Bachelor thesis

FUNGAL AIR CONTAMINATIONS IN ENTOMOPATHOGENIC NEMATODE FORMULATIONS

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A study about the relationship between the process of formulating entomopathogenic nematodes and fungal air contaminations tested at Koppert BV

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Preface

My name is Luuk Jungerling, in the context of the completion of my fourth year of the Bachelor Applied Biology I have done this thesis on the topic of entomopathogenic nematodes.

In this preface, I would like to take the opportunity to thank all the people who made the data collection of this thesis an instructive and great experience. Starting with my two supervisors from Koppert; Felipe Cortes and Evelien Weerdenburg. By guiding me in the lab activities and with their constructive feedback, I was able to improve myself as a biologist and researcher, for which I am grateful. Apart from them, I want to thank the other colleges from the lab and production team who always tried to help me whenever I would have question or problems. Furthermore, I want to thank the other interns for offering help on busy days and conversations on slower days. Lastly, I want to thank Koppert BV for providing me with an opportunity to help finish my bachelor education.

Apart from the people at Koppert I want to thank my supervisors from Aeres. Namely my old and new mentor Herman Offereins and Eelco Peetsra for their feedback and help. As this is my last project done for Aeres I also want to thank the school, its personnel and the students for all the help and fun they have given me throughout the years.

CONTENT

SUMMARY

Fungal contaminations cause problems in different industries worldwide. One of these is the production of entomopathogenic nematodes (EPNs) which are used as biological pesticides. How these contaminations occur is often unclear. Because the production and formulation process is done partly in the open air, it is hypothesized that the contaminations are airborne. To study this possibility research was done at one of the largest biocontrol companies in the world, Koppert Biological Systems. Koppert offered an excellent testing location because they produce bacteria and fungi in the same room as the EPNs. These microorganisms affect the microbial air load, making the effect of air contaminations easier to measure. Two different nematode species were researched, the *Steinernema feltiae* and the *Steinernema carpocapsae*. To test the hypothesis this research question was designed:

Research question: *Is there a relationship between the species and the amount of microbial air load during the formulation process of entomopathogenic nematodes and the later development of fungal colonies during storage at 4°C?*

To answer the research question four sub-questions were designed and tested over time.
Sub-question 1: *How is the quality of the product affected by contaminations?*
Sub-question 2: *What is the effect of the microbial air load during formulation on invisible contamination inside the EPN formulations?*
Sub-question 3: *What is the effect of the microbial air load during formulation on the development of visible fungal colonies?*
Sub-question 4: *Which microbial species are seen most often in the EPN formulations?*

The sub-questions tested 3 EPN formulations made under different presumed microbial air loads:
1) Formulation under laminar flow hood providing filtered air devote of air contaminants
2) Formulation inside the Koppert factory when there were only nematodes being processed
3) Formulation inside the Koppert factory when Trichoderma products were being processed

Sub-question 1: The contaminations did not affect the quality of the product, regardless of the treatment or nematode species. **Sub-question 2:** No apparent effect was found between the air samples and the invisible contaminants cultured on growth medium. Only one fungi species was seen inside the EPN formulations and in the air samples. However, it was also seen in the treatment made in the laminar flow hood with the clean air so this contaminant could also be in the product before the formulations process. **Sub-question 3:** There was no clear effect found between the fungal air contaminants and the later development of visible fungi. Between the *Steinernema feltiae* and *Steinernema carpocapsae* a large difference in replicates affected by visible fungi growth was found. What causes this is still unknown, but it might be interesting to research in follow-up studies. **Sub-question 4:** The contaminations that were identified were all common and non-dangerous, where these occurred is yet to be discovered.

Research question:
No apparent relation was found between the air samples and the amount or types of contaminations in the final product for both nematode species and treatments. This was confirmed by the treatment made in the flow hood. Because the filters provided air devote of contaminants but still the replicates of this treatment had similar contamination levels as the other treatments. This also means that before the formulations process starts there are already contaminations in the EPN product.

SAMENVATTING

Schimmel contaminaties veroorzaken wereldwijd problemen in verschillende sectoren. Een van deze sectoren is de productie van insect parasitaire nematoden die worden ingezet als biologisch bestrijdingsmiddel. Hoe deze contaminaties ontstaan is vaak onduidelijk. Doordat het productieproces deels open in de fabriek wordt uitgevoerd is een hypothese dat contaminaties uit de lucht komen. Om dit te onderzoeken is dit onderzoek uitgevoerd bij een van de grootste producenten van biologische bestrijdingsmiddelen ter wereld, Koppert Biological Systems. Koppert bood een goede locatie om dit te testen omdat zij bacteriën en schimmels produceren in dezelfde ruimte als hun nematode producten. Deze micro-organismen hebben een effect op de hoeveelheid microben in de lucht waardoor het effect hiervan beter te meten is in het nematode product. **Onderzoeksvraag:** *is er een relatie tussen de soorten en het aantal micro-organismen in de lucht tijdens het formulatie proces van insect parasitaire nematoden en de latere ontwikkeling van schimmels in de nematode producten opgeslagen bij 4°C?*

Om dit te beantwoorden zijn er 4 deelvragen onderzocht gedurende een tijdsperiode van 3 maanden.
Deelvraag 1: *Wat is het effect van contaminaties op de kwaliteit van het product?*
Deelvraag 2: *Wat is het effect van micro-organismen in de lucht tijdens het formulatie proces op contaminaties die niet zichtbaar zijn met het blote oog?*
Deelvraag 3: *Wat is het effect van micro-organismen in de lucht tijdens het formulatie proces op contaminaties die zich tot zichtbare kolonies ontwikkelen in het product?*
Deelvraag 4: *Welke micro-organismen worden het meest gezien in de nematode producten?*

De deelvragen testen twee soorten nematode, de *Steinernema feltiae* en de *Steinernema carpocapsae* en de formulaties zijn gemaakt onder verschillende lucht condities:
Luchtconditie 1: Formulaties gemaakt in een laminaire stroming kap met gefilterde lucht zonder micro-organismen in de lucht
Luchtconditie 2: Formulaties gemaakt in de fabriek van Koppert wanneer alleen nematode producten werden geproduceerd
Luchtconditie 3: Formulaties gemaakt in de fabriek van Koppert wanneer Trichoderma producten werden geproduceerd

Deelvraag 1: De contaminaties hadden geen effect op de kwaliteit van het product. Er was ook geen verschil tussen de kwaliteit van verschillende nematode soorten of luchtcondities. **Deelvraag 2:** Er is geen duidelijk verschil gevonden tussen de luchtmonsters en de opgekweekte micro-organismen gevonden in het nematode product. Slechts een schimmelsoort is in de lucht en product monsters gevonden. Maar deze is ook gevonden in de producten gemaakt in de laminaire stroming kap dus deze contaminatie kan ook al voor het formulatie proces zijn opgedaan. **Deelvraag 3:** er is geen duidelijk effect gevonden tussen de contaminaties aanwezig in de lucht en de ontwikkeling van zichtbare schimmel kolonies in het product. Tussen de *Steinernema feltiae* en de Steinernema *carpocapsae* is een groot verschil gevonden in het aantal zichtbare kolonies. Wat dit veroorzaakt is nog niet duidelijk maar kan interessant zijn voor vervolgstudies. **Deelvraag 4:** De geïdentificeerde contaminaties waren allen algemeen en ongevaarlijk waar deze in het product zijn gekomen is nog niet duidelijk**.**

Research question:
Er is geen relatie gevonden tussen de micro-organismen in de lucht en de hoeveelheid en soorten micro-organismen gevonden in het eindproduct. Dit geldt voor de verschillende luchtcondities en de twee geteste nematode soorten. Dit wordt bevestigd door de nematode geformuleerd in de flow stroming kap. Doordat de filters voor schone lucht zorgen maar vergelijkbare contaminatieniveaus tonen als de andere luchtcondities. Dit betekent ook dat voor het formulatie proces begint het product al is besmet met contaminaties.

1 INTRODUCTION

1.1 BIOLOGICAL PESTICIDES

Fungal contaminations cause major problems in different industries. In the food sector, multiple species can cause loss of products (Pitt & Hocking, 2009) and health risk due to fungal emitted toxins for both humans and animals (Kabak, Dobson, & Var, 2006). Fungi create health risks in hospitals (Cole & Cook, 1998) and other working environments (Palmas & Meloni, 1998). Another industry facing problems due to fungal contaminations is that of nematode production. To combat fungal contaminations most companies producing nematodes at antifungal chemicals. However, these chemicals might be subjected to regulations increasing the difficulty to sell nematode products in certain countries. In collaboration with Koppert BV, one of the largest biological control companies in the world, this research was carried out. The aim was to gain knowledge on where the contaminations occur to improve the production process. This could lead to a decrease in the use of antifungals or even making them unnecessary solving the problem of these regulations. A by-product of this research will be identifying the commonly found contaminations so Koppert BV can better target them in their research into new antifungal products that are not subjected to regulations. This will be further discussed later in the introduction, but first the biology and production of nematodes are explained.

1.2 ENTOMOPATHOGENIC NEMATODES

Nematodes are transparent roundworms found in soil and water. The body structure is relatively simple, and they are mostly only visible under a microscope (Figure 1, Nuetzelinge, 2011). Nematodes which can infect host insects are called insect-parasitic nematodes or entomopathogenic nematodes (EPNs). The EPNs are a member of two families: Steinernematidae and Heterorhabditidae (Dilman, et al., 2012). They are associated with two symbiotic bacteria *Xenorhabdus* and *Photorhabdus*, which are pathogenic to a variety of their insect hosts (Vashisth, Chandel & Sharma, 2013). They have a symbiotic relationship with bacteria. The nematodes search and enter hosts, inside the host the bacteria get released and rapidly multiply in the host's hemolymph which can be compared to a mixture of the blood and lymphatic fluids. The host then dies because of hemolymph poisoning, usually within 24 to 48 hours after infection. The nematodes consume the bacteria and insect tissue, then multiply.

These symbiotic bacteria are species of *Xenorhabdus* and *Photorhabdus* which are gram-negative bacteria belonging to the family Enterobacteriaceae (Rougon-Cardoso et al., 2016). The bacteria are classified gram-negative due to their stronger outer layer compared to gram-positive bacteria (Takeuchi et al., 1999), this protects them against the environment posed by the insides of the nematodes and insect host. The position of the symbiotic bacteria in the EPNs are below the pharynx in an intestinal vesicle or interiors and mid-intestine (Stevens & Lewis 2017). The pharynx is the part of the throat that is behind the mouth. These positions allow the bacteria to be quickly released after entering a host. EPNs can protect crops both above and below the ground (Lacey & Georgis, 2012). Appendix 7.1 contains a list of pests that can be combated by EPNs.

Figure 1: Steinernema feltiae*, real size about 1mm, by Neutzelinge.*

1.3 INFECTION CYCLE OF NEMATODES

When nematodes are used as biopesticides, they follow their natural way of reproducing (Figure 2, Arthurs, 2018). One of the significant advantages of using EPNs is how they will multiply themselves via the pests and so effectively increase their number. It is even possible for the nematodes to permanently establish a population at the site where they are introduced, protecting the crops for a more extended period of time (Hazir, Kaya, Stock & Keskin, 2004). In situation 1 the juvenile nematode searches for a host insect to infect while living in the soil. *Steinernema carpocapsae* find a host by waiting for passing insects. The *Steinernema feltiae* on the other hand actively roam beneath the soil looking for a host (Singh & Upadhyay, 2018). In situation 2, the nematode enters the body of the host via a natural opening. It then releases the symbiotic pathogenic bacteria. In situation 3, the bacteria multiply in the hemolymph of the host and kill the insect within 48 hours. Then the development of the infective juveniles to adults takes place, making the nematodes able to produce eggs (Gaugler, 2002). The adult nematodes inside the host complete one to several generations by feeding on the bacteria and nutrients within the dying host as shown in situation 4. Only when all the host's available nutrients have been consumed does the new generation of juveniles emerge, situation 5. All are carrying the symbiotic bacteria with them which has also multiplied inside the body of the host insect (Devi, 2018). Situation 1 to 3 takes approximately 3 days, steps 4 and 5 takes another 2-3 days (Gaugler, 2002).

