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BIOMIX: Assessing the impacts of tank-mixing on biopesticide efficacy

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DISCLAIMER

This work involves tests on a number of commercial biopesticide products and investigates the impacts of simulated tank-mixing on pest mortality following application. These experiments were carried out in controlled laboratory conditions during a short-term project involving only two crop species and two species of target insects. Our results and conclusions may not be representative of pest control efficacy that might be achieved following application in horticulture and agriculture. Further field testing may be required in order to make specific recommendations for any particular commercial product. This report focusses on the potential effects of tank-mixing on biopesticides that contain live pathogens targeting insects; we have not considered other types of biopesticides such as those containing molecules derived from living organisms.

1. Abstract

Biopesticides formulated from live insect pathogens offer control options for insect pests as part of integrated pest management (IPM) programmes that can help reduce reliance on chemical insecticides. These environmentally sustainable biopesticides can limit the ecological harm associated with some chemical products. One ecological benefit of biopesticides is that they can have relatively higher target-specificity than many conventional chemical products, leaving non-target insects unaffected. However, this specificity brings a practical drawback: a wider range of products may be needed to protect crops against the full range of insect pests that threaten production. Farmers and growers may be able to achieve improved efficiency of biopesticide application by tank-mixing products and applying them simultaneously. However, few biopesticide tank-mixing guidelines exist. Furthermore, when insects are simultaneously affected by two different pathogens, both synergistic and antagonistic interactions can occur, meaning the effect of biopesticide tank-mixing on control-efficacy may be unpredictable.

We surveyed the biopesticide tank-mixing practices of UK horticultural growers: the information we received indicated that use of tank-mixes containing two products based on live pathogens that target insects is not widespread. Where products were tank-mixed, growers had not seen obvious impacts on efficacy. In laboratory experiments we simulated tank-mixing for pairwise combinations of four commercial biopesticides targeting either whitefly (*Trialeurodes vaporariorum*) or tomato leafminer (*Tuta absoluta*). Products targeting these pest insects were unaffected by mixing with a second biopesticide; moreover, combining two products targeting the same insect did not increase the pest mortality achieved. We undertook a literature survey and meta-analysis to assess general trends in published literature on whether pathogens, parasites and parasitoids cause antagonistic or synergistic effects when they infect arthropod hosts at the same time. Analysis of over 1100 effects clearly demonstrated that strong antagonism was rare; on average, mortality increased slightly during combined infections, but the effect was less than additive.

We caution that our experimental data and most studies in our meta-analysis were conducted under laboratory conditions, and may not be representative of results in the field. Nevertheless, this research provides an evidence base that tank-mixing of biopesticides is unlikely to frequently compromise their pest control efficacy. Horticultural growers may be able to exploit this result to improve biopesticide application efficiency. Farmers, growers, agronomists and biopesticide producers should use this knowledge to consider the role of tank-mixing in the design of biopesticide-based IPM programmes. As biopesticides begin to be deployed in open field settings in the UK, those conducting field trials may want to use these findings when considering the benefits of applying products as tank-mixes.

2. Introduction

2.1. Biopesticide tank-mixing

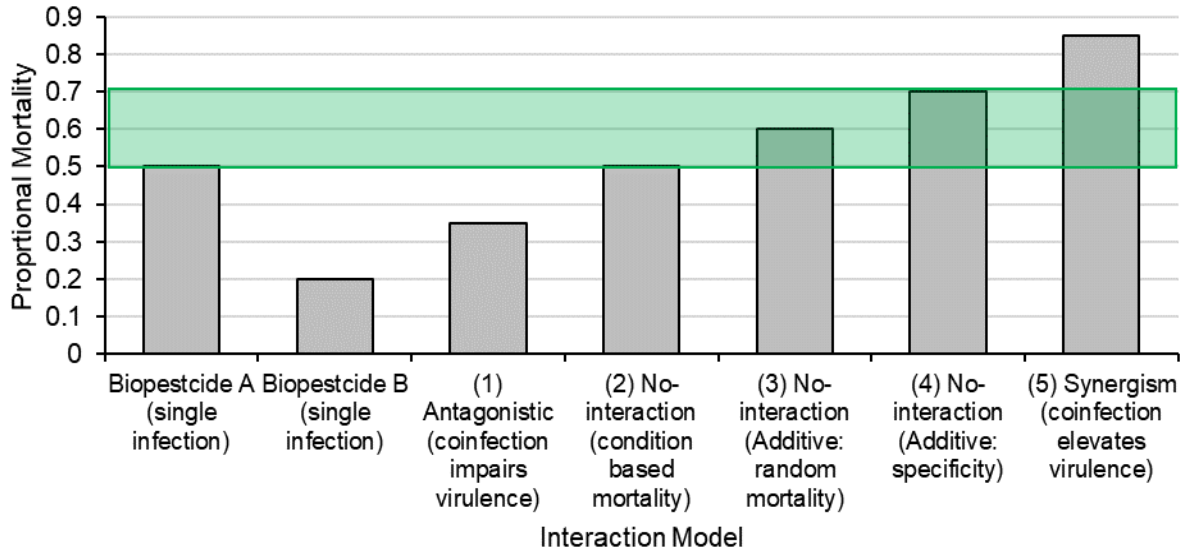
The environmentally sustainable future for agricultural crop protection lies in increased use of biopesticides and other biorational products (Fenibo et al., 2021). One major benefit of biopesticides is their target specificity: they generally kill a relatively narrow species range, with few off-target effects for beneficial insects. However, this benefit is a practical drawback for farmers because multiple biopesticide products need to be applied to defend a crop against all pest species threatening yield. Spraying crop protection products has high labour costs, causes greenhouse gas emissions from machinery, and interrupts other farm activities (Lal, 2004).

One solution to maximise biopesticide application efficiency is tank-mixing. Guidelines for tank-mixing synthetic pesticides are well established, primarily based on direct chemical interactions during spraying and combined effects on pest physiology (Gandini et al., 2020). However, there are no similar guidelines for biopesticides. In this study we focus on microbial biopesticides formulated from live pathogens (rather than those containing biologically derived molecules). Whilst it is unlikely that the pathogens in individual products will directly interact with one another, co-application by tank-mixing means individual pests are simultaneously exposed to multiple pathogens targeting different pest species. Experimental literature shows that simultaneous coinfection by multiple pathogens can have unpredictable consequences for pest mortality, with synergistic and antagonistic effects reported (Mideo and Reece, 2012). Whilst some biopesticide or pathogen mixes may enhance efficacy (Hodgson et al., 2004), others might impair pest control (Li et al., 2021). To ensure biopesticide efficacy is maximised and to avoid eroding industry confidence through poor performance, we identified an urgent need to determine microbial biopesticide compatibility for tank-mixing.

Most UK biopesticide use is currently in protected horticulture, but the future now heralds escalating uptake in open field settings. Questions of biopesticide tank-mix compatibility will become increasingly relevant for UK farming as this transition to biopesticide-based pest control in arable agriculture occurs. In this project we addressed these concerns through three studies: (1) a survey of biopesticide tank-mixing practices amongst horticultural growers, (2) a laboratory assessment of biopesticide compatibility for two pests of importance to protected horticulture in the UK, and (3) a meta-analysis of coinfection effects from existing published literature, which allows us to broaden the scope of our study and facilitates general predictions.

2.2. Defining antagonism and synergy

This study's core aim was to assess whether tank-mixed microbial biopesticides are likely to interact antagonistically such that their efficacy is impaired. Alternatively, biopesticides might generally not interact, or might interact synergistically to deliver improved pest mortality. However, care is needed to clearly define antagonistic and synergistic effects (LeBlanc and Wang, 2006). A standard null expectation from pesticide research is that if two control agents do not interact with each another, pest mortality following co-application will be additive compared to mortality under single treatments. However, the term additive is used inconsistently in the literature. The dominant definition envisages that two agents each kill a random but overlapping subset of the host population (McVay et al., 1977); however, some studies consider an additive effect to be the arithmetic sum of the mortalities in the two single treatments. Whilst many studies in this research field attempt to identify whether pathogens interact with one another during coinfection, in reality it can be difficult to determine this by studying mortality alone (Fig 1).



Interaction model	Type	Description	Virulence prediction
1	Antagonistic	Pathogen B reduces mortality caused by pathogen A	AB < A
2	No interaction - condition-based mortality	Both pathogens kill the weakest subset of the population; pathogen A and B target the same individuals	AB = A
3	No interaction – additive random mortality	Pathogens A and B both kill the population at random (overlapping subsets of the population die)	AB = A+B*(1-A) (McVay et al., 1977)
4	No interaction – additive specificity	Pathogens A and B each kill different subsets of the population (subsets which do not overlap)	AB = A + B
5	Synergism	Combined infection results in greater than additive mortality	AB > A+(B*(1-A)) or AB > A + B

Figure 1. Defining additive effects. Possible scenarios for the outcome of simultaneous coinfection with pathogens from two microbial biopesticides. Here, biopesticide pathogen A is more virulent than biopesticide pathogen B. Models 2, 3, and 4 all involve examples where the biopesticide pathogens do not interact in the target host. The green bar indicates the range of mortalities that could occur for different scenarios in which pathogen interactions are absent.

