overcome the insect's cuticle barrier and to interact with its distinct defense mechanisms creates a strong selective pressure leading to variation in fungal isolates and specialization on certain hosts. Nevertheless, not all fungal isolates are equally specialized (Ortiz-Urquiza and Keyhani 2013). Estimating the effect of a potential fungal control agent on host range and non-target organisms should thus be an important element of risk assessment (Mudgal et al. 2013).

The reference isolate in this study, *M. brunneum* BIPESCO 5, was one of the first entomopathogenic fungi approved for use as an insecticide and the first commercially used *Metarhizium* isolate in Europe (Mudgal et al. 2013). For its registration, a peer review on pesticide risk was conducted (European Food Safety Authority 2012). This peer review identified appropriate risk assessment of non-target arthropods as a gap in the then-current procedures and listed the "risk of epizootic disease" as a critical concern. Since then, studies have aimed to close this gap. Tests on soil-dwelling non-target arthropods have been conducted in the laboratory (de Azevedo et al. 2019; Saito and Brownbridge 2016), in the field (Babendreier et al. 2015; Fischhoff et al. 2017), and under semi-natural conditions (de Azevedo et al. 2018). However, studies vary in product formulation, concentration, exposure method, and length as well as endpoints and quality control, complicating their comparison and thus subsequent risk evaluation. To harmonize test setups and better take into account the biology of EPF, we identified the following three major issues that require adaptation within existing protocols developed for chemical products by OECD (2016a) and Bakker et al. (2003).

4.1 Quality control

In an experimental setup, positive controls are added to verify that the experimental method is suitable to detect the intended effect (Johnson and Besselsen 2002). The OECD (2016a) protocol for non-target testing with *G. aculeifer* suggests the use of a well-researched acaricidal substance, such as dimethoate or boric acid. If the test conditions are adequate for the acaricidal substance, mite mortality will increase, and the assumption is that the conditions are then also adequate to detect effects, if present, of the test substance itself. Nevertheless, this approach is not sufficient because adequate conditions for chemicals are not necessarily adequate conditions for biologicals. In contrast to inanimate control agents, the efficacy of biocontrol organisms depends on their viability and their virulence. The stability of these two characteristics is often difficult to achieve and is a major concern for fungal biocontrol agents because their biological activity is readily affected by conditions such as temperature, UV exposition, and the nutrients available to them (Kim et al. 2019). It is thus a prerequisite for any non-target test with biocontrol organisms that viability testing is carried out simultaneously to the experiment to determine whether sufficient viable propagules are present that can exhibit the effect desired in the test. It is also necessary to confirm the virulence of fungal isolates, and biocontrol agents in general, in the selected experimental setup. Among other factors, growth rates and virulence of *M. brunneum* vary considerably within one isolate depending on the temperature (Bugeme et al. 2008; Li and Feng 2009) or soil type and moisture levels (Jaronski 2007). To demonstrate the virulence of a specific isolate under the given test conditions, the protocol should include a species previously shown to be generally susceptible to EPF, such as *Galleria mellonella* L. larvae (Saito and Brownbridge 2016), or the specific biocontrol target species, e.g., the wireworm *A. obscurus* in our case. If the EPF do not exhibit the expected virulence on the target species in the experimental setup, the experiment would be invalid and should be adapted.

In the presented experiment, viability was assessed through germination tests of conidia. The minimum germination threshold was set to 95% for all samples in accordance with de Azevedo et al. (2019) to ensure that sufficient infective propagules were present. In performing this simple test, misleading trials using a nonviable test agent can be avoided. Fungal spores passed this threshold in our setup. Moreover, the tested isolate *M. brunneum* ART2825 was able to cause mortality and mycosis in the susceptible species in this setting, with substantially elevated *A. obscurus* mortality in both fungus-treated groups.

4.2 Test duration

Infections with entomopathogenic fungi begin with a conidium attaching to the cuticle of the insect host (Boucias et al. 1988). It then penetrates the host and fungal hyphae grow into the body cavity, destroying hemolymph and various tissues (Charnley 2003; Sun et al. 2016), eventually leading to the death of the host. The lethal time varies depending on incubation temperature, fungal isolate (Ekesi et al. 1999), and host species. Estimated 95% confidence intervals (CI) of lethal times for two target species of *M. brunneum* ART2825 are 10.5–31.6 days post-inoculation for *A. lineatus* and 11.03–16.7 days for *A. obscurus* when dipped into a conidial suspension of 0.03% (v/v) aqueous Tween® 80 and fungal conidia (10^8 conidia/ml) and incubated at 23°C (Eckard et al. 2014). The results with *A. obscurus* as susceptible species in our study exceed these lethal times (95% CI 21–35 days after the start of the test for the higher concentration). Reasons may be that incubation temperature was lower (22°C) and that the method of exposure (conidia incorporated in sand) represents a less invasive approach than dipping host insects in spore solutions. However, our approach might better resemble natural infection conditions, under which mortality might be lower. It is important to note that the results of our test with