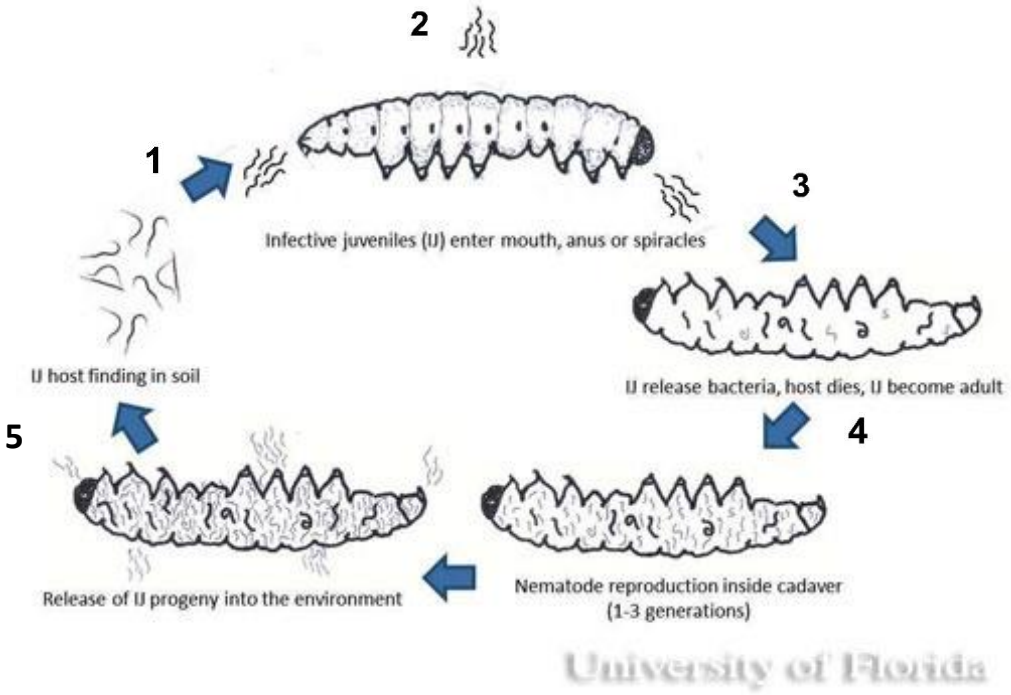

Figure 2: Development infective juveniles inside a host-insect. By Steven Arthurs

1.4 PRODUCTION OF ENTOMOPATHOGENIC NEMATODES

EPNs are produced using in vivo and in vitro methods (solid or liquid fermentation). In vivo production of EPNs appears to be the appropriate method for laboratory use and small-scale field experiments. Where a lack of capital, scientific expertise or infrastructure cannot justify big investments into in vitro culture technology in vivo production is also appropriate. This applies to niche markets and small growers. Biocontrol companies use in vitro technology because large scale production is needed at reasonable quality and cost (Shapiro-Ilan, Han, & Dolinks, 2012). The nematodes are produced in fermenters in a liquid media and made ready for commercial use in a process called “formulation”.

The formulation is a process that transforms living entities into a product that can be applied by practical methods (Niemietz, Chandhok & Schmidt, 2015 ; Heriberto, Jaime, Carlos & Jesusita, 2017). This formulation process consists of 2 steps. First, the nematodes are prepared from storage or directly from the fermenters. Secondly, they get mixed with several compounds (also known as ingredients) providing the nematodes with an environment they can survive in for up to a year. This survival time of the EPN is also known as formulation shelf life. The product must easily suspend in water, so it can be applied using a multitude of farm machinery (Shapiro-Ilan, Han & Dolinks, 2012). This means that the ingredients must be able to dissolve in water as well and don’t cause any harm when applied.
The formulation is one of the most critical factors for the successful use of EPNs as microbial insecticides. Because this product consists of living organisms, it is essential for them to survive and remain infective until the customer uses them. The nematode formulations are regularly researched by companies to find improvements for optimising the production process and the commercial product.

1.5 PRESENTATION OF THE PROBLEM

In some of the EPN products visible fungal colonies develop after 1 to 3 months of storage at 4 ^{o}C. These fungal contaminations occur somewhere during the production and formulation process.
To avoid this fungal development, companies include antifungal ingredients in the formulations which have the potential to preserve the products free of visible fungi for up to 1 year. The fungal growth in most cases does not seem to have a significant effect on the efficacy of the EPNs. However, the visible fungal growth in the product makes it less appealing to the customers. This research focusses on how this might be prevented by improving the production and formulation process. This will be done by practical research and comparing literature of other industries encountering fungal contaminations. On the production of nematodes on an economic scale, little literature is available due to research primarily being carried out by private companies and the still relatively small sector. The companies secretively are partly caused by the fact that organisms cannot be patented making it harder to protect their products.

Because the production process is done partly in the open air, it is hypothesized that the contaminations are airborne. To study this possibility research was done in collaboration with Koppert BV, situated in Berkel en Rodenrijs, the Netherlands. The formulation process of EPNs at Koppert is carried out in 2 separate production rooms where no measure is taken to remove airborne microbes. In these same rooms, other products of Koppert also get manufactured or packaged. These products consist of fungi and bacteria which are also used for biocontrol purposes. The production process of these microorganisms has an impact on the microbial load in the air of the factory. Because of the presence of these atmospheric contaminations (microbial air load), the production area of Koppert offered an excellent setting to test the hypotheses of fungal air contaminations.

Koppert wants their EPN formulations to be usable for at least 4 months after being formulated when kept in cooled storage at 4^{o}C - 8^{o}C. By adding multiple ingredients, the nematodes can survive this period.
The manufacturing of EPN products by Koppert consists (stated in a simplified way) of three steps:

1-Multiplication of the nematodes by liquid fermentation (upstream processing),
2-Concentration of the nematodes into a technical product (downstream processing),
3-Stabilization and final product packing (formulation).

During the upstream process, the EPNs grow and multiply in a clean environment inside a liquid medium. In the downstream process, the EPNs will be concentrated into a technical product (nematode paste) by separating the growth medium and dead nematodes. During step three, the technical product will be formulated for longer survival of the EPNs.

Aspects relevant to this project are the identification (to the species level) of the most common visible fungal contaminants, the quantification of the contaminants in the final product (visible and not visible) and the impact these different species of contaminants may have in the storage of the EPN products. This research will help to give insight on how the contamination happens and can help Koppert to better target the selection of type and dose of antifungal preservatives for the EPN products.

1.6 RESEARCH QUESTIONS

Previous experience with the nematode formulations shows various degrees of fungal development during storage at 4°C. Some formulations seem barely affected, but others can have mould after a month. Additionally, studies conducted using air samples have shown that the air load of microbial CFU (colony forming units) inside the factory also varies across the time measured in days of the week. Suspected is that these air loads vary because of other Koppert products being processed. Due to time constraints, only the formulation process is being studied in this research for the *Steinernema carpocapsae* and *Steinernema feltiae* species.

To study the relationship between the microbial load of the air during EPNs formulation and fungal development, a research plan was designed. This resulted in a research question which was compartmentalized in four sub-questions as shown below.

Research question: *Is there a relation between the species and the amount of microbial air load during the formulation process of entomopathogenic nematodes and the later development of fungal colonies during storage at 4°C?*

The quality of nematodes is measured by the number of living nematodes and their efficacy over time. Koppert uses a 90 per cent mark as the limit of these quality standards. For this testing they have a separate Q&R department, who test samples of every batch using standardized protocols. These same tests were done on the formulations made for this study to see if contaminations affect quality.

Sub-question 1: *How is the quality of the product affected by contaminations?*

In the formulation different types of contaminations are present most are fungal spores or bacteria. Not all of these will develop into colonies visible with the naked eye. Thus, it is vital to test these 'invisible' contaminations. This is done by plating the formulations on different growth media. These plates were then compared to the air samples plated on the same types of growth media to find a possible relation.

Sub-question 2: *What is the effect of the microbial air load during formulation on invisible contamination inside the EPN formulations?*

The larges problem posed by the fungal contaminations is the visible colonies formed in the product during storage. Different formulations were made under different presumed air loads. Over time these were visually assessed and compared to air samples taken during formulation to see if there was a relationship. Because the treatments were made under different conditions, comparing them could also give an indication of how the contaminations occur.

Sub-question 3: *What is the effect of the microbial air load during formulation on the development of visible fungal colonies?*

From the plated formulation from sub-question 2 and the visual assessment from sub-question 3 isolations were made. The isolations and later identifications were only made from the most commonly found contaminants. This is done to see if they are dangerous for humans or the product. They can also be used by Koppert in their research on new antifungal products in future research.

Sub-question 4: *Which microbial species are seen most often in the EPN formulations?*

2 MATERIAL & METHOD

To answer the research question EPN formulations were made under different circumstances. These formulations are then tested over time to answer the specific sub-questions. It is hypothesized that the fungal air load is affected by the processing of other biocontrol products made in the same factory for Koppert. Four treatments were designed for formulating EPNs to study if there is a relationship between the air load and later development of fungi inside EPN formulations. Each treatment differs in the air load (Table 1).

Treatment 1; formulations for this treatment were made in a laminar flow hood, this is a cabinet with filtered air. The HEPA filter has holes of 0,3 µm, so bacteria (4-8 µm) and spores (1-10 µm) cannot enter the air. This treatment was used as a negative control to compare to the different air loads in the production room. Treatment 2; this treatment was made in the production area of Koppert BV when there were no other production processes going on. This was presumed to make for a general air load. Treatment 3; This was made in the production area of Koppert BV when Trichoderma fungal products were being dried or packaged in the same room. These processes would cause for more fungal spores in the air, this was confirmed by tests done at Koppert beforehand and general literature (Madelin, 1994).

Three replicas were made for each treatment. During the formulation of each replica, a control group was made using the same materials. To this control treatment, the antifungal that is used in the standard formulation of Koppert's nematode products was added. These treatments were made for both the *Steinernema carpocapsae* and *Steinernema feltiae*.

Table 1: Summary of the different treatments

ID	Treatments	The process inside the factory during formulation	Air load	# replicas
1	Flow hood formulation	Not affected by factory processes	No fungal air load due to the clean air from a laminar flow hood	3
2	Only nematode formulation	Nematode products being formulated and packaged	The general level of fungal air contaminants in the packing area	3
3	Only Trichoderma formulation	*Trichoderma harzianum* product being dried or packaged	Trichoderma spores from the processing added to the general packing area air load	3
control	Standard formulation with antifungal products	Same process as the controlled treatment	Same air as the treatment being controlled	9

2.1 AIR SAMPLING DURING FORMULATION

During the formulations process, air samples were taken at the side of formulation conform the protocol in Figure 3. The protocol is standard for this machine and adapted in the different growth media used for the specific needs for this research. 3 different types of growth media were used in triplet for every measurement. Two of these have added antibacterial substances to focus on fungal growth, the SAB growth medium and the PDA growth medium. The two different media allow for different species to thrive so more could be detected. The third medium has antifungal compounds to promote bacterial growth, the TSA medium. This was not the focus for this research but was added to make it more well-rounded and help Koppert get a better view on contaminations in their production area and microbiology laboratory. This air sampling is done to verify if the processes in the factory indeed affect the microbial air load. Additionally, the samples will be useful to see if there is a match between the contaminations present in the air and the fungal contaminations found in the product. This will be compared to the samples for both sub-question 2 ad 3.

Protocol air sampling

Materials and solutions:

- Merck 100 Air Sampler
- 3x SAB oxoid plate
- 3x TSA natamycin plate
- 3x PDA igepal plate

Experimental procedure:

1 Remove the lit of the Merck 100 Air Sampler and take away the second cap (with holes).
2 Place a plate inside the machine.
3 Use a SAB or PDA plate for growing fungi and a TSA natamycin plate for growing bacteria.
4 Place the cap without lit back on the machine over the plate.
5 Press yes to start up the machine, press yes, a second time to start the air sampling
6 The setting should be 100L air in 1 minute. Press a third time, yes to start the sampling.
7 Wait 1 minute (which is visible on the machine) and take the cap away.
8 Take out and seal the plate.
9 Incubate the air samples at 23°C and wait three to four days before measuring the results.
10 Take a photo of the plate showing the necessary data, both from the top and bottom of the dish.
11 Place the Petri scales on a Colony Counter to count the amount of fungal and bacterial colonies

Figure 3: Protocol used to sample the air during the formulation process

2.2 SUB-QUESTION 1 QUALITY CONTROL

To control the quality of the EPN formulations every formulation will be regularly tested on the survival rate and efficacy of the EPNs. The survival rate of the nematodes is expressed in percentages of living nematodes measured following the protocol in Appendix 7.4. The efficacy is tested using the protocol shown below (Figure 4), by a bioassay on mealworms (Figure 5). To execute step 7 of this protocol first the survival rate must be calculated. Koppert uses these standard protocols to test the EPNs of every batch, for the product to be sold both tests need to be above 90 per cent. These quality control test show if the EPNs are affected by contaminations and if the formulation process was done correctly. The results of the EPNs survival rate and efficacy will statistically be tested using a two-way ANOVA comparing both the different treatments and the nematode species. This will be tested after a few days of formulating, after 2 months in cold storage and after 3 months in cold storage.