For the purposes of this study, we chose to focus on whether biopesticide tank-mixing might impair efficacy; therefore, we principally compared pest death rates in combined treatments to the ability of the more effective agent to kill the target pest when applied singly (Model 2 above). However, we also sought to identify whether elevations of pest mortality following mixed pathogen application were consistent with additive effects, therefore we compared death rates in combined treatments to those expected under an additive model involving random mortality (Model 3 above).

Another issue when determining whether effects are additive or synergistic is the relative concentrations of products in single and combined treatments, termed 'concentration additivity'. In our experimental work we focussed on the scenario where tank-mixed biopesticides were always applied at recommended concentrations; the concentration of individual products was the same when they were used singly or in combination (therefore the total combined product concentration in mixed treatments was higher). When we surveyed previously published literature, we recorded whether mixed treatments were at higher combined concentrations or whether combined treatments had a total concentration equalling single treatments; we accounted for this in our analysis. Whilst tank-mixed application of biopesticides will simultaneously expose pests to two or more pathogens, some studies investigate the impacts of sequential exposure to different pathogens; again, we assessed whether sequential vs simultaneous exposure influenced mortality outcomes in our analysis. This has relevance for crop protection because even in the absence of tank-mixing, IPM regimes may involve application of more than one biopesticide to a crop at different times to control multiple pest species.

2.3. Study design

This study is principally focussed on horticulture because this is the sector with the highest biopesticide use in the UK. Our laboratory study investigated how tank-mixing affects biopesticide efficacy for two key pests of glasshouse horticulture: whitefly (*Trialeurodes vaporariorum*) and tomato leafminer (*Tuta absoluta*). Both these pests can be controlled with biopesticides (in addition to biological control agents). We selected two biopesticides that target *T. vaporariorum*, Botanigard and Mycotal; these are both fungal agents (*Beauveria bassiana* and *Lecanicillium muscarium* respectively) for which spores adhere to the insect cuticle, germinate, penetrate the cuticle, and then grow inside the insect body to cause death. In contrast, for *T. absoluta* we chose Dipel, which contains a mixture of live *Bacillus thuringiensis* spores and four different *B.t.* toxins, and Tutavir which contains Phthorimaea operculella granulovirus. Both the *T. absoluta* agents must be consumed from contaminated foliage. We chose these pests and biopesticides because in tomato horticulture, growers might need to control both pests simultaneously and could potentially do this by tank-mixing products targeting each of them. Our overall study is also motivated to fulfil the future demands of arable agriculture, where there may be a requirement to control a wider range of pests

with a wider range of biopesticides. Nevertheless, this horticulture-focussed study represents a first test case. We also aimed for our study to provide more general predictions for whether tank-mixing is likely to be a suitable strategy for efficient biopesticide application in the future. In order to make these general predictions, we surveyed published literature for studies that involved simultaneously or sequentially co-infecting arthropod hosts with two different parasitic agents. We included in the remit of this literature survey any relevant study on arthropods which involved coinfection with microbes (e.g. fungi, bacteria and viruses) and also with macro-parasites (e.g. nematodes and parasitoid wasps). We then undertook a meta-analysis to identify whether coinfection generally enhanced or impaired the ability of individual parasites to kill hosts, and also whether these trends were influenced by characteristics of the parasites concerned. We aim that these findings will be useful to those designing trials for current horticultural IPM programmes, and for those in the future planning how biopesticides can best be applied in open-field settings.

3. Materials and methods

3.1. Survey of biopesticide tank-mixing amongst UK horticultural growers

During March 2022, we surveyed a number of horticultural growers in the UK to ascertain their experience of tank-mixing biopesticides. Contact was made with growers principally by telephone, but also by email using online information. For each grower contacted, we requested information on three topics: (1) whether they used biopesticides for crop protection; (2) whether they tank-mixed these biopesticides to apply them in combination; (3) whether they believed this tank-mixing influenced pest control efficacy. We also consulted the most recent pesticide usage survey for edible protect crops (Ridley et al., 2021).

3.2. Laboratory trials of the effects of tank-mixing on biopesticide efficacy

3.2.1. Insects and plants for experiments

Trialeurodes vaporariorum originated from Bioline AgroSciences and had been maintained as a large colony (at least 4000 adults) at Stirling University for approximately six months prior to these experiments. *Tuta absoluta* were supplied as pupae from Andermatt Biocontrol AG. During experiments *T. vaporariorum* were reared on organic black beauty aubergine plants and *T. absoluta* on organic roma tomato plants (seed from Tamar Organics). These host plants were chosen due to the high proportion of foliage and high oviposition for both pest species. Plants were grown in plant-growth rooms or chambers at 24˚C, 70% RH, with a 16:8hr light-dark cycle. Seeds were sown in a mixed peat sowing compost, with seedlings re-potted into fertiliser enriched compost. All plants were fertilised fortnightly. Plants were between 10-12 weeks from sowing when used in the experiment,

by which time aubergine plants had at least four large leaves and tomato plants had reached a minimum height of 30cm. All *T. vaporariorum* culturing and experiments took place at 26˚C, 70% RH, 16:8hr L:D; the same conditions were used for *T. absoluta* except that the temperature was 24˚C.

3.2.2. Preparation of insect-infested plants

To infest plants with juveniles of the two pest species, adults were transferred to mesh-caged plants in controlled environment plant growth chambers. *Trialeurodes vaporariorum* adults were collected from our laboratory colony using a mechanical pooter; approximately 75-100 were put into each individual plant cage with one aubergine plant. Adult *T. vaporariorum* were left for four days to lay eggs before being removed from the cages; plants were then left for a further 10 days before treatments were applied, by which time most nymphs had reached second instar. We received *T. absoluta* from Andermatt Biocontrol AG as pupae, and these were housed in a climate-controlled room until emergence. Adults were kept for up to 24 hours whilst being fed 40% sucrose solution before transfer to tomato plants. We introduced 25-30 adult *T. absoluta* into mesh cages containing nine tomato plants; they were given three days to mate and lay eggs before being removed, then plants were left for four more days for larvae to develop to L1.

3.2.3. Biopesticide application

Trialeurodes vaporariorum L2 nymphs and *Tuta absoluta* L1 larvae were exposed to biopesticide treatments whilst on plants. Four biopesticides were used: Botanigard® WP (*Beauveria bassiana*, strain GHA; Certis), Mycotal® (*Lecanicillium muscarium* strain Ve6; Koppert), Dipel® DF (*Bacillus thuringiensis var kurstaki*; Valent Biosciences), Tutavir® (Phthorimaea operculella granulovirus; Andermatt). These biopesticides were applied as both single and paired treatments in all combinations, resulting in a total of 11 treatments (Distilled water control, 4 single applications, and 6 mixed applications). Biopesticides were prepared in distilled water at manufacturers' recommended concentrations (Botanigard: 6.25g/litre; Mycotal: 1g/litre; Dipel: 2.5g/litre; Tutavir 1ml/litre). For combined treatments, both products were at their individual recommended concentrations. Plants were placed in a fume-hood, then treatment formulations were sprayed onto plants using a Sparmax DH-125 airbrush sprayer attached to a compressor at 50psi. The airbrush was cleaned before and after each treatment, spraying through first with ethanol, then distilled water, and then flushing through with biopesticide formulation before spraying commenced. Leaves were sprayed individually to runoff, following manufactures' guidelines. Once sprayed, the plants were placed in individual plant cages to avoid insect contamination, then cages were placed back into the controlled environment chambers. For *T. vaporariorum*, the trials were set up over five experimental blocks, whereas for *T. absoluta* there were two blocks. The minimum number of treatments per block was four, and all blocks contained a Botanigard treatment to assist statistical detection of block effects.

3.2.4. Data collection and analysis

Pest mortality and development were recorded by systematically searching plants for live and dead insects ten days after biopesticide application for *T. vaporariorum*, and seven days after application for *T. absoluta*. Data were analysed in R version 4.1.3 (R Core Team, 2022). Mortality variation between treatments was assessed using generalised models with a two-vector response (number dead, number alive) and a quasibinomial error distribution (to account for overdispersion). Plant was the replicate in all analyses. Initial models contained two categorical fixed factors, treatment and block, alongside their interaction. Significance of model terms was determined on deletion using likelihood ratio tests. Differences between treatment categories were determined from the significance of individual parameter estimates.

3.3. Meta-analysis: effects of parasite coinfection on virulence in arthropods

3.3.1. Inclusion criteria

The criteria for inclusion in our meta-analysis were as follows: (1) host species is an arthropod; (2) parasite species are organisms which cause mortality and fitness loss in their host, including microbes, parasitoids, and nematodes; (3) coinfection mortality is reported, i.e. two pathogens, including strains of the same species, combined either simultaneously or sequentially; (4) mortality is reported for at least one of the single pathogens; (5) mortality is reported (survival or mortality) as a proportion or raw numbers; (6) precise sample sizes are reported; (7) study is in the English language; (8) study is in the peer-reviewed literature. We therefore excluded theoretical models, studies reporting only LD50 values rather than mortality, field studies assessing host abundances, studies involving transgenic organisms such as *B.t.* crops, or studies involving predators of the host. We also excluded papers with missing key data (e.g. sample sizes or mortality), as time constraints prevented us from contacting the authors.