EPF application at field concentration showed no statistically significant differences in *A. obscurus* mortality after 14 days, whereas after 36 days, mortality in the treated group was significantly higher than in the control group. This underlines the importance of adopting an extended observation time span and test duration. The test duration of the standardized OECD protocol is 14 days (OECD 2016a). If we had followed this recommendation, we would have missed the effect of the entomopathogenic fungus on *A. obscurus* mortality and would have concluded that the setup did not work.

However, simply prolonging the exposure period of the experiment is not possible, as it interferes with the protocol. In the OECD experimental setup, adult female mites are exposed to the control agent after the start of the egg-laying period to be able to measure the reproductive output at the end of the test. At 20°C, juveniles will reach the deutonymph stage on average after 2 weeks (Smit et al. 2012) and can be discriminated from adults. In a prolonged setting, however, juveniles would complete their development, and the distinction between offspring and their initially exposed parent mites would become impossible. Separating adults from juveniles after 2 weeks is also not feasible, as the retrieval of mites through heat extraction and fixation in 70% ethanol as described in OECD (2016a) leads to the death of the test subjects. For this reason, our proposal follows the 5-week setup proposed by Bakker et al. (2003). The division into a 2-week exposure period and a 3-week observation period allows the observation of mites from protonymph to reproductive adults for the selected endpoints.

4.3 Selection of endpoints

Mortality rates and reproductive success are set as endpoints in the two current protocols to assess lethal and sublethal effects on the non-target organism. While these endpoints may be sufficient for chemical substances that degrade over time, they are not sufficient to evaluate the effects of biological agents. EPF propagate through infected hosts, build up their populations, and promote further secondary infections (Meyling and Eilenberg 2007). For example, it has been proposed that further persistence of *M. brunneum* in soil correlates with the occurrence of its host insect, *Diabrotica virgifera virgifera* (Pilz et al. 2011). Similarly, a significantly prolonged survival time of the EPF *Beauveria brongniartii* is observed when its host insect, the cockchafer *Melolontha melolontha*, is present (Kessler et al. 2004). Mycosed non-target organisms may elicit the same phenomenon and slow down the expected decrease of fungal inoculum in the soil over time. We therefore propose to add mycosis as another endpoint to the protocol. In our tests, mycosis was not observed on *G. aculeifer*, regardless of concentration or fungal isolate; whereas for *A. obscurus* , mycosis was clearly visible on 90 and 100% of all dead individuals in the field concentration group and the high concentration group, respectively.

Mortality and oviposition rates were similar in control and ART2825-treated mite groups, suggesting that the isolate had no adverse effect on the mites tested. This would make further risk calculation unnecessary (OECD 2014). For the case of an observed impact, however, it will be necessary to classify the risk in order to enable well-founded decisions in the registration process. Missing risk assessment procedures can cause gaps in data requirements and lead to the prolongation of the registration process (Köhl et al. 2019). Dose–response-dependent ecological endpoints, such as the NOEC (no observed effect concentration) or the EC_{50} (half-maximal effective concentration), are commonly used to contribute to decision making. With only two concentrations tested, this protocol does not allow the calculation of such endpoints. However, dose–response relationships are considered less adequate for microbial plant protection agents (OECD 2014). Infection rates of pathogens, such as EPF, typically do not follow a dose–response curve. The number of infectious particles usually needs to exceed a certain threshold to overcome the host's immune defense. Furthermore, not all infections lead to death; host recovery is also possible (Anttila et al. 2017). Therefore, even if the number of infections increases with a rising concentration, it will not be evident in a setup that relies on visible disease symptoms and will not be represented in dose–response-dependent endpoints. Thus, we would recommend the formulation of a risk assessment scheme as the next important step to facilitate and accelerate registration and implementation of EPF as biological control agents.