Protocol Bioassay on mealworms

Materials and solutions:

- Formulation
- Stemi 2000 C Stereo Microscope
- Tap water
- Stirring rod
- Magnetic stirrer
- Microscope slide with 3 holes (5x)
- Pipette what type
- 25*3 mealworms (for every formulation tested)
- 3 bioassay cups with lids containing air holes
- Potting soil

Experimental procedure:

1 Weigh 20g of formulation and fill this up to 2000 grams with tap water (100x suspended)
3 Place a stirring rod in the suspension and let it stir for 5 minutes on a magnetic stirrer
4 Take 5 grams of suspension while it is still being stirred, add 95g tap water (20x suspended) put it in a tube of 120 ml with a cap
5 Fill 5 object slides each containing 3 holes with 10µl of the suspension
6 Count the living and dead nematodes using a microscope
7 Calculate the amount of nematode suspension needed to have 3125 nematodes per cup:
8 Put 25 mealworms in every cup and fill up with potting soil
9 Add 5ml liquid to every cup = amount of nematodes suspension needed +tap water

Figure 4: Protocol used for the quality control of the efficacy of the nematodes

Figure 5: Materials needed for a bioassay on mealworms to test EPN efficacy

2.3 SUB-QUESTION 2 INVISIBLE FUNGI

Samples were plated from the formulations of treatments 2 and 3 to find non-visible contaminations. Only these two treatments were used due to time constraints, these were chosen to be able to make a comparison between the treatments made in the production area. A new protocol was designed and tested for improvements to research this (Figure 6), this protocol is now still in use at Koppert BV and visualized in Figure 7. The same growth media are used for the plating as are used for sampling the air. TSA natamycin (antifungal) plates were used to grow bacteria. This was done to serve as an extension of the research to airborne contaminations. SAB gentamicin-chloramphenicol and PDA igepal were used to grow fungi. These media have added antibiotics so fungal colonies have a better chance of developing. The plates will be visually compared to the air samples to find out if there is any relation between the air load during formulation and the amount and type of contaminations. When fungal colonies are often found, they can be isolated for sub-question 4.

Protocol Plating formulation for invisible contaminations

Materials and solutions:

- Nematode formulation
- Tap water
- Water bath
- Pipetman
- 3x SAB oxoid plates
- 3x PDA igepal plates
- 3x TSA natamycin
- L-spatula
- Colony counter
- Tube 120 ml with cap

Experimental procedure:

1 Weigh 10 grams of nematode formulation and fill this up to 50 grams with tap water
2 Put the suspension in 120 ml tube in a water bath of 45C° for 1 hour to kill all the nematodes
3 Plate 100ul of the suspension on 3 TSA natamycin, SAB oxoid and PDA igepal plates
4 Store the plates at 23°Cfor 2-5 days, count colonies when able
5 Use a colony counter to count each plate and document the results.
6 Use the (average) counted number of colonies to calculate the CFU, CFU is given per ml.
For example:

Number of colonies counted	48 *10^5(suspension of 5 times)	x
Volume	0,1 ml (100 microliters)	1ml

X= (48*10^5)/0,1 *40 (for the initial suspension) = CFU = 1.920.000.000 = 1,9*10^9/ml or per gram

Figure 6, Protocol designed to plate formulations to research non-visible contaminations

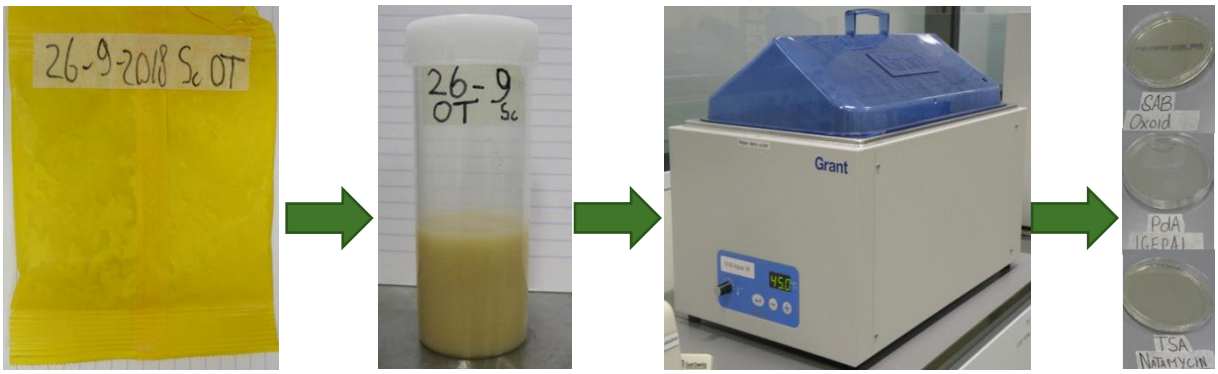

Figure 7: From left to right, first a sample of 10 grams of EPN is suspended with 40 grams of tap water, this is heat treated for 1 hour at 45°C using a water bath, finally 100µl is plated on multiple growth media and put in an incubator at 23°C

2.4 SUB-QUESTION 3 METHOD MONITORING VISIBLE FUNGI

The packages in which the EPN product is stored will be visually asses each month on the appearance of fungal colonies. Pictures and notes will be taken to follow this development over time. To answer the sub-question the visible colonies will be compared to the air samples taken during formulation to find a possible relation between them. The data on the effect of different treatments might help Koppert in scheduling the production of different biological control agents in a way to reduce contaminations.

2.5 SUB-QUESTION 4 IDENTIFICATION OF MOST SEEN CONTAMINATIONS

The contaminations that were most often seen were isolated for both the visible fungi and microscopic fungi of sub-questions 1 and 2. After successful isolation, the species will be determined by visually assessing if they are known species. Multiple employees of Koppert familiar with microorganisms and Koppert's products will assist in this assessment. When the species cannot be determined visually, and the organism is common, or otherwise of interest, it will be sent for identification to Baseclear. Baseclear is a private research facility specialized in identifying microorganisms using 16S rDNA. This was not done for every isolation due to budgetary constraints. The isolations were compared to the air samples to verify if there is any relationship between the contaminants present in the EPN formulations and in the air.

2.6 PRACTICAL METHOD

The formulation process was done following the lab protocol in Appendix 7.6. This protocol is used in all the nematode formulations made in the lab for research at Koppert BV. First, the nematode paste gets made inside fermenters in the factory. A few kilograms of nematode technical product (pure nematodes concentrate or "paste") is sent to the lab (Figure 8). This is used for experiments and quality control tests; the rest will be formulated and sold as a product. The number of nematodes per gram is calculated (Appendix 7.4) so that every pack of EPN formulation contains approximately 250 million nematodes. Based on the nematodes per gram the amount needed of the other ingredients is calculated. Both the paste and the ingredients then get weighed and mixed in the second step (Figure 9). These ingredients increase the shelf-life of the EPNs from a few days to up to a year. To finalise the product the nematodes, get packaged and sealed (Figure 10). For every formulation made during this research three bags of nematodes were made, one clear bag to observe for sub-question 3 and two yellow bags for taking the samples for sub-question 1 and 2. When enough nematode paste was available, the packages contained 250 million nematodes. When there weren't enough nematodes available, packages of 125 million were made.

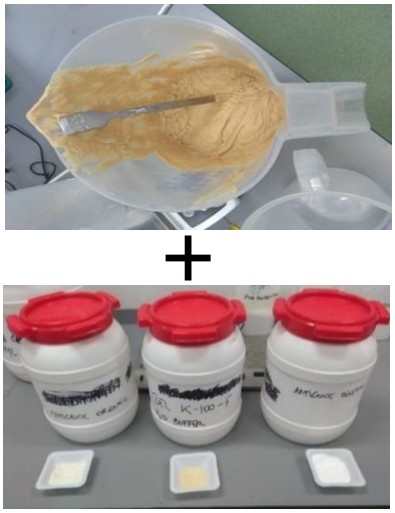

Figure 8: Nematode paste from the factory is mixed with different components to increase shelf-life

Figure 9: All the components of the formulations are put together (left) than they are mixed(right) to create the formulation

Figure 10: The formulation in packages of 250 million nematodes, ready for storage or use

3 RESULTS

The results for the sub-questions are shown in this chapter. For every treatment a minimum of three replicas was made. This was completed for both the *Steinernema carpocapsae* and *Steinernema feltiae.*

3.1 RESULTS SUB-QUESTION 1 QUALITY CONTROL

The efficacy of the nematodes was tested by performing a bioassay using mealworms. Al the treatments were tested a few days after formulating, 2 months after formulating and 3 months after formulating. The average of the tested treatments over time were all above 90% on average (Table 2), which is the limit Koppert uses. The two-way ANOVA test was used to test if there was any difference between the nematode species and the different treatments with their controls. This was done separately for both the survival rate and efficiency over time. None of the treatments was significantly different (Appendix 7.7)

Table 2: Average per cent of dead mealworms per treatment

***S. carpocapsae* average % dead mealworms**				***S. feltiae* average % dead mealworms**			
Treatment ID	0 months	2 months	3 months	**Treatment**	0 months	2 months	3 months
1	93	99	97	**1**	93	96	91
Control 1	89	97	97	**Control 1**	93	96	95
2	99	100	93	**2**	97	96	95
Control 2	97	97	96	**Control 2**	96	97	99
3	100	92	97	**3**	89	97	95
Control 3	97	97	97	**Control 3**	91	96	99

The survival rate of the nematodes over the time span of 3 months stayed above 90% for every treatment (Table 3).

Table 3: Average survival rate of nematodes per treatment

***S. carpocapsae* average % living nematodes**				***S. feltiae* average % living nematodes**			
Treatment ID	0 months	2 months	3 months	**Treatment ID**	0 months	2 months	3 months
1	92	99	99	**1**	93	99	98
Control 1	96	99	99	**Control 1**	93	96	98
2	96	99	100	**2**	95	99	98
Control 2	96	98	99	**Control 2**	96	95	98
3	94	98	100	**3**	93	97	97
Control 3	96	98	99	**Control 3**	93	95	97

3.2 RESULTS SUB-QUESTION 2 INVISIBLE FUNGI GROWTH AND AIR SAMPLES

All the plated formulations of TSA were completely overgrown by one type of yellow bacteria. This was isolated and send for identification for sub-question 4. In both, the plates for *Steinernema feltiae* and *Steinernema carpocapsae* a green fungus (blue circles) is seen in one-third of the samples and a pink yeast (pink circles) in two-thirds of the samples. Apart from these there are no other fungi often seen. These two fungi were both isolated and sent for identification. The green fungus was also seen in some of the air samples.

3.2.1 Plated formulations and air samples *S. feltiae* during nematode processing treatment 2

In the plated samples (left) a pink yeast (pink circles) can be seen, apart from that there are some white and yellow bacteria. The air samples (right) show only some bacterial colonies and do not look like the plated samples (Figure 11).