3.3.2. Literature search

We used Web of Science to search the published literature, from any time until the present, using the databases ‘Core Collection’, ‘Biosis Citation Index’, ‘Current Contents Index’, ‘Data Citation Index’, ‘Medline’, ‘SciELO Citation Index’, and ‘Zoological Record’. We used a combined “topic” search (which includes title, abstract, author keywords and Keywords Plus) of two term lists (1) and (2) (see Appendix 1. For these search term lists we selected the names of potential parasites (microbes, biopesticides and other biocontrol agents) and arthropods (species and other groups) based on the relevant literature and discussions with colleagues. In cases where pathogens were searched by species (e.g. *Bacillus thuringiensis*), the genus name was not used alone because of the large number of irrelevant results returned in trial searches. This search was conducted on 18 January 2022, returning 5,457 records, of which 73 duplicates were excluded (Fig 2).

3.3.3. Screening process

Screening of titles and abstracts was performed within Endnote X9.2 (Fig 2). We screened the titles of 5384 remaining papers and excluded 4115 records which were clearly irrelevant or were not primary literature, leaving 1269 remaining. To assess the validity of this process, two screeners independently reviewed a randomly selected set of 100 titles, for which there was 96% agreement. Abstracts of the remaining records were screened, and 564 were excluded as they did not meet the inclusion criteria. Two screeners independently reviewed a random set of 50 abstracts, for which there was 92% agreement. The remaining 705 full texts were then screened, 567 of which were excluded: 51 of them were unavailable, and 34 were not in English (see Figure 2 for all reasons for exclusion). A small subset (35) of the papers were not used due to time constraints. For the final meta-analysis, data was extracted from 138 papers, yielding 1138 effect sizes.

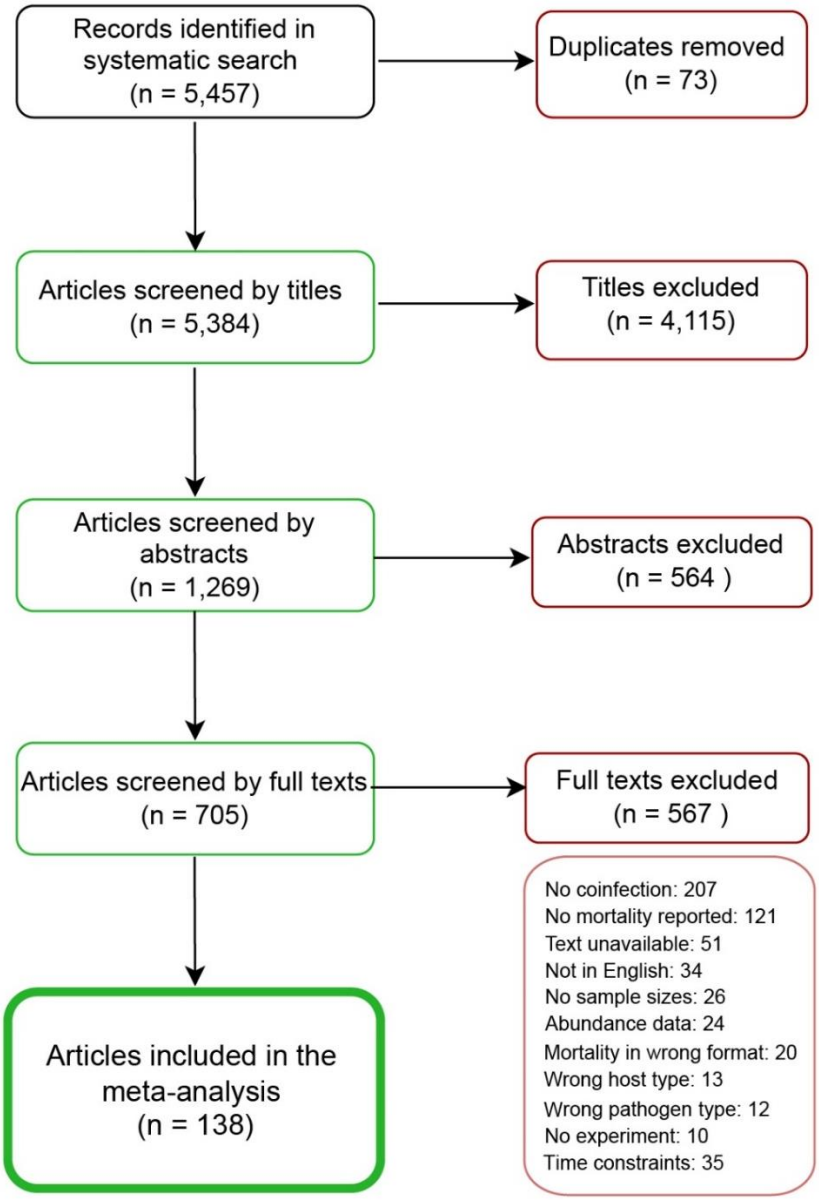


Figure 2. Flowchart for screening of literature.

3.3.4. Data extraction and calculation of effect sizes

To assess the extent to which coinfection alters mortality risk we calculated risk ratios between death rates in the combined and single treatments. We extracted mortality data and sample sizes for the single treatment of each pathogen, and their combined treatment, resulting in three mortality proportions for each effect size. Data were extracted from the main text of each paper, either from tables or from figures, using WebPlotDigitiser 4.5. In cases in which mortality was presented at multiple timepoints (e.g. survival curves), we chose the timepoint at which the more virulent pathogen treatment reached 50% mortality, or the endpoint time given if this threshold was never reached. For each study, we also collected data for use as moderator variables (potential explanations of variation in coinfection effects), including the host and parasite species, the dose used in the combination relative to the single treatment, and the timeframe of treatment combination (whether infections were simultaneous or sequential).

We calculated two types of risk ratio for use in the meta-analysis. The first risk ratio measures whether the combination of two pathogens causes equal mortality to the more virulent pathogen alone: Combined mortality / More virulent single mortality. A risk ratio of 1 indicates no difference between them, and a risk ratio of 2 indicates a doubling of virulence by the combination treatment. Values below 1 suggest an antagonistic effect. The second risk ratio measures whether the observed combination mortality is equal to the expected mortality based on the two pathogens. We calculated expected mortality following using the following formula: Parasite 1 mortality + Parasite 2 mortality x (1 - Parasite 1 mortality) (McVay et al., 1977). A value of 1 indicates an additive effect of the two pathogens, whilst values over 1 suggest synergism.

3.3.5. Statistical analysis

Statistical analysis was performed in R version 4.0.5 (R Core Team, 2022) using the package metafor (Viechtbauer, 2010), and all analyses were performed separately on each of the two types of risk ratio (effect size). To estimate the overall summary effects, we fitted models using *rma.mv*, with a restricted maximum likelihood estimator (REML) and a random effects structure of effect size nested within paper, to account for non-independence of effect sizes across studies. We assessed these models for publication bias using both the visual inspection of funnel plots and Egger's regression test (Egger et al., 1997). We also fitted these models with fixed effects (moderators) of the following variables: host type, parasite relatedness, infection timeframe (simultaneous vs sequential), dose, mortality caused by the more virulent parasite, and mortality asymmetry between parasites. An interaction term was included between the latter two moderators. Parasite relatedness was defined as one of three possibilities: two strains of the same species, two species of the same broad type (e.g. bacteria, fungi, nematodes, viruses), or two parasites of different types.

To test whether pathogens vary in their combined effect on mortality, we fitted separate models for studies involving each of the five most commonly used pathogens: bacteria, fungi, nematodes, parasitoids, and viruses. These were fitted as above, but with a random effect of paper

identity, rather than a nested effect structure. Fixed effects models were also fitted as above for each subgroup.

All models were tested for between-effect and between-paper heterogeneity using *var.comp* to calculate the I^2 heterogeneity statistic from the rma model.

4. Results

4.1. Survey of biopesticide tank-mixing amongst UK horticultural growers

Protected horticulture represents a significant share of total biopesticide use in the UK, in part due to successful IPM regimes and extensive use of beneficial insects in glasshouses (predators, parasitoids and pollinators). The pesticide usage survey (Ridley et al., 2021) provides a general overview of pest control activity in the sector. For tomato crops, biopesticide agents containing *Bacillus thuringiensis var. kurstaki* are the most commonly employed, whereas for cucumber *Lecanicillium muscarium* strain Ve6 is dominant. Products based on *Beauveria bassiana* are also used (strains GHA and ATCC-74040). Not all growers who we contacted were able to tell us about their biopesticide (or other pesticide) application practices. The majority of respondents who reported biopesticide use (or other biorational products) did not tank-mix the products. However, of those that did employ tank mixing, two reported that they occasionally or regularly tank-mixed of biopesticides targeting insects alongside other agents; these were both physical control products (e.g. Majestic) and anti-fungal agents. We did not receive any reports of growers currently tank-mixing pairs of biopesticides based on pathogens that both targeted insect pests.

4.2. Laboratory trials of the effects of tank-mixing on biopesticide efficacy

We tested the impact of simulated tank-mixing on the effectiveness of biopesticides targeting two leading pests of UK glasshouse horticulture: whitefly (*Trialeurodes vaporariorum*) and tomato leafminer (*Tuta absoluta*). For each pest, we assessed mortality caused by single and all pairwise combined treatments of four biopesticides, two of which target *T. vaporariorum* (Botanigard and Mycotal) and two of which target *T. absoluta* (Dipel and Tutavir).