4.4 General applicability

The three issues discussed here (quality control, test duration, and choice of endpoints) concern not only the testing of EPF but also other microbial control agents. Viability and virulence are key for the effectiveness of microbials and depend on the quality of the inoculum. The speed of kill should be taken into consideration when setting test durations. While bacteria are usually fast-acting, viruses, such as EPF, may have longer lethal times (Ravensberg 2011). For example, mean times to death of 8.6 to 14.0 days have been reported, depending on the granulovirus isolate (Gómez Valderrama et al. 2018). The choice of endpoints should reflect the mode of action, whether it relies on pathogenicity or toxicity. In contrast to other microbials, the main insecticidal activity for many entomopathogenic bacteria is based on toxic proteins (Glare et al. 2017). Additionally, potential proliferation and transmission pathways need to be considered in setting endpoints. Besides the release of infectious

Table 3 Short summary of the proposed risk assessment test for non-target species

Test species	*Gaeolaelaps aculeifer* (non-target organism) Susceptible species (variable, e.g., target organism)
Test phases	Exposure to EPF (14 days) Observation (21 + 7 days)
Exposure	10 protonymphs per exposure vessel (plastic, diameter 4.5 cm, height 6 cm, filled with 30 g (dw) quartz sand moistened with 2-ml distilled water, sealed with perforated lids and Parafilm®), incorporation of EPF conidia in the sand
Observation	Mites are transferred to observation vessels: standard Petri dish with vents (100 × 15 mm), 16 ml of 20% water agar, sealed with Parafilm®
Measurements	Mortality assessed for non-target and susceptible species on days 14 and 35 Mycosis determined 7 days after detection of cadaver based on morphological characters Oviposition: eggs/female mite counted every 3 days from day 21 to day 36
Endpoints	Cumulative mortality rate in percent Mycosis visible on cadavers Oviposition rate: eggs/female/day
Test duration	36 days + 7 days for observation of mycosis
Test conditions	Darkness, 22°C, 70% RH Water source: measurement of water content in cups (weekly by weighing), rewatering if necessary Food: species appropriate (e.g., *Ephestia kuehniella* eggs for *G. aculeifer*), available ad libitum renewed twice a week, supplied on the surface
Quality control	Determination of germination ability of the fungus Temperature recorded daily and adjusted if necessary Synchronization and randomization of test individuals Virulence against susceptible species
Validation criteria	Pending: assessment through ring testing
Data assessment	Mortality: linear mixed-effects model, family "binomial", fitted by the Laplace approximation (package "lme4" (version 1.1-21)) Multiple comparisons of treatment levels by Tukey contrasts, package "multcomp" (version 1.4-10, 2019; Hothorn et al. 2008). Oviposition: *t*-tests using Satterthwaite's method for linear model Statistical software R (version 3.5.1; R Development Core Team 2019) Random factors: boxes and test run $\alpha = 0.05$

particles through cadavers, main transmission pathways may also include transmission through feces in microsporidia (Goertz and Hoch 2008).

The identified concerns and proposed solutions can in general function as a starting point to adapt other existing protocols to microbial agents for the control of arthropods. Acknowledging sensitive life stages when choosing a test subject is valuable as they are also known in bacteria (Bravo et al. 2011) and viruses (Sporleder et al. 2007). Regarding the application method, disparities in host invasion should be considered. While fungi can enter through the cuticle of the arthropod host, other microorganisms mostly need to be ingested (Lacey 2017). Incorporating the inoculum in the substrate as proposed in this protocol may thus not be a suitable application method in such cases. Overall, flexibility to modify the test setup is necessary for the diverse requirements of microbial biocontrol agents, and this protocol specifically may be adapted for testing EPF on other soil micro- and macro-arthropods.

5 Conclusion

The proposed protocol in this study (see Table 3 for a summary) is a first step to adapt the existing OECD protocol to pathogens and thus to harmonize the evaluation of environmental risks for EPF used as biocontrol agents. The selected non-target species for this protocol, the predatory mite *G. aculeifer*, showed no negative impact of the test isolate *M. brunneum* ART2825. To further improve the protocol, clarify test details, and set validation criteria, a ring test with a standardized EPF is recommended. The next steps should include the formulation of concrete guidelines for more taxonomic groups and the development of strategies by the cooperation of expert groups, policymakers, and stakeholders to clearly define acceptable risks.

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Availability of data and material Data are available from the corresponding author upon request.

Code availability Not applicable.

Declarations

Ethics approval No approval of research ethics committees was required to accomplish the goals of this study because the experimental work was conducted with unregulated invertebrate species.

Consent to participate Not applicable.

Consent for publication Not applicable.

Conflict of interest The authors declare no competing interests.

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