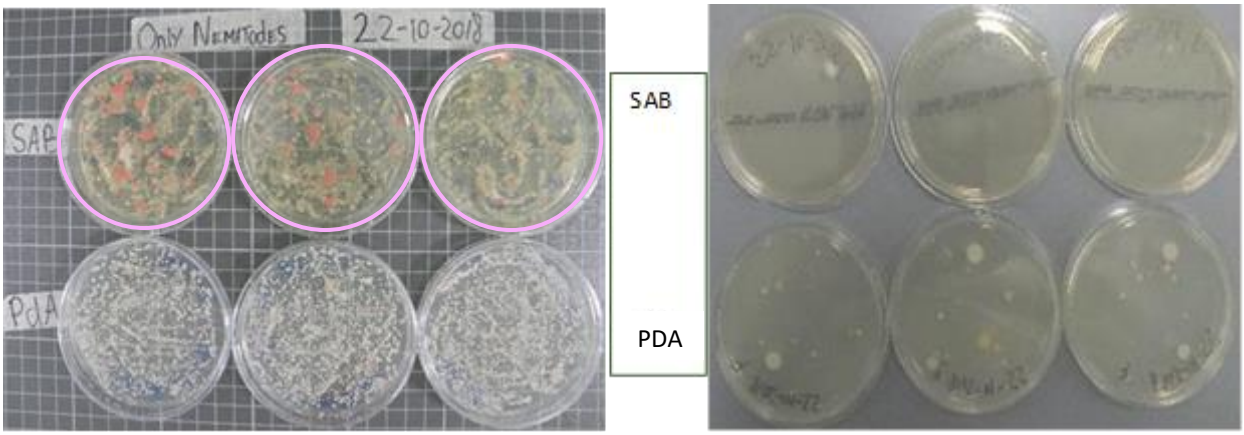

Figure 11: Plated formulation (left) and air sample (right) made during nematode processing for S. feltiae

The plated samples (left) again show a pink yeast (pink circles), apart from that there are some white and yellow bacteria as well. The air samples (right) show green fungi colonies (blue circles) and some bacterial colonies but do not look like the plated samples (Figure 12).

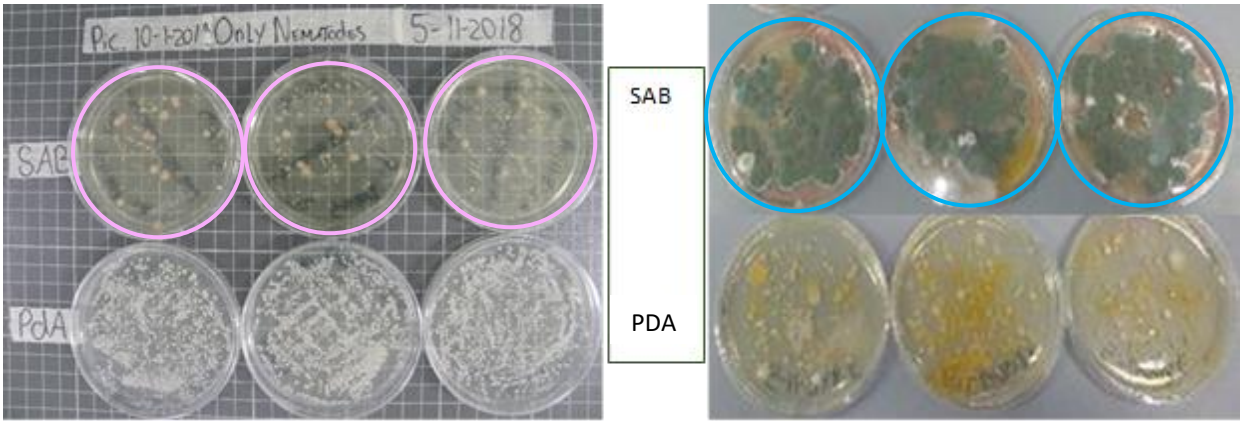

Figure 12: Plated formulation (left) and air sample (right) made during nematode processing for S. feltiae

Plated formulations and air samples *S. feltiae* during nematode processing, treatment 2

In the plated formulation (left) the same green fungi (blue circles) as in the air samples (right) can be seen, also there are white, yellow and orange bacterial colonies. Apart from this the air samples show some white and black fungi (Figure 13).

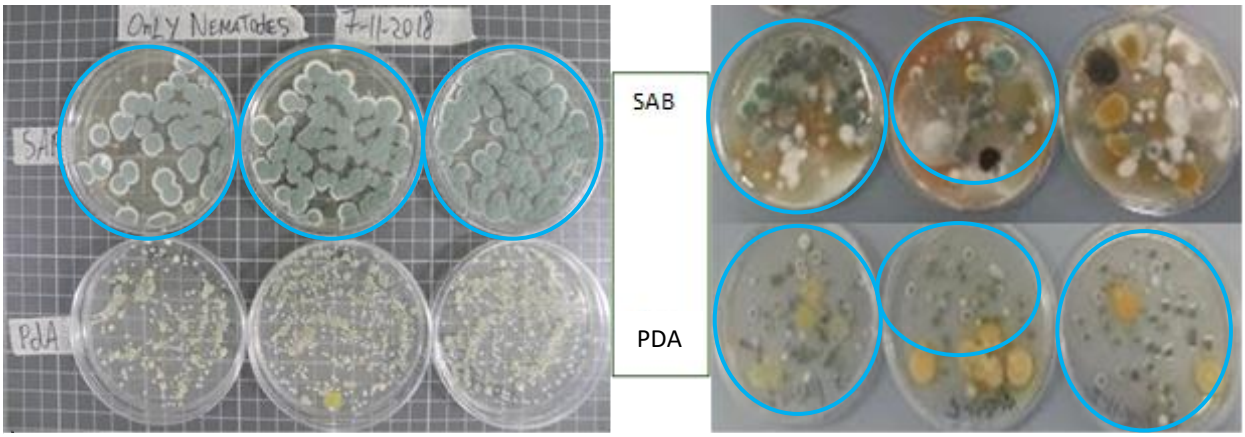

Figure 13: Plated formulation (left) and air sample (right) made during nematode processing for S. feltiae

3.2.2 Plated formulations and air samples *S. feltiae* during Trichoderma processing, treatment 3

The contaminations in the plated formulations (left) are not seen in the air samples (right). Only the pink yeast (pink circles) is seen as fungi in the plated material, the air samples show multiple fungi. The fungi that are seen more commonly are the green colonies (blue circles) the others are not seen in other samples from this treatment. As with all the samples there are multiple types of bacteria growing despite the antibacterial properties of the growth medium (Figure 14).

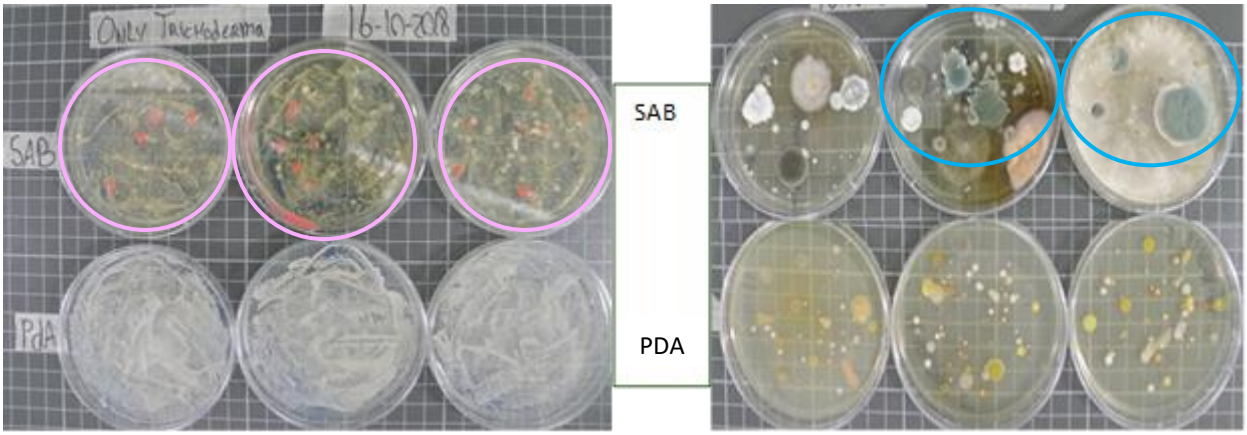

Figure 14: Plated formulation (left) and air sample (right) made during Trichoderma processing for S. feltiae

Plated formulations and air samples *S. feltiae* during Trichoderma processing, treatment 3

The plated formulation (left) shows some bacterial growth, the air samples (right) show unfamiliar fungal colonies and one of the green fungal colonies (blue circle), seen in other formulations (Figure 15).

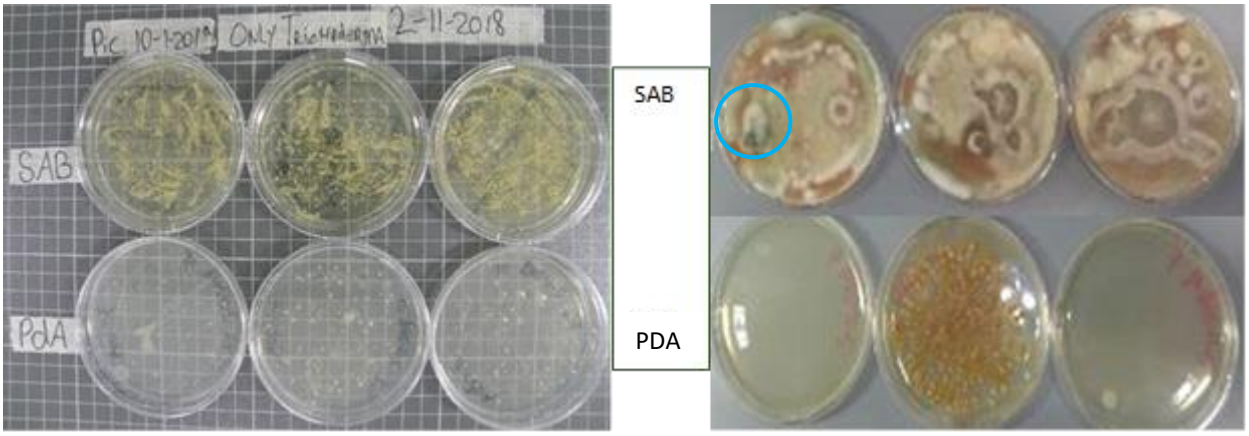

Figure 15: Plated formulation (left) and air sample (right) made during Trichoderma processing for S. feltiae

The plated formulation (left) shows only the pink yeast (pink circles) and some bacteria, the air samples (right) show a few bacterial colonies and the green fungi (blue circles). They only share some of the white bacteria that are seen (Figure 16).

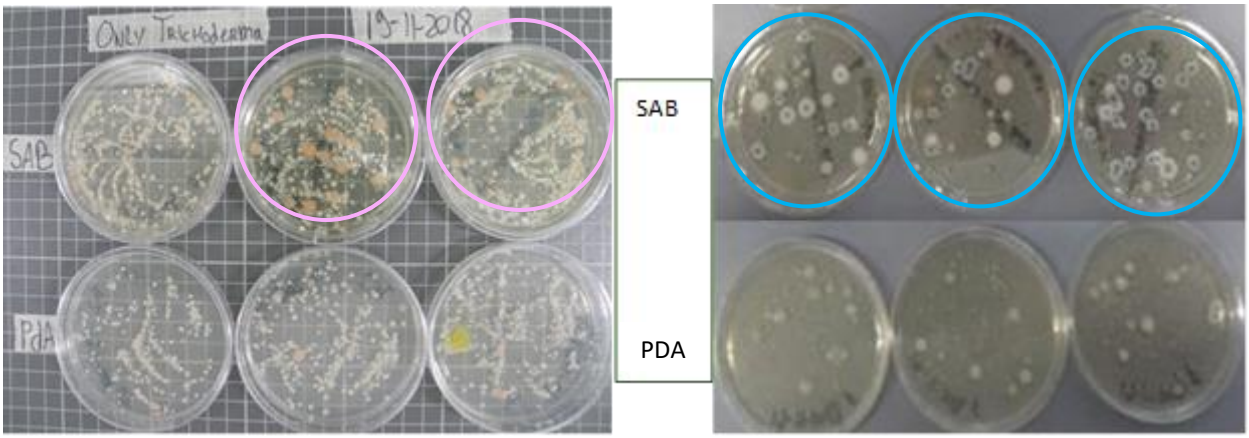

Figure 16: Plated formulation (left) and air sample (right) made during Trichoderma processing for S. feltiae

3.2.3 Plated formulations and air samples *S. carpocapsae* during nematode processing, treatment 2

The contaminations in the plated formulation (left) are not seen in the air samples (right). Again, the pink yeast (pink circles) and green fungi (blue circle) are seen. Apart from this the plated formulations have some white and yellow bacterial colonies as have the air samples (Figure 17).