4.2.1. Effects of biopesticide tank-mixing on *T. vaporariorum control*

For *T. vaporariorum* we assessed survival of 18,329 nymphs on 90 independent aubergine plants. Mean number of plants per treatment was 8.2 (range 5 – 9); mean number of nymphs per plant was 203.7 (± 12.4 SE). Across all treatments containing biopesticides that target *T. vaporariorum* (Botanigard and Mycotal single treatments, or their combinations with other products) average mortality of nymphs was 51.1% (n = 58 plants; Fig 3). Average nymph mortality in the control treatment was low (5.4%; 95%CI 1.6%—16.5%); neither Dipel nor Tutavir elevated mortality

significantly relative to water-sprayed controls (t = 1.60, P = 0.12; t = 0.15, p=0.88, respectively Fig 3F). The experiment was conducted over five experimental blocks; mortality differed considerably between blocks ($\chi^2_{(4,75)}$ = 707.4, P < 0.001) but treatments effects did not vary significantly between blocks (block x treatment interaction: $\chi^2_{(23,75)}$ = 374.5, p=0.068).

When applied singly, there was almost identical mortality caused by Botanigard (59.3%; 95%CI 46.9—70.6%) and Mycotal (59.1%; 95%CI 45.7—71.3%; Fig 3B). The efficacy of Botanigard and Mycotal at killing *T. vaporariorum* nymphs was neither enhanced, nor impaired by co-application alongside Dipel or Tutavir; in no case was the mortality in these combined treatments different from single application (Fig 3A, C, D, E). Whilst Botanigard and Mycotal both caused substantial *T. vaporariorum* mortality when applied singly, when these two products were combined, mortality elevation was only slight and not significant (+4.5%; t = 0.68, p=0.50; Fig 3B).

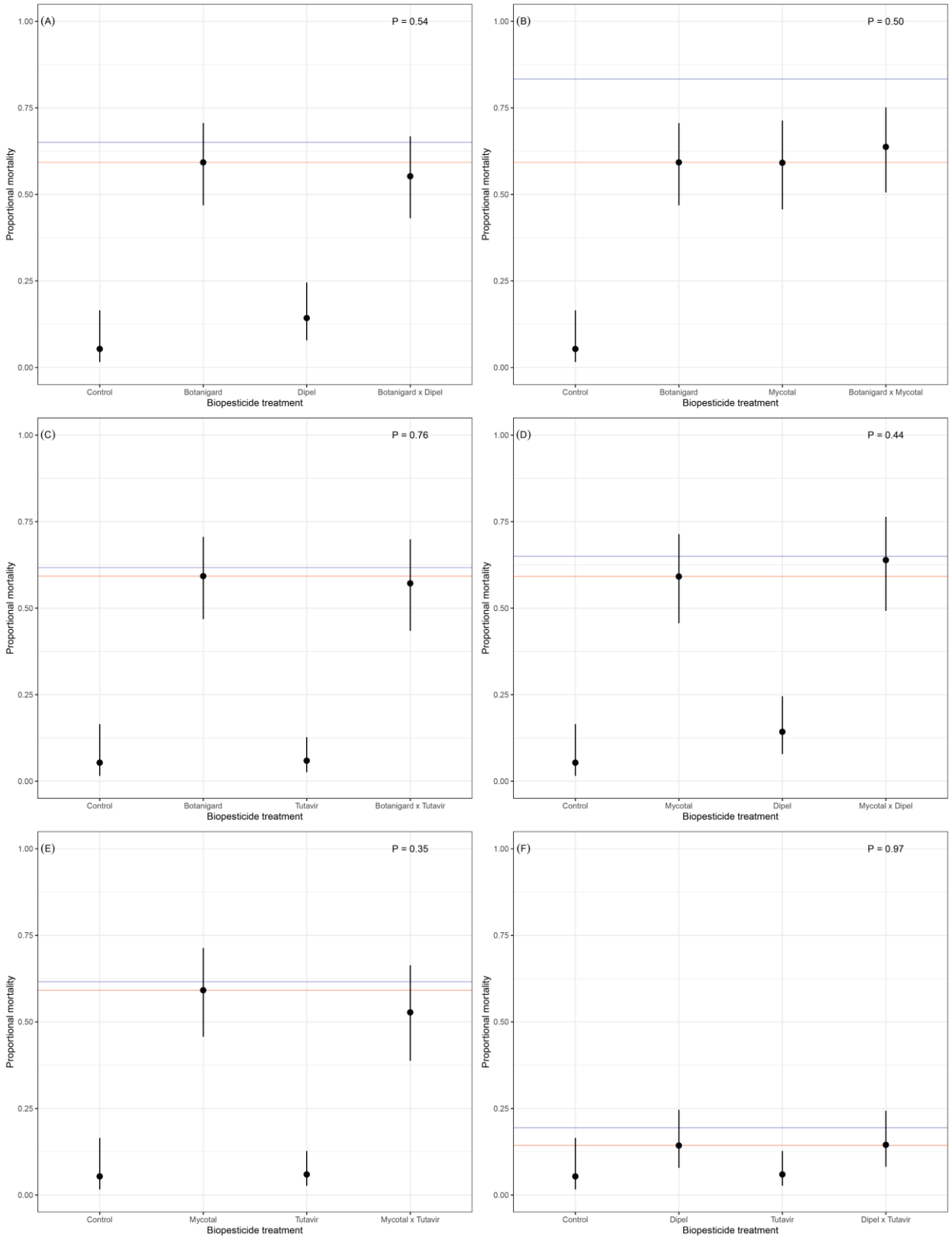


Figure 3. Mortality of *T. vaporariorum* nymphs on aubergine plants 10 days after experimental application of different biopesticide treatments. Each sub-plot shows mortality associated with single application of two different biopesticides, alongside the combination treatment. The red line indicates mortality caused by the most effective biopesticide in the pair, whereas the blue line indicates the mortality rate that would be predicted if the two biopesticides had an additive effect in combination (McVay et al., 1977). The P values for the comparison between mortality in the combined treatment with the more virulent of the two single treatments are shown on each plot: in no cases was mortality significantly affected by biopesticide mixing. Points show model predictions of mean mortality in each treatment group with 95% confidence intervals.

4.2.2. Effects of biopesticide tank-mixing on *T. absoluta control*

We assessed survival of 1441 *T. absoluta* larvae on 35 separate tomato plants (mean larvae per plant 41.2 ± 2.2 SE). Across 11 experimental treatments, average plants per treatment was 3.2 (± 0.1 SE). Mean mortality across all treatments containing products that principally target *T. absoluta* (Dipel and Tutavir singly or in combination mixes) was 83.2% (n = 19 plants). Mortality in the control treatment was only 4.6% (95%CI 1.2%—15.6%). The experiment was conducted over two replicate blocks; mean mortality did not differ between the blocks ($\chi^2_{(1,24)}$ = 0.007, p=0.96).

In single application, Tutavir caused the highest *T. absoluta* mortality (89.1%; 95%CI 76.0—95.4%), followed by Dipel (75.2%; 95%CI 62.8—84.4%); Botanigard also caused moderate mortality (28.4%; 95%CI 19.4—39.5%) (Fig 4). Mortality in the Mycotal treatment was low (13.2%; 95%CI 0.68—24.0%) and not greater than the controls (t = 1.14, p=0.16). Application of mixed combinations of these biopesticides had little impact on their ability to kill *T. absoluta*. For Tutavir and Dipel, mixing with Botanigard or Mycotal never caused significant virulence alterations (all P values >0.35; Figs 4A, B, D & E). Despite Tutavir and Dipel both causing high mortality when applied singly, mixing these products barely changed the mortality rate (-0.1%; t = 0.02, p=0.99; Fig4C). Whilst Botanigard caused 28.4% mortality singly, this reduced to 13.1% when combined with Mycotal, although this change was not significant (t = 1.82, p=0.08; Fig 4F).

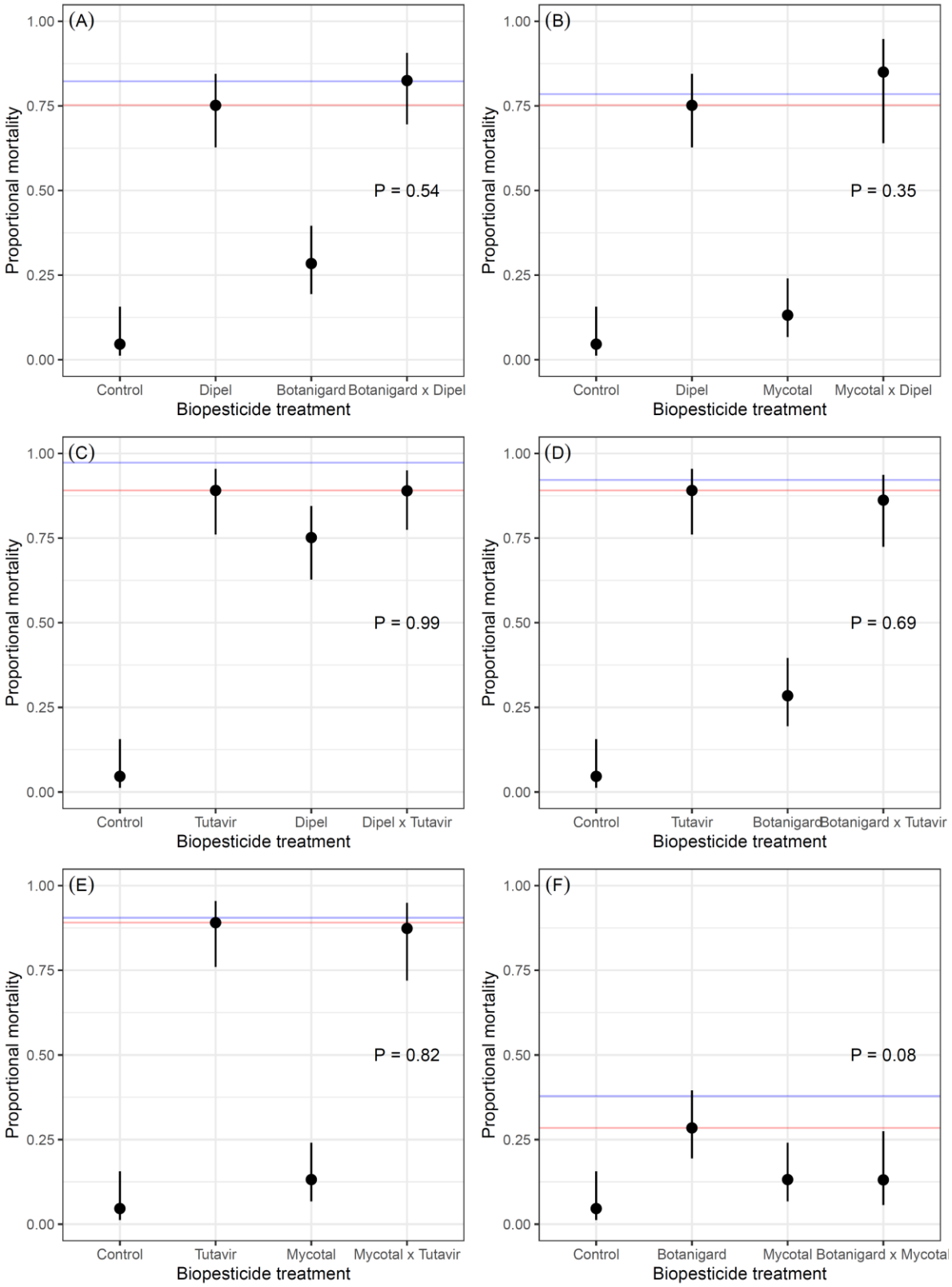


Figure 4: Mortality of *T. absoluta* larvae on tomato plants seven days after experimental application of different biopesticide treatments. Each sub-plot shows mortality associated with single application of two different biopesticides, alongside the combination treatment. The red line indicates mortality caused by the most effective biopesticide in the pair, whereas the blue line indicates the mortality rate that would be predicted if the two biopesticides had an additive effect in combination (McVay et al., 1977). The P values associated with the comparison between mortality in the combined treatment with the more virulent of the two single treatments are shown on each plot: in no cases was mortality significantly affected by biopesticide mixing. Points show model predictions of mean mortality in each treatment group with 95% confidence intervals.

4.3. Meta-analysis: effects of parasite coinfection on virulence in arthropods

4.3.1. Overview of literature used

We extracted data from 138 papers, calculating a total of 1133 effect sizes. For each effect size we calculated two risk ratios (see methods for details): risk ratio 1 quantified the change in mortality risk under coinfection compared with the most virulent parasite infecting singly; whereas for risk ratio 2 this comparison was relative to the predicted additive effect of the two parasites. We refer to each of these effect sizes here as a study. 1103 of these studies were conducted on insect hosts, with the remaining 30 on crustaceans. Of the insect studies, the vast majority of hosts were Lepidoptera (59%) or Coleoptera (27%). Diptera and Hemiptera made up an equal number of studies (3%) and the remainder of studies were on hosts from Ixodida, Thysanoptera, Hymenoptera, Orthoptera, and Blattodea. Fungal parasites were used in 53% of studies, bacteria in 47%, nematodes in 31%, viruses in 29%, microsporidia in 7% and parasitoid wasps in 3%; other parasites used were mites, ooymycetes, Plasmodium, protozoans, tachinid parasitoids, and trypanosomatids (making up <2% of studies in total). These percentages add up to more than 100% because many studies used more than one type of parasite. The most common parasite combinations were Bacterium-Virus (23%), Fungus-Nematode (19%), Fungus-Fungus (16%), and Bacterium-Fungus (14%). See Figure 5 for all parasite combinations and their effect size estimates.

Most combination treatments (73%) were performed by exposing the host to both parasites simultaneously as opposed to sequentially. The dosage of the combination treatment was also equal to that in the single treatments in most cases (84%). Almost all studies (97%) involved bacteria, fungi, nematodes and/or viruses, of which 18% did not report the origin of either parasite used in their treatments. For those studies which reported parasite information, 33% (295) used a commercial product for at least one of the parasites, and 2% (18) used two commercial products in combination.

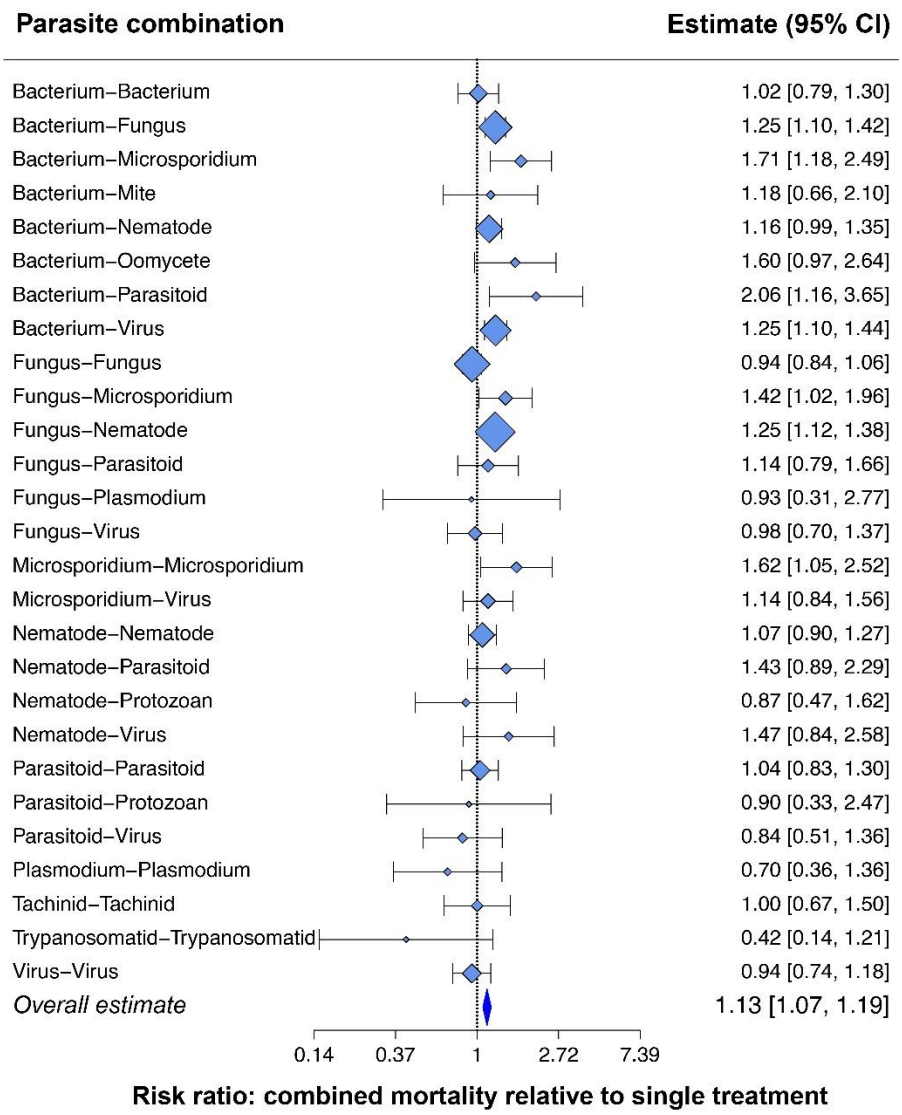


Figure 5. Average risk ratio effect sizes for coinfection involving different pairings of parasitic agents. The risk ratio (RR1) reports the change in mortality under coinfection relative to the mortality rate for the most virulent parasite when infecting singly. A risk ratio of 1 demonstrates that coinfection does not alter the mortality rate compared to the most effective pathogen. On average, coinfection slightly elevated mortality risk. The sample size is 1133 effect sizes from 138 published papers. See Appendix 2 for risk ratio 2, where coinfection effects are assessed relative to a predicted additive effect of the two pathogens.

4.3.2. Summary estimates of overall effects

We found a significant effect of coinfection on host mortality compared to that caused by the more virulent parasite (Risk ratio 1: combined mortality/more virulent pathogen mortality). Overall, adding a second parasite increases mortality by 13% (estimate = 1.13, 95% CI = 1.07 – 1.19, p<0.0001, n: 1133; Fig 5). We found no evidence of synergism, as would be seen if there was an increase in mortality beyond expectations based on additive effects of the two parasites (Risk ratio 2: combined mortality/expected mortality). On the contrary, adding a second parasite caused slightly less than expected for additive mortality (estimate = 0.95, 95% CI = 0.91-0.99, p = 0.03, n=1133 (See Appendix 2). Both models show a high degree of heterogeneity within the data (I^2 for RR1: 91.7%; I^2 for RR2:

94%), which suggests that the variation among studies cannot simply be explained by sampling error, but rather reflects other differences among studies and systems that are not explained in our model. We found no evidence of publication bias within the dataset (Egger's test = 0.06, p>0.05, see Appendix 3 for funnel plot).