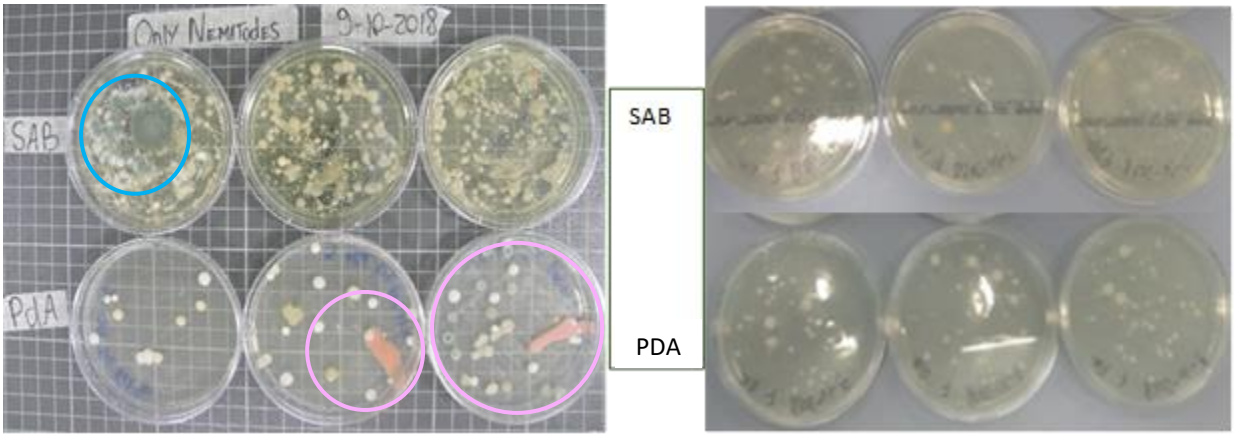

Figure 17: Plated formulation (left) and air sample (right) made during nematode processing for S. carpocapsae

Many white fungal colonies were found inside the plated formulations (left) excreting reddish compounds, these were not seen before. Apart from this a pink yeast colony (pink circle) was found. The air samples only show yellow and white bacteria (Figure 18).

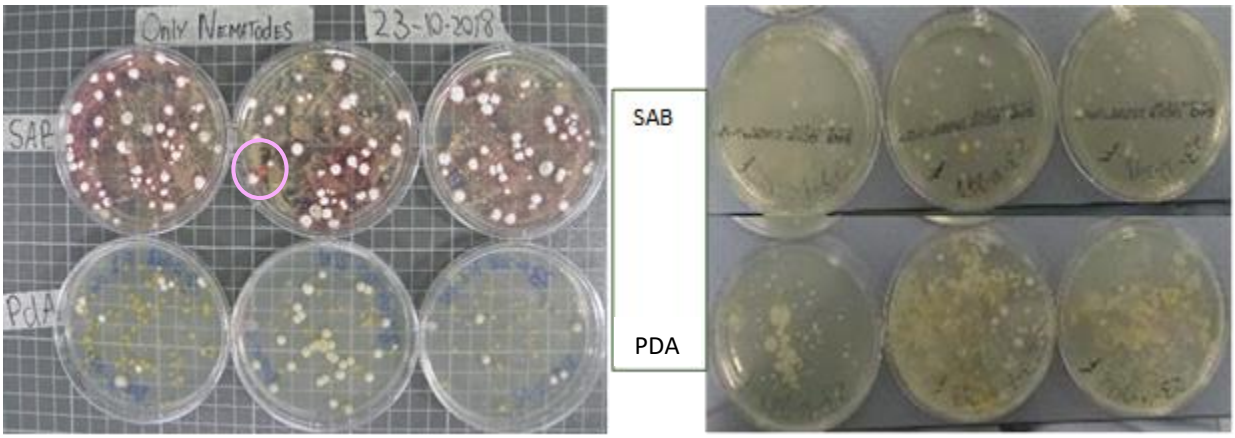

Figure 18: Plated formulation (left) and air sample (right) made during nematode processing for S. carpocapsae

Plated formulations and air samples *S. carpocapsae* during nematode processing, treatment 2

The plated formulation (left) shows only some white and yellow bacteria, the air samples (right) show the green fungi (blue circles). The air samples also show yellow and white bacteria (Figure 19).

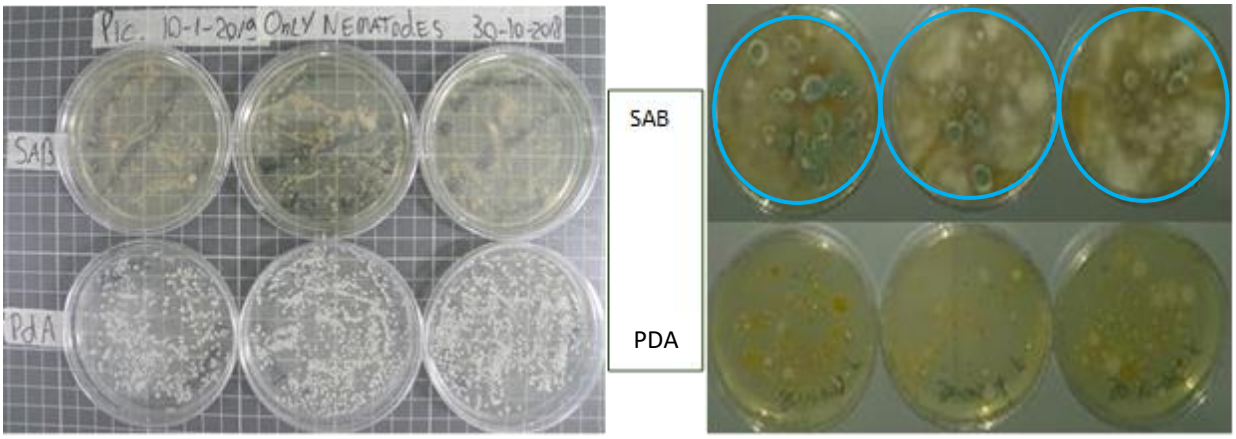

Figure 19: Plated formulation (left) and air sample (right) made during nematode processing for S. carpocapsae

3.2.4 Plated formulations and air samples *S. carpocapsae* during Trichoderma processing, treatment 3

Apart from the familiar green fungi (blue circles) and the pink yeast (pink circles) a white fungal colony is seen in the plated formulations (left). The air samples (right) show multiple fungi, most are the green fungi, but some white and brownish fungi can also be seen, these do not occur in other samples from this treatment (Figure 20).

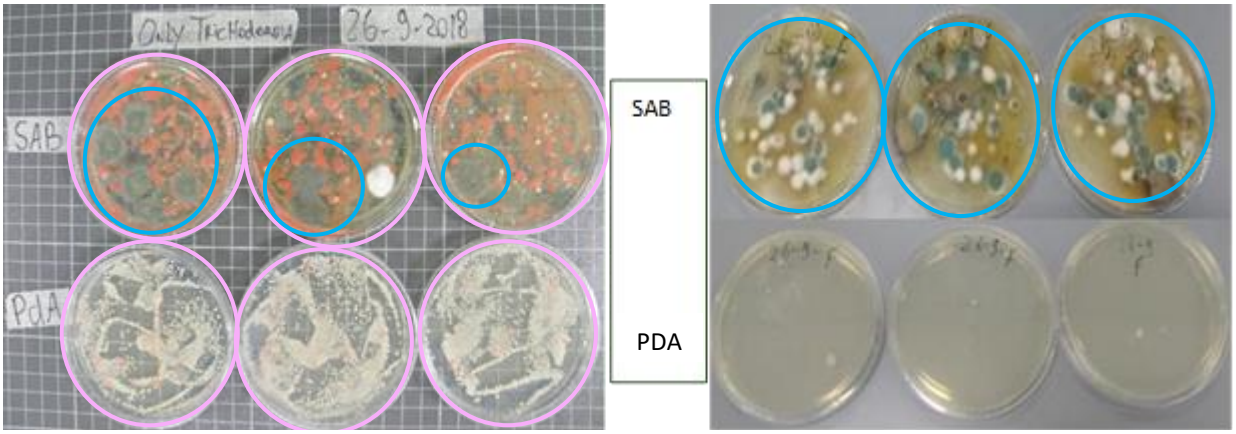

Figure 20: Plated formulation (left) and air sample (right) made during Trichoderma processing for S. carpocapsae

Plated formulations and air samples *S. carpocapsae* during Trichoderma processing, treatment 3
The pink yeast (pink circles) was seen in in the plated formulation (left). The air samples only show one green fungal colony. Apart from this there are white and yellow bacteria (Figure 21).

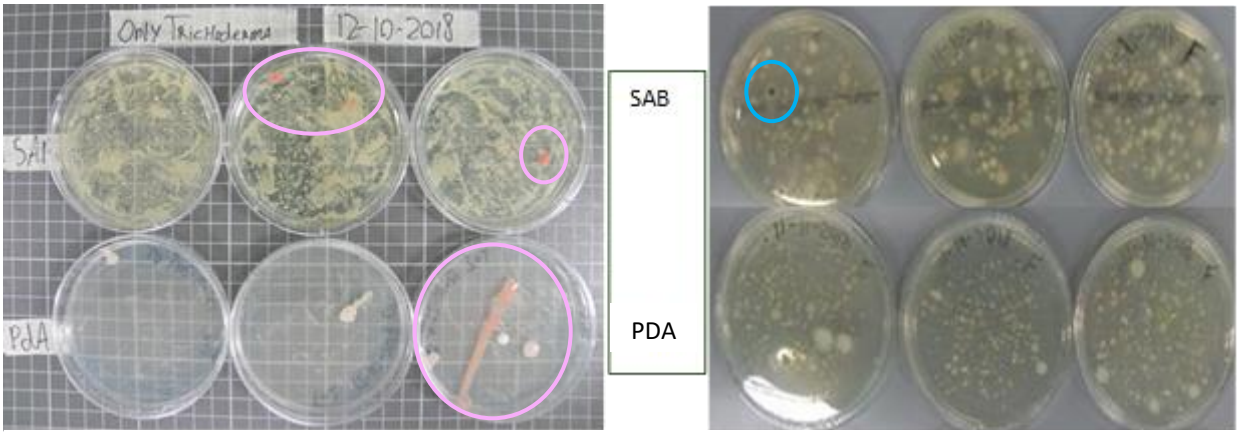

Figure 21: Plated formulation (left) and air sample (right)made during Trichoderma processing for S. carpocapsae

Both the plated samples (left) and the air samples (right) show the green fungi (blue circles). Apart from these fungal developments a black fungus is seen in one of the air samples. Both the samples show yellow and white bacteria (Figure 22).

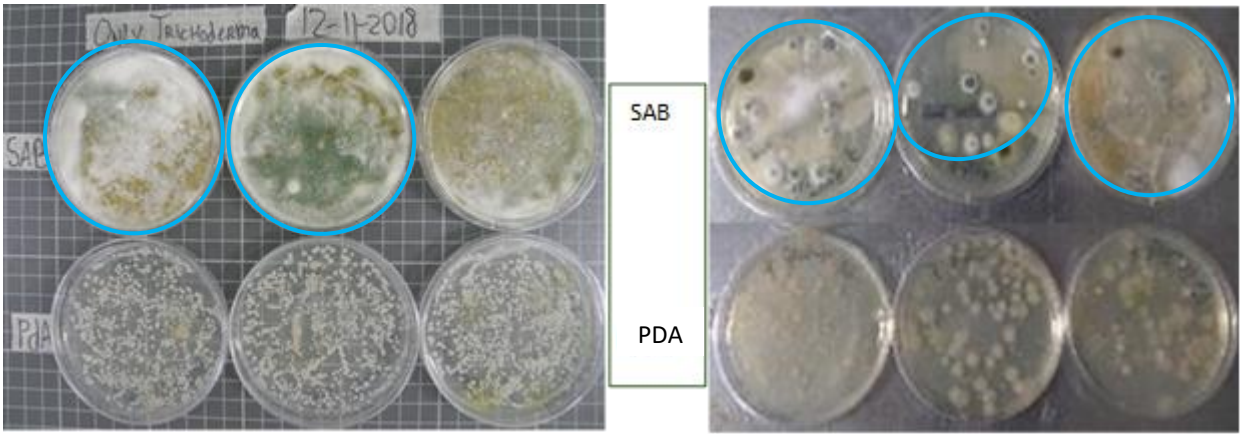

Figure 22: Plated formulation (left) and air sample (right) made during Trichoderma processing for S. carpocapsae

3.3 RESULTS SUB-QUESTION 3 MONITORING VISIBLE FUNGI

For every replica a control was made with the same ingredients. To this formulation, an antifungal was added. None of these control treatments shows any visible fungi growth for both the *S. feltiae* and the *S. carpocapsae.* The pictures of the control were therefore not added to the results. All the pictures of the formulations shown were made after 3 months of storage at 4°C.

3.3.1 Treatment 1, formulations made inside a laminar flow hood for S. *feltiae*

For the formulations made in the flow hood with *S. feltiae,* none of the formulations shows any visible fungi growth in the clear packages (Figure 23).

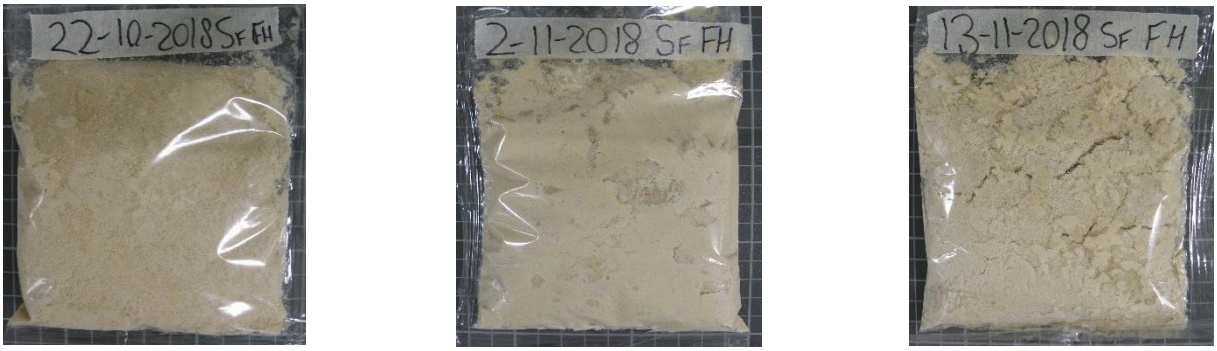

Figure 23: Clear packages made inside the flow hood with S. feltiae *after 3 months of storage at 4°C*

3.3.2 Treatment 2, formulations made during nematode processing for S. *feltiae*

One of the formulations with *S. feltiae* shows the black fungus (black) in the clear packages and the yellow tarnish(red). The other formulations are clean of visible fungi growth (Figure 24).

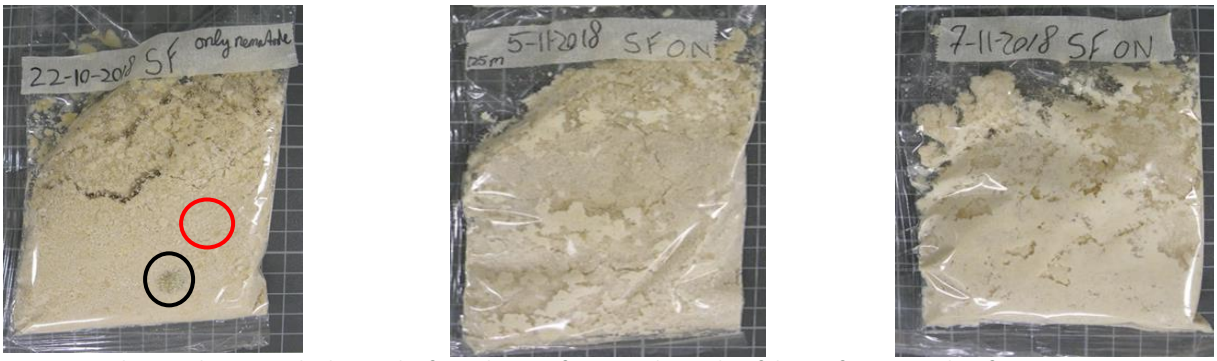

Figure 24: Clear packages made during the formulation of nematodes with S. feltiae *after 3 months of storage at 4°C*

3.3.3 Treatment 3, formulations made during Trichoderma processing for S. *feltiae*

None of the formulations shows any visible fungi growth in the clear packages for the formulations made in the packing-area during the drying and packaging of Trichoderma with *S. feltiae* (Figure 25).

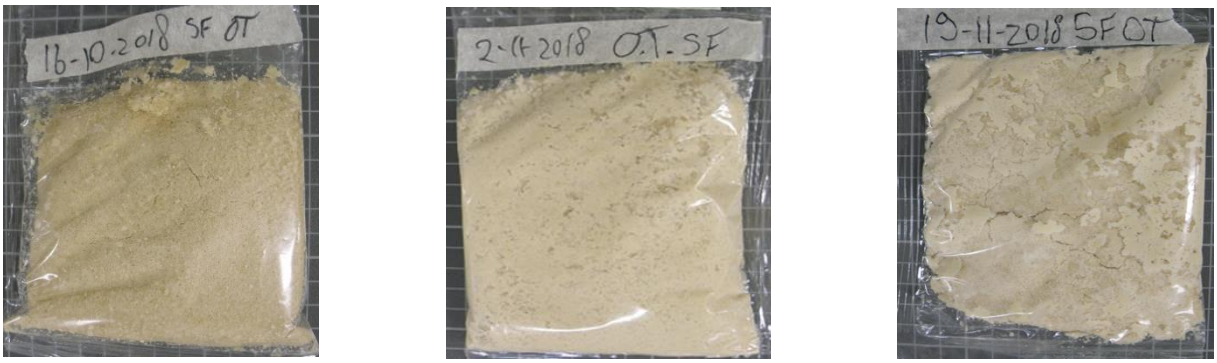

Figure 25: Clear packages made during the drying/packaging of Trichoderma *with* S. feltiae *after 3 months of storage at 4°C*

3.3.4 Treatment 1, formulations made inside a laminar flow hood for *S. carpocapsae*

Two of the formulations made inside the flow hood show the small blue fungi (blue). One formulation shows the yellow tarnish (red). The formulation from 30-10-2018 doesn't show any fungal growth (Figure 26).

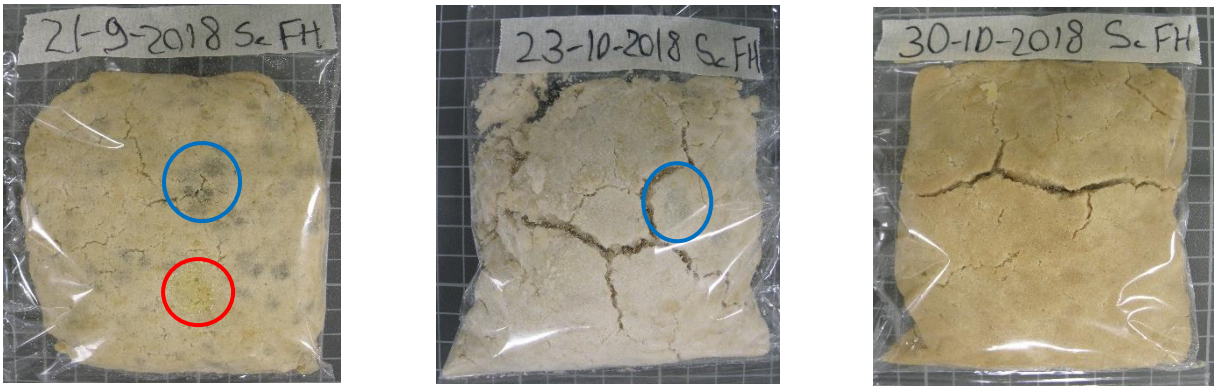

Figure 26: Clear packages made inside the flow hood with S. carpocapsae *after 3 months of storage at 4°C*

3.3.5 Treatment 2, formulations made during nematode processing for *S. carpocapsae*

Formulation 9-10-2018 shows a brown fungus (brown), formulation 23-10-2018 and 9-10-2018 show the yellow tarnish (red), the blue fungi (blue) and a black fungus (black). The formulation from 30-10-2018 doesn't show any fungal growth (Figure 27).

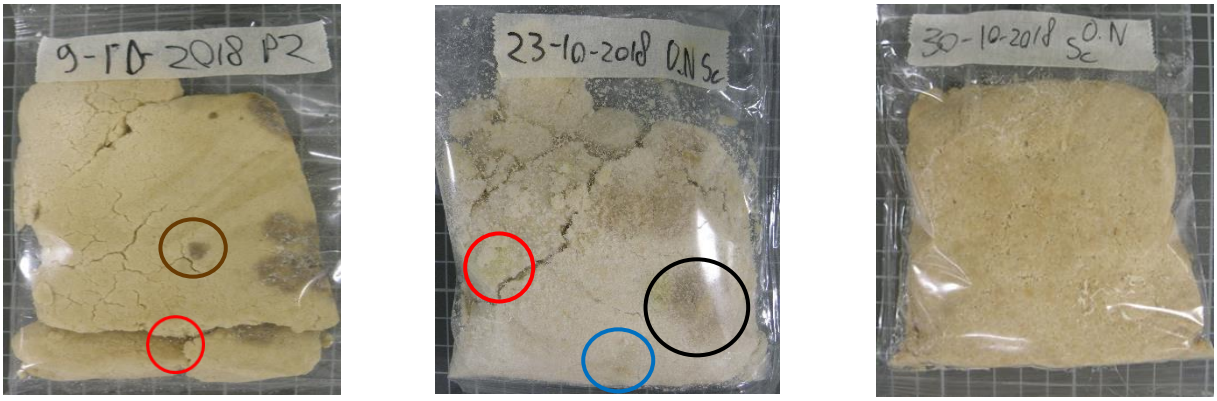

Figure 27: Clear packages made during the formulation of nematodes with S. carpocapsae *after 3 months of storage at 4°C*

3.3.6 Treatment 3, formulations made during Trichoderma processing for *S. carpocapsae*

The formulation of 26-9-2018 shows a black fungus (black). The formulation from 12-11-2018 shows A brown fungus (brown) the formulation of 12-10-2018 doesn't show any fungal growth (Figure 28).

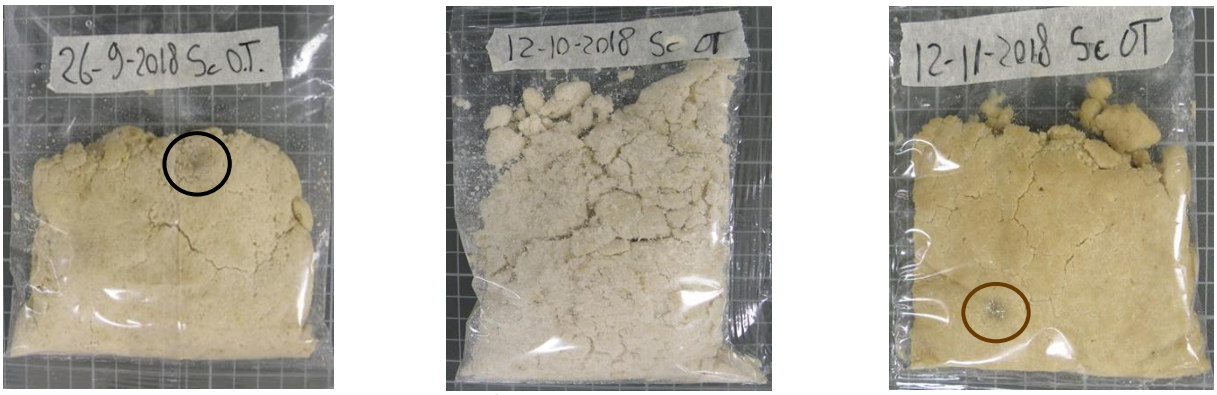

Figure 28: Clear packages made during the drying/packaging of Trichoderma *with* S. carpocapsae *after 3 months of storage at 4°C*

3.4 RESULTS SUB-QUESTION 4 IDENTIFICATION OF MOST SEEN CONTAMINATIONS

Some of the isolations were identified by Baseclear with an accuracy of 99.99% and some were identified by experts working with microorganisms at Koppert BV. Due to budgetary and time constraints not, all common contaminations were identified. All the isolations were multiplied and stored to be used in future research on new antifungal products.

3.4.1 Isolations of sub-question 4

Four different fungal species were observed in the clear EPN packages. Three of the *S. carpocapsae* and one *S. feltiae* formulation show the yellow tarnish (red). This has been isolated and is probably a fungus of the *Trichoderma* genus (Figure 29), this was determined by the head researcher on Koppert's Trichoderma products, Erik van de Zilver. Three of the *S. carpocapsae* formulations show the small blue fungi (blue). This has been isolated and appears to be the same fungi isolated from the plated formulations, fungi from the *Penicillium* genus (Figure 30) as identified by Baseclear with 99,99% accuracy.