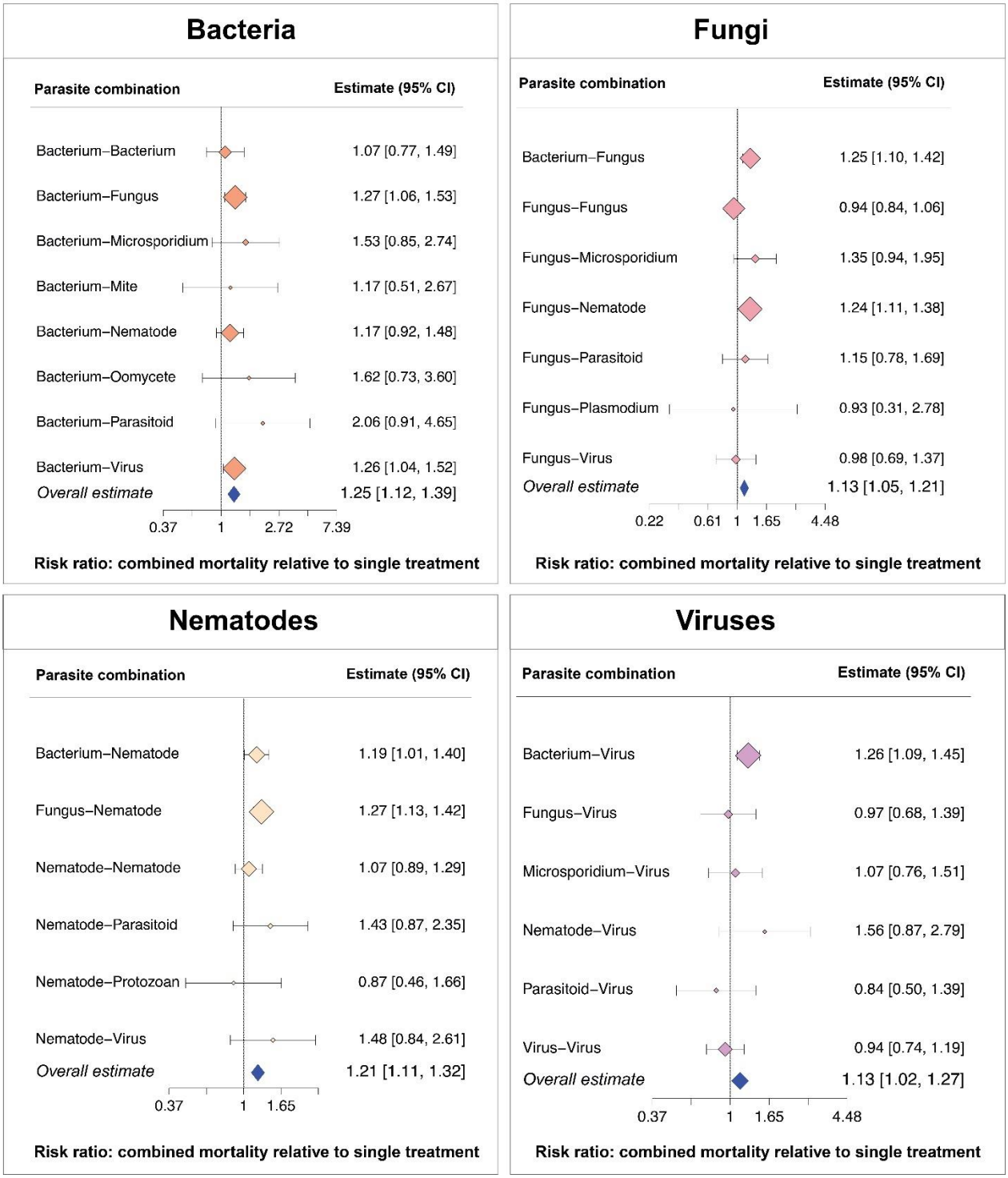


Figure 6. Average risk ratio effect sizes for coinfections involving the four types of organism most commonly used in biopesticides. The risk ratio (RR1) reports the change in mortality under coinfection relative to the mortality rate for the most virulent parasite when infecting singly. A risk ratio of 1 demonstrates that coinfection does not alter the mortality rate. Coinfection elevated mortality risk more strongly for parasite combinations involving either bacteria or viruses more than it did combinations involving for fungi or nematodes.

4.3.3. Effects of moderators

When the fixed effect models were fitted with all moderators available, we found no effects of host type, dosage (whether coinfection involved half the dose of each individual agent) or timeframe (simultaneous vs sequential) on either risk ratio. These moderators were therefore removed from the final model. We found a significant negative effect of “pathogen virulence” (i.e. mortality caused by the more virulent parasite) on combined mortality in the model for risk ratio 1 (RR1 estimate = 0.59, 95% CI = 0.53-0.67, p<0.0001, n = 1133), meaning that as the virulence of the more virulent parasite increases, the extent that adding a second parasite elevates virulence becomes smaller. However, there was no such effect in the model for risk ratio 2. In both risk ratio models, we found a significant negative effect of parasite virulence asymmetry on combined mortality (RR1 estimate = 0.08, 95% CI = 0.05-0.12, p<0.0001; RR2 estimate = 0.31, 95% CI = 0.21-0.47, p<0.0001, n=1133). As virulence asymmetry increases (i.e. the second parasite has low relative virulence), the effect of adding the second parasite decreases. We also found a significant positive interaction between parasite virulence and asymmetry in both models (RR1 estimate = 12.36, 95% CI = 8.08-18.91, p<0.0001, n=1133; RR2 estimate = 3, 95% CI = 1.96-4.58). This interaction suggests that when the first parasite is highly virulent, adding another highly virulent parasite (low asymmetry) increases mortality by a small amount; whilst adding a low virulence parasite is ineffective. When the first parasite is of low virulence, mortality can be increased more effectively by adding a parasite of similar virulence (low asymmetry), rather than a parasite of very low virulence. Heterogeneity (I^2) was 88.3% for the RR1 model, and 63.3% for the RR2 model.

We found a significant effect of the taxonomic distance between the two parasites on combined mortality in both models (whether the coinfecting parasites were strains of the same species, species of the same parasite type, or a different parasite type) (Fig 7). When parasite virulence and asymmetry are unaccounted for, combining two strains of the same species results in a non-significant decrease in overall virulence relative the more virulent parasite (RR1 estimate = 0.92, 95% CI= 0.78-1.08, p=0.31, n=1133) and a significant decrease in virulence relative to expected additive mortality (RR2 estimate = 0.8, 95% CI = 0.68-0.93, p<0.01, n=1133). Combining two species of the same type similarly does not increase mortality beyond the more virulent of the pair (RR1 estimate = 1.01, 95% CI = 0.94-1.08, p=0.83, n=1133) and produces less mortality than predicted under an additive interaction (RR2 estimate = 0.89, 95% CI = 0.83-0.95, p<0.001, n=1133). By contrast, combining two different types of parasite increases virulence (estimate = 1.23, 95% CI = 1.16-1.31, p<0.0001, n=1133) and produces mortality as expected for an additive interaction (RR2 estimate = 1.01, 95% CI = 0.96-1.06, p=0.74, n=1133). See figure 7 for all estimates of the effect of phylogenetic distance. When parasite virulence and asymmetry are accounted for, there remain significant differences between types. The effect of combining different types of parasite produces significantly greater mortality than when different strains of the same parasite species coinfect (RR1 estimate = 1.32, 95% CI = 1.12-1.58, p<0.001, n=1133; RR2 estimate = 1.28, 95% CI = 1.08-1.5, p<0.01, n=1133).