Figure 29: Formulation made in the flow hood S. carpocapsae *(left), isolation of yellow tarnish (right)*

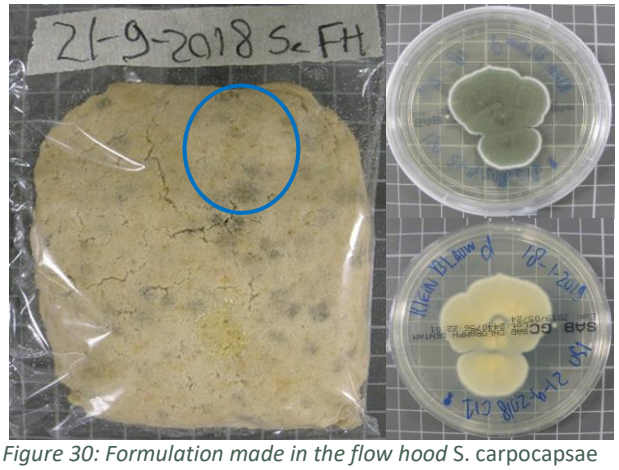

Figure 30: Formulation made in the flow hood S. carpocapsae *(left), isolation of the blue fungi (right)*

Two of the *S. carpocapsae* formulations show the black fungi. This has been isolated, but the species is not yet determined (Figure 31). Two of the *S. carpocapsae* formulations show the big brown (brown) fungi (Figure 32). This also has been isolated, but the species is not determined. These isolations are stored for now but can be identified or used later if they become of interest, for example if they are seen in future research.

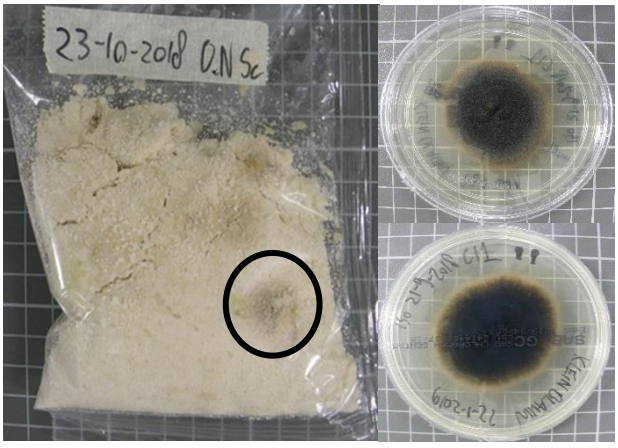

Figure 31: Formulation made during nematode processes S. carpocapsae *(left), isolation of black fungi (right)*

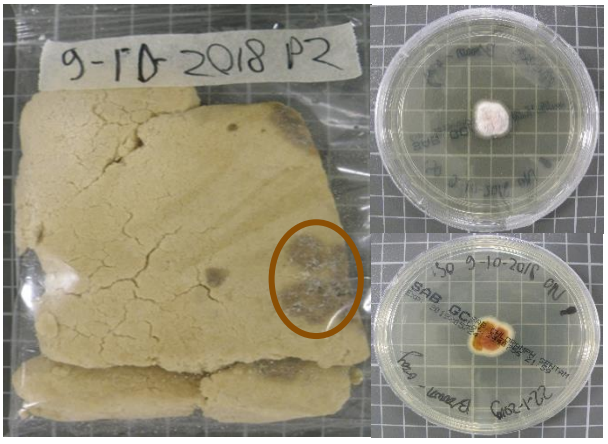

Figure 32: Formulation made during nematode processes with S. carpocapsae *(left), isolation of brown fungi(right)*

3.4.2 Isolations of sub-question 3

These isolations were all identified by Baseclear. Multiple species of the *Penicillium* genus (Figure 33) were found inside the isolation. The isolations were made from the green encircled fungal colonies seen in the results from sub-question 3. These green fungi were seen in one-third of the plated formulations. Many of its species are very common, found on stored foods of human beings and other animals, but also in animal dung, building materials, indoor air, and several other habitats (Frisvad & Samson, 2004)

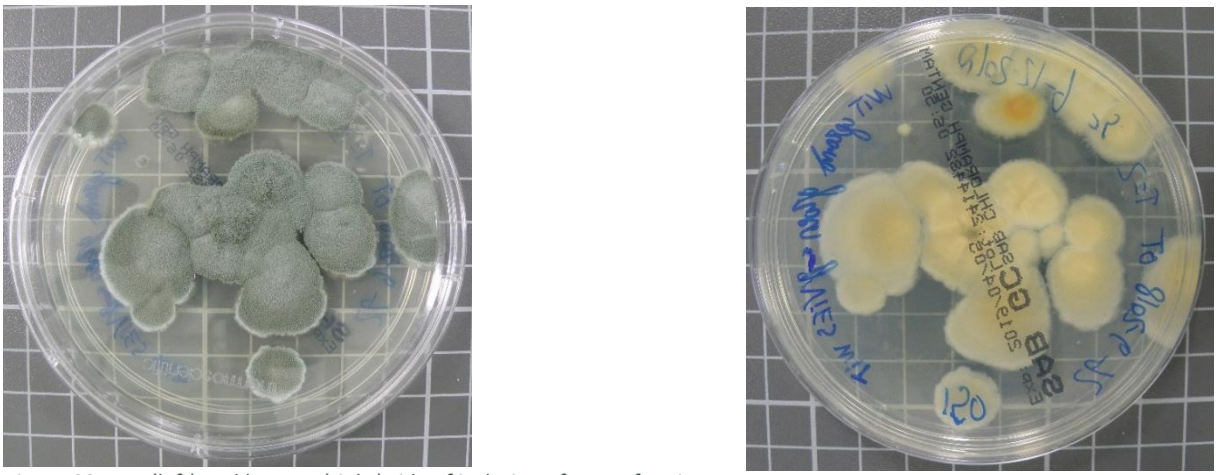

Figure 33: Top (left) and bottom (right) side of isolation of green fungi

The pink yeast (Figure 34) is a *Rhodotorula mucilaginosa.* It was seen in two-thirds of the plates. It is a common fungus which can spread itself through the air, water, and soil (Wirth & Goldani, 2012). It is isolated from the pink encircled fungal colonies from sub-question 3.

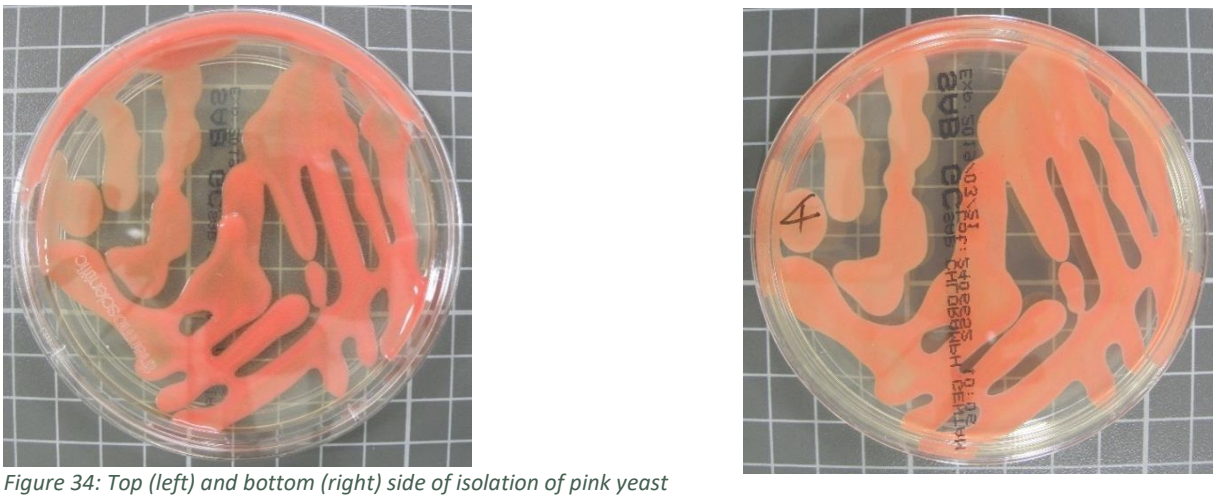

Figure 34: Top (left) and bottom (right) side of isolation of pink yeast

Isolations of sub-question 3

These isolations were made from the plated formulations of sub-question 3. All the formulations that were plated on the TSA growth medium were utterly overgrown by these bacteria (Figure 35).

Figure 35: Plated formulation made during the processing of nematodes (left) and plated formulation made during the processing of Trichoderma (right)

The isolation (Figure 36) was identified as *Carnobacterium maltaromaticum* by Baseclear and is the only species found on the TSA plates. This bacterium is mostly found in meat and fish and is generally non-dangerous (Casaburi et al., 2011)

Figure 36: Top (left) and bottom (right) side of the yellow bacteria isolation found in every plated formulation

4 DISCUSSION

Due to the limited amount of available literature on contaminations in EPNs, fungal countermeasures were sought in other sectors. Many types of research are done to prevent fungal air contaminations in different settings. Solutions that decrease the amount of fungi in hospitals are the use of antifungal products (Rogawansamy, Gaskin, Taylor, & Pisaniello, 2015), the use of air filtration (Fox, Chamberlin, Kulich, Rae, & Webster, 1990) and wearing protective clothes (Araujo, Cabral & Rodrigues, 2008). However, most of these counter measurements cannot be implemented in EPN production on an industrial scale, yet they may serve as inspiration. Antifungal products are common in EPNs but can be restricted by legislation or biological standards important for branding the product. Other factors that might affect the amount of fungus that was found are the season and contaminated water resources (Şimşekli, Gücin, & Asan, 1999) (Bedient, Rifai, & Newell, 1994). Water is regularly tested at Koppert making it an unlikely contamination site. By doing research in different seasons the effect of outdoor contaminations could be quantified in a follow-up study.

4.1 DISCUSSION SUB-QUESTION 1

The results of the quality control tests show that the formulations were correctly made. The standard tests came back positive above the quality standard of 90% used by Koppert. Due to time constraints, the formulations were not tested after 4 months. However, these nematode products can stay alive for up to a year. Based on these results and expectations of the lead researcher, Felipe Cortes, the quality would stay constant for the 4-month measurement. *S. carpocapsae* shows more visible fungal colonies than *S. feltiae* but this doesn't affect the results of the standardized tests. A two-way ANOVA test confirmed that there was no significant difference between the species and treatments for both their survival rate and efficacy.

4.2 DISCUSSION SUB-QUESTION 2

There is high variability in contaminations between the replicas of the same treatments in both the plated formulations and air samples. When the plated formulations are compared against the air samples, there is no clear relationship between the air load and the types of contaminations inside the formulations. It was expected that more contaminations in the air samples would mean more contaminations in the product and that it would be similar species. Because this is not the case the hypotheses that the contaminations came from the air is not supported.

In all the samples bacterial colonies are seen despite the antibacterial properties of the growth medium. These yellow, white and orange bacterial colonies are deemed of no interest by employees of Koppert. These employees work in Koppert's lab with different microorganisms and see these types of colonies often. The results of the plated product might be affected by the abundant growth of bacteria, possibly overgrowing fungal colonies (Nagano et al., 2008). This also made it impossible to count the CFU of the plates. A quantification of contaminations and subsequently a statistical analysis could not be carried out but would be an excellent addition to future research. To quantify the results in follow-up studies other growth media could be used with better antibacterial properties. The plates could also be checked more frequently during their time in the incubator to halt growth when some colonies start overgrowing each other.

For the plating of the formulations a new protocol was developed (Appendix 5.5) this new protocol had some drawbacks. A detection limit of 50 CFU/gram was achieved, if a sample had less contaminations, it could not consistently be found. This detection limit could not be lowered due to the viscosity of the nematode product. Only a random sample of 10 gram of the formulation is plated

while most formulations weight 60-100 gram. The samples are also heat-treated for 1 hour at 45°C; this could affect the contaminations that grow on the plates. This temperature was chosen after several tests on the lethal temperature for these nematodes. Both species die at this temperature and duration. All the nematodes need to be killed because otherwise they would move in the growth medium spreading contaminations. Most fungal spores are not affected by this temperature (Krishnamurthy, Khurana, Soojin, Irudayaraj & Demirci, 2008; Ballestra & Cuq, 1998). This new protocol is still in use at Koppert as an improvement of the old protocol.