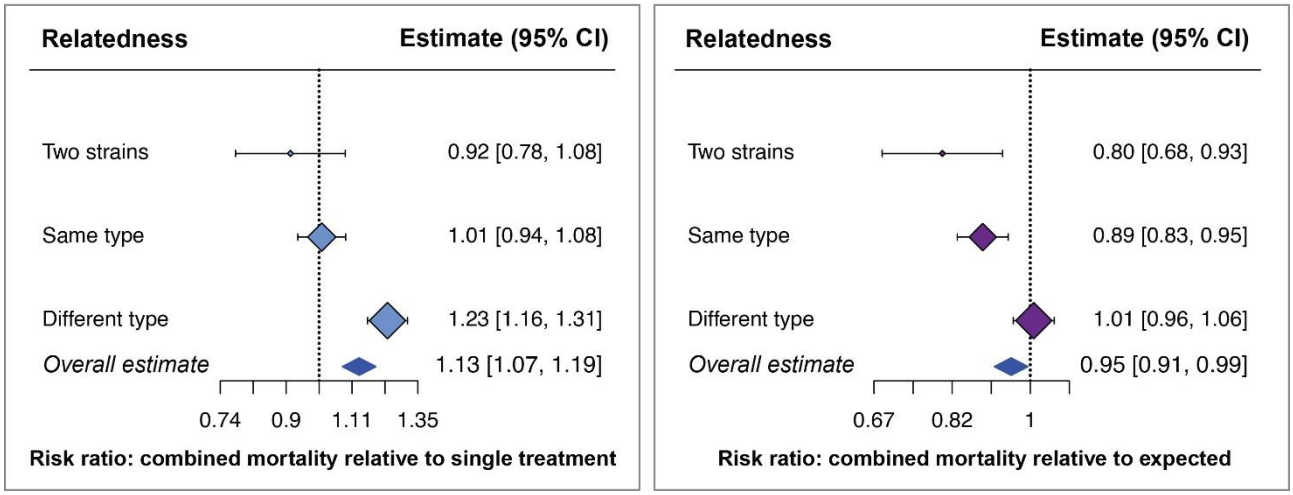


Figure 7. Average risk ratio effect sizes for coinfections involving parasites that differ in their taxonomic distance: either two strains of the same species, two species of the same type (e.g. two species of fungus) or two different types of parasite (e.g. fungus and bacterium). The left plot reports the risk ratio as the change in mortality under coinfection relative to the mortality rate for the most virulent parasite when infecting singly (RR1). The right plot reports the risk ratio as the change in mortality under coinfection relative to the predicted mortality based on an additive effect of the two parasites. A risk ratio of 1 demonstrates that coinfection does not alter the mortality rate. On average, coinfection only elevated mortality risk strongly for parasite combinations involving different types of parasites. Combining different strains of the same species marginally (but not significantly) impaired mortality relative to the more virulent parasite in single infections.

The impact of coinfection was not affected by whether studies used commercial products or other naturally occurring parasites (no effect in either model when they were fitted for all data which included bacteria, fungi, nematodes and viruses, and for which parasite provenance was reported). When the four parasite groups (bacteria, fungi, nematodes and viruses) were analysed separately, we found similar effects of parasite virulence and asymmetry to those found in the full dataset. The effect of taxonomic distance varies between types of parasite: there is no effect of taxonomic distance between the coinfecting pair both for bacteria and nematodes for RR1. However, coinfection-induced changes in virulence do vary dependent on whether different types or strains are combined in coinfections for fungi (RR1 estimate = 1.28, 95% CI = 1.05-1.56, $p<0.05$, n=604) and viruses (RR1 estimate = 7.77, 95% CI = 1.8-33.1, $p<0.01$, n=324). Using RR2, none of the subgroups showed significant effects of phylogenetic distance.

5. Discussion

This project has generated two unique data sets that provide an evidence-base for decisions on applying combinations of biopesticides in agriculture. This project aimed to determine whether farmers and growers are likely to be able to make efficiency savings by tank-mixing biopesticides without compromising their pest control efficacy. Although we have undertaken no field trails in this brief project, our results allow us to predict that tank-mixing, or other forms of co-application, will generally have small impacts, if any, on biopesticide efficacy and that strong antagonistic interactions are unlikely to be common.

There are reports in published research literature of particular pathogens and parasites where combined infection causes both strong antagonistic or synergistic effects on target host mortality (Mideo and Reece, 2012, Hodgson et al., 2004, Choisy and deRoode, 2010). It remains possible that some microbial biopesticides and other biocontrol agents may be similarly affected. However, our laboratory study of four commercial biopesticides targeting two crop pests found no evidence of strong interactions between these products. Furthermore, our meta-analysis of over 1100 published results of parasite/pathogen infection combinations from 138 independent studies suggests that most coinfection effects are small and on average slightly positive relative to the mortality caused following single infection. Similarly, our survey of UK horticultural growers, although small in extent, uncovered no reports from growers that biopesticides act antagonistically following tank-mixing.

Our study of tank-mixing effects on biopesticide efficacy in whitefly control used two fungal biopesticides targeting *T. vaporariorum*: Botanigard (*Beauveria*) and Mycotal (*Lecanicillium*). The efficacy of these products did not change either when they were mixed with each other, or when they were mixed with biopesticides targeting other pests (Tutavir and Dipel). It is likely that *T. vaporariorum* nymphs were unaffected by Tutavir and Dipel: both attack through the gut following consumption of contaminated foliage, so immobile nymphs may not have been infected. Furthermore, the granulosis virus in Tutavir is quite strongly specific to lepidopteran species closely related to its native host *Phthorimaea operculella* (Ben Tiba et al., 2019). However, both Botanigard and Mycotal killed approximately 50% of nymphs when applied singly, therefore it is interesting that combined treatment containing both these agents at their individually recommended concentrations provided no substantial improvement in insect control.

A more powerful test of how tank-mixing affects efficacy comes from our work on *T. absoluta*: in this case there is the potential for all four biopesticide products to have infected larvae, either by ingestion (Tutavir and Dipel) or by cuticle penetration (Botanigard and Mycotal), and because three of our biopesticides (Tutavir, Dipel and Botanigard) caused significant mortality when applied singly.

Nevertheless, pairwise co-application of these products never caused a significant increase or decrease in *T. absoluta* mortality relative to the most effective product on its own.

Whilst field trials would be necessary to verify whether these results are valid for commercial settings, our data suggest that horticultural growers attempting to simultaneously control *T. vaporariorum* and *T. absoluta* may be able to apply biopesticides targeting each of these pests as a tank-mix without any efficacy-loss. Furthermore, our data suggest that simultaneous co-application of two products that target the same pest would be unlikely to provide an appreciable benefit compared to applying just one.

This project aimed to make general predictions as to the likely consequences of tank-mixing biopesticides in addition to the specific tests of horticultural biopesticides that we have undertaken. We did this by carrying out an extensive literature search of studies that have investigated the impact of simultaneous coinfection or sequential infection by parasites in arthropods. After screening over 5000 published papers, we identified 138 studies predominantly focussing on insects (but also some crustaceans) and which investigated a mix of biopesticides, biological control agents and natural host-parasite associations. This data set produced over 1100 comparisons of the effects of coinfection compared to single infection. On average, mortality in coinfection treatments was 13% greater than for a single infection with the parasite that caused the highest individual mortality. Nevertheless, this level of enhanced mortality was slightly (and significantly) less than would be predicted for an additive effect where the two parasites kill overlapping subsets of the target population at random.

Virulence trends under coinfection differed depending on the type of parasite involved. Focussing on organisms commonly used as biopesticides (bacteria, fungi, nematodes and viruses), coinfections involving bacteria and nematodes tended to have stronger positive effects on virulence than did those involving fungi and viruses (although none exceeded an additive effect). Ecological niche theories predict that the ability of parasites to coexist in a single host is influenced by the extent to which their niches within their host's body overlap (Rynkiewicz et al., 2015); such overlap is likely to be stronger for parasites that are more similar to one another. Furthermore, host immune responses may overlap more strongly between more closely related parasites, meaning they are more likely to suffer indirect effects during coinfection from host immune activation (Fenton and Perkins, 2010, Venter et al., 2022). Our data support these predictions, a finding that has important applied consequences. Across our whole data set, coinfections involving two strains of the same parasite species tended to produce lower virulence than was the case for single infections with the more virulent strain. Coinfections involving different species of the same type of parasite (such as two fungi, or two viruses) tended to cause mortality at the same rate as the most virulent parasite. However, coinfections involving different types of parasite (for example a bacterium and a fungus)

tended to have higher virulence: combined virulence that was approximately additive relative to the single treatments. Therefore, if tank-mixing of biopesticides is to be adopted in agriculture, then mixing products containing different types of pathogen/parasite is likely to result in better pest control outcomes than mixing different products containing closely related pathogen strains.

We also assessed the impact of the degree of difference in virulence between two parasites on how virulence changed under coinfection. Mixing two highly virulent parasites together in a combined infection tended to increase mortality the most; whereas mortality increases became smaller if the degree of virulence difference was high. Therefore, in general, we predict that combining a poorly performing biopesticide alongside an effective product is unlikely to improve combined performance. Also, from the perspective of biopesticide product design, mixing low virulence strains of pathogen alongside a more virulent strains is unlikely to increase product efficacy.

Our survey of horticultural growers in the UK did not record any growers who reported that they tank-mixed pairs of microbial biopesticides that both targeted insect pests. The only records of microbial biopesticide tank-mixing we received involved mixes with physical control agents or anti-fungal products. Our research work suggests that future horticultural trials to assess the potential for tank-mixing of microbial biopesticides would be worthwhile and could benefit growers.

In summary, our project has found laboratory evidence that the performance of four horticultural biopesticides in driving pest mortality is not impaired by tank-mixing. An extensive literature survey and meta-analysis has shown that strong antagonism (and synergy) between arthropod parasites (ranging from microbes through to nematodes and parasitoid wasps) is rare, and that on average coinfection results in almost additive effects. We caution that our experimental work all derives from the laboratory and may not reflect the effects of tank-mixing in ‘real-world’ use; therefore, field testing would be required to verify that our results also apply in agricultural settings. We suggest that those designing IPM programmes should consider recommendations for tank-mixing, and that biopesticide producers should undertake research to validate the use of their products in tank-mixes for ease of grower use. This project was conceived with a forward-looking agenda, considering the future challenges of using biopesticides extensively in open-field agriculture. The evidence base we have generated suggests that future field trials of biopesticides to control multiple pests in arable crops should consider including tank-mixed treatments to make application regimes more efficient for famers. Tank-mixing application efficiencies may remove barriers to uptake of biopesticides that might otherwise hinder their adoption and prevent their ecological benefits being fully exploited.

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8. Appendices

8.1. Appendix 1: Meta-analysis search terms

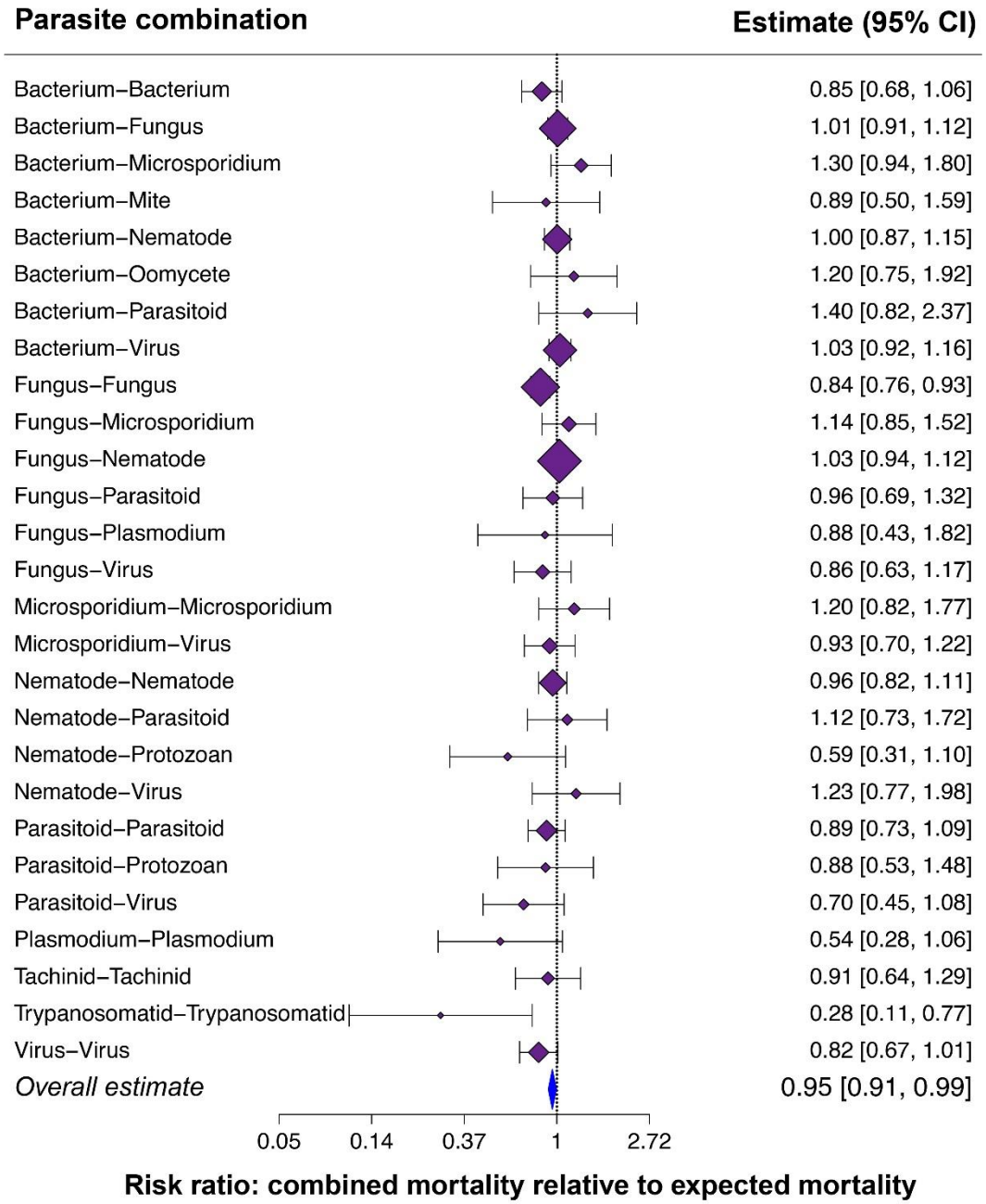
Search terms used for literature search in the meta-analysis. We used two search term lists. Search list 1 identified literature based on taxonomic group of the target arthropod in association with key words associated with coinfection. Search list 2 identified literature based on key words associated with biopesticides and other biocontrol agents alongside terms describing coinfection or tank-mixing.

Search list 1
((arthropod OR crustacea* OR chelicerat* OR hexapod* OR myriapod* OR arachnid* OR eurypterid* OR merostomat* OR pycnogonid* OR chilopod* OR diplopod* OR pauropod* OR symphyl* OR branchiopod* OR cephalocarid* OR multicrustacea* OR oligostraca* OR remiped* OR copepod* OR insect* OR collembola OR proturan* OR dipluran* OR archaeognatha* OR zygentoma* OR ephemoptera* OR odonat* OR phasmid* OR plecopteran* OR dermaptera* OR dictyopteran* OR embiopteran* OR grylloblattaria* OR mantophasmatod* OR orthoptera* OR zoraptera* OR hemiptera* OR phthirapteran* OR psocoptera* OR psocod* OR thysanoptera* OR coleoptera* OR diptera* OR hymenoptera* OR lepidoptera* OR mecoptera* OR megaloptera* OR neuropteran OR raphidioptera* OR Siphonaptera* OR strepsiptera* OR trichoptera* OR isoptera* OR blattod* OR mantod* OR heteroptera* OR sternorryncha* OR auchenorryncha* OR symphyt* OR apocrit* OR parasitica* OR aculeat* OR drosophila OR daphnia OR tribolium OR bee OR wasp OR ant OR sawfly OR moth OR butterfly OR mosquito OR fly OR flies OR blackfly OR borer OR spider OR mite OR beetle OR tick OR bug OR flea OR louse OR locust OR grasshopper OR cricket OR millipede OR centipede OR cockroach OR thrips OR aphid OR leafhopper OR termite OR whitefly OR "scale insect" OR coccid OR mealybug OR weevil OR lacewing OR springtail OR caterpillar OR maggot OR silkworm) AND (virulence OR mortality OR survival OR kill* OR death) AND (co-infection OR coinfection OR dual-infection OR "dual infection" OR mixed-infection OR “mixed infection” OR "multipl* infect*" OR "within-host competition" OR "intrahost competition" OR "pathogen competit*" OR "competitive interactions" OR "within-host interaction*" OR "intrahost interaction*" OR "parasite-parasite" OR "pathogen-pathogen" OR "parasitoid-parasitoid" OR "simultaneous* infect*" OR "sequential infection" OR “super-infection” OR superinfection))
Search list 2
((parasitoid OR ectoparasitoid OR endoparasitoid OR mycoinsecticid* OR "bacterial insecticid*" OR "viral insecticid*" OR biopesticid* OR "biochemical pesticid*" OR microsporidi* OR "microbial insecticid*" OR entomopath* OR "insect pathogen" OR "microbial control" OR "microbial biocontrol" OR iflavirus OR baculovirus OR granulovirus OR nucleopolyhedrovirus OR "NPV" OR "GV" OR "nuclear polyhedrosis virus" OR "granulosis virus" OR "Bacillus thuringiensis" OR

"Bacillus sphaericus" OR "Clostridium bifermentans" OR "Saccharopolyspora spinosa" OR "Streptomyces avermitilis" OR " Pseudomonas alcaligenes" OR "Pseudomonas aureofaciens" OR "Serratia entomophila" OR "paenibacillus" OR Aschersonia OR Beauveria OR Metarhizium OR Lecanicillium OR Nomuraea OR Nosema OR Hirsutella OR Verticillium OR Isaria OR Paecilomyces OR Xenorhabdus OR Photorhabdus OR Steinernema OR Heterorhabditis) AND (virulence OR mortality OR survival OR kill* OR death) AND (co-infection OR coinfection OR dual-infection OR "dual infection" OR mixed-infection OR “mixed infection” OR "multipl* infect*" OR "within-host interactions" OR "within-host competition" OR "intrahost competition" OR "pathogen competit*" OR "competitive interactions" OR "parasite-parasite" OR "pathogen-pathogen" OR "parasitoid-parasitoid" OR "simultaneous* infect*" OR "sequential infection" OR super-infection OR superinfection OR "tank-mix*" OR tank-mix OR synerg* OR additive OR antagonis*))

8.2. Appendix 2: Plot of meta-analysis effect sizes relative to a predicted additive effect based on the two parasites infecting independently.

The comparison to an additive effect (risk ratio 2) was calculated following model 3 (see introduction) which uses a previously published approach (McVay et al., 1977). This plot contrasts with Figure 5 which was instead plotted by calculating effect sizes relative to the mortality caused by the more virulent pathogen when infecting singly.



8.3. Appendix 3: Funnel plot of effect sizes from meta-analysis.

Funnel plot for the full dataset for risk ratio 1 (coinfection mortality relative to the more virulent of the two parasites on its own) from a random effects model. Each datapoint is an individual study and the log risk ratio estimate for each study is shown against its log standard error. The y-axis therefore indicates study size, with smaller studies towards the bottom of the plot. Studies which fall outside the funnel can be indicative of publication bias; however only a small proportion of the 1133 effects fall outside this range. We found no significant evidence of publication bias (Egger's test = 0.06, p>0.05).