4.3 DISCUSSION SUB-QUESTION 3

None of the control formulations containing the antifungal products show visible fungal growth. *Steinernema feltiae* shows less fungal growth than *Steinernema carpocapsae*. Why there is such a difference in visible contaminations is unknown. When looking at the plated formulations from sub-question 2 this difference in contaminations was not found. Of the four types of visible fungi growing in the *Steinernema carpocapsae* plates, only 1 was also seen in the air samples, the *Penicillium sp*. These species are one of the most commonly found air contaminants (Araujo, Cabral, & Rodrigues, 2008). The *Penicillium sp.* and *Trichoderma sp.* were found in every treatment, even the flow hood treatment. This serves as an indicator that contaminations occur before the formulations process and might not be airborne, underlying the need for a broader approach in follow-up studies.

Every treatment had 2 replicas showing visible fungi growth for *S. carpocapsae, t*his indicates there is no apparent difference between the treatments. Thus, the processes in the packing-area during formulation and the later development of fungi seem unrelated. The *S. feltiae* had only one replica affected by visible fungi growth. The black and brown fungi are only seen in formulations made in the packing-area, so these contaminations might have occurred there but were not seen in the air samples. Only qualitative data was collected on the visible fungal contaminations in future research it would be interesting to collect quantitative data. This might be done by counting the separately forming colonies or measuring contamination in the per cent of the package covered in visible colonies. However only the front and back of the EPNs are visible. Because of this only a two-dimensional measurement can be made while the product is three-dimensional increasing the difficulty for obtaining data.

4.4 DISCUSSION SUB-QUESTION 4

The contaminations that were often found were isolated. Only a part of the isolations has been sent for identification yet. Not all were sent on account of the monetary costs. Because only contaminations that were often seen are isolated a large group of replicas was made. Harvesting them from multiple samples and comparing isolation visually. By taking isolates from the original isolations it was ensured that only one species was apparent in the plates ultimately sent to Baseclear. The identification accuracy of Baseclear all proceeded 99% which means they are accurate up to the species level of the microorganism. Both the isolations of fungi that were sent and were not sent for identification have been multiplied for the testing future antifungal products by Koppert.

The isolations of the plated formulations from sub-question 2 only show common non-dangerous contaminants. The pink colonies are *Rhodotorula mucilaginosa* yeast and the yellow bacteria found on all the TSA plates is the *Carnobacterium maltaromaticum.* These species were not seen in the air samples. The green fungus was seen in some of the air samples and plated formulation. However, there was not a clear relation between air samples and plated formulation from the same formulation date. This green fungus appears to be the blue looking fungi seen in the packages.

Isolations were made from the visible fungi of sub-question 3, only the blue coloured fungus was sent to Baseclear. This was identified as various fungi from the *Penicillium* family, which is also a common non-dangerous fungus. The yellow tarnish seen in the packages was identified by the lead researcher on fungi production at Koppert as being of the *Trichoderma* genus. The same type of fungi that is being processed during the Trichoderma treatment. The black and brown fungi have yet to be identified.

4.5 DISCUSSION RESEARCH QUESTION

The results of this research could have been affected by the activities going on before formulation takes place in areas connected to where the formulations took place. Namely in the downstream processing room, here the fermenters with nematodes and fungi are. Harvesting and storage activities might affect the microbial air load of this area which then in turn affects the microbial air load in the formulation area. This was not considered for this research. No clear differences were found for the two treatments made during the processing of different Koppert products in the factory. It might be that these do not affect the microbial air load as much as was thought.

In follow-up research I would be interesting to take air samples during other phases of the production process, apart from the formulation step to research the hypothesis of airborne contaminations more broadly. Phases that could possibly be sites for airborne contaminants are the separation of nematodes from the growth media after liquid fermentation and the later sieving of nematodes from water in cold storage. There could also be opted for other types of air sampling methods with different equipment. For example auto sampling in certain areas of the factory or the use of liquid entrapment instead of using different growth media (Carvalho et al., 2008). These methods would be more costly. It might be of interest to do more research on the sites which are subjected to other types of contaminations than the air such as storage tanks. These tanks are not regularly cleaned and were found to be contaminated multiple times in the past by different types of microorganisms.

5 CONCLUSION

Research aim:

The aim of this study was to test the hypothesis that: fungal contaminants in entomopathogenic nematode products occur via airborne contamination. the following research question was designed to answer this: *Is there a relation between the species and the amount of microbial air load during the formulation process of entomopathogenic nematodes and the later development of fungal colonies during storage at 4°C*? To test this air samples were taken during the formulation process and compared to contaminants later found in the product. Both for contaminants that would grow into colonies visible to the naked eye and contaminants only apparent after culturing samples of the nematode product. To answer the research question and test the hypothesis the following four sub-questions were researched and answered.

Sub-question 1: *How is the quality of the product affected by contaminations?*

The quality of the EPN products is not affected by any visible or invisible contamination. The statistical test shows that both the number of surviving nematodes and their efficacy remain unchanged after 3 months of storage at 4C°. Statistical analyses also show that there is no difference between the quality of the different treatments and the different species.

Sub-question 2: *What is the effect of the microbial air load during formulation on invisible contamination inside the EPN formulations?*

Fungi of the *Penicillium* genus were the only fungi found in both the air samples and the product. However, these fungi were also seen in the treatment made in the laminar flow hood, so these contaminations can occur before formulation. The amount of contaminations varies between replicates of the same treatments without apparent differences between the treatments themselves. There is also no apparent effect of the different nematode species regarding these invisible contaminations. If air samples show an abundance of contaminations, it does not mean the plated formulations will also show this abundance, and vice versa. This indicates that the air load during formulation does not have a substantial effect on the number of invisible contaminations in the final product.

Sub-question 3: *What is the effect of the microbial air load during formulation on the development of visible fungal colonies?*

The microbial air load tested by the air samples did only show one of the fungi species that was seen as visible contamination in the nematode packages. Using this experimental set up at Koppert there does not appear to be a clear effect of the fungal air contaminants on the later development of visible fungi. The *Steinernema feltiae* only had one replicate out of the 3 treatments affected by visible fungal growth while *Steinernema carpocapsae* had six affected. What causes this is still unknown but it might be interesting to research in follow-up studies.

Sub-question 4: *Which microbial species are seen most often in the EPN formulations?*

The common fungal contaminations were identified using the knowledge of Koppert employees and the Baseclear laboratory. All that were identified are common and non-dangerous. Multiple fungi species were identified: species of the *Trichoderma* genus, the *Penicillium* genus and the *Rhodotorula mucilaginosa*. One bacteria species was also isolated due to its consistent abundance in the product, the *Carnobacterium maltaromaticum*. It is not yet known where these contaminations occur.

Research question: *Is there a relation between the species and the amount of microbial air load during the formulation process of entomopathogenic nematodes and the later development of fungal colonies during storage at 4°C?*

No apparent relation was found between the air samples and the amount or types of contaminations in the final product. Multiple air loads were tested using the different treatments all yielding similar results. The treatment made in the flow hood underlined that there was no relation by having similar results as the other treatments while the filters provided air devote of contaminants. It can be concluded that the microbial air load during formulation is not the primary way of contamination and there are already contaminants inside the nematode product before the formulations process starts.

Recommendations

Because the origin of most of these contaminations in the products of Koppert remains unknown Follow-up studies should be done. To prevent the product from becoming mouldy which would turn off customers in the short term it would be best to focus research on new antifungal products that can be added to the nematodes. It is essential that these new antifungal products conform to regulations for the intended market. The antifungal products are not entirely fail-safe and might not work against all types of fungi. The isolations collected during this research can help in testing out different antifungal products. In the long term it would be better to improve the process to prevent contaminations from happening.

To find these improvements, future research should take a broader approach to research the nematode production process. Due to time constrains only the formulation process was researched in this study. Apart from the investigated sites other possible contamination sites have been disclosed. Especially contamination sites which are not affected by airborne contamination might be of interest. The research methods should be adapted to measure quantitative data on the number of contaminations so statistical analyses can be applied more efficiently. The overall time for research might also be increased to provide data up to the 4-month storage mark, which is aimed for limit for Koppert's nematode products. Why *Steinernema feltiae* shows fewer contaminations than *Steinernema carpocapsae* might also provide insight on how to prevent fungal contaminations in the future and deserves more indebt research.

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7 APPENDIX

7.1 LIST OF PESTS AND KOPPERT PRODUCTS TO COMBAT THEM

For seven of the shown pests, either one or multiple nematode species could be used to manage them.
These seven in order from top to bottom: Thrips, Sciarid fly, Caterpillar, Shore fly, Vine weevil, Red palm weevil and White Grubs.

7.2 PROTOCOL 1: HOW TO SAMPLE AIR AND MEASURE AIR CONTAMINATIONS

Protocol ID	
SOP version	
Title	How to sample air and measure air contaminations
Author	Luuk Jungerling

Background:

Materials and solutions:
- Merck 100 Air Sampler
- 3x SAB oxoid plate
- 3x TSA natamycin plate
- 3x PDA igepal plate

Experimental procedure:
1 Remove the lit of the Merck 100 Air Sampler and take away the second cap (with holes).
2 Place a plate inside the machine.
3 Use a SAB or PDA plate for growing fungi and a TSA natamycin plate for growing bacteria.
4 Place the cap without lit back on the machine over the plate.
5 Press yes to start up the machine, press yes, a second time to start the air sampling
6 The setting should be 100L air in 1 minute. Press a third time, yes to start the sampling.
7 Wait 1 minute (which is visible on the machine) and take the cap away.
8 Take out and seal the plate.
9 Incubate the air samples at 23°C and wait three to four days before the results.
10 Take a photo of the plate showing the necessary data, both from the top and bottom of the dish.
11 Place the Petri scales on a Colony Counter to count the amount of fungal and bacterial colonies.

Note:

7.3 PROTOCOL 2: PLATING FORMULATIONS FOR NON-VISIBLE FUNGAL CONTAMINATIONS

Protocol ID	
SOP version	
Title	Plating formulations for non-visible fungal contaminations
Author	Luuk Jungerling

Background:

Materials and solutions:
- Nematode formulation
- Tap water
- Water bath
- Pipetman
- 3x SAB oxoid plates
- 3x PDA igepal plates
- L-spatula
- Colony counter
- Tube 120 ml with cap

Experimental procedure:

1 Weigh 10 grams of nematode formulation and fill this up to 50 grams with tap water
2 Put the suspension in 120 ml tube in a water bath of 45C° for 1 hour to kill all the nematodes
3 Plate 100ul of the suspension on 3 SAB and PDA igepal plates
4 Store the plates at 23°Cfor 2-5 days, count colonies when able
5 Use a colony counter to count each plate and document the results.
6 Use the (average) counted number of colonies to calculate the CFU, CFU is given per ml.
For example:

Number of colonies counted	48 *10^5(suspension of 5 times)	x
Volume	0,1 ml (100 microliters)	1ml

X= (48*10^5)/0,1 *40 (for the initial suspension) = CFU = 1.920.000.000 = 1,9*10^9

Note:
To use the CFU calculation, ideally, you need to have a colony count between 30-300 if you don't have this amount you can change the suspension factor in coming experiments.

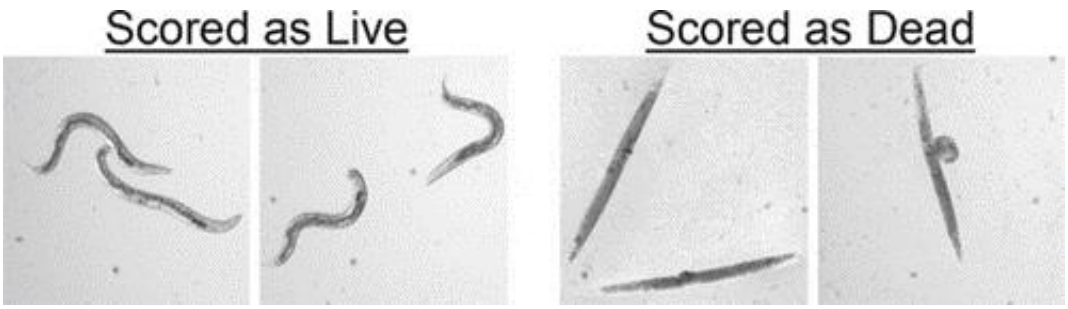
7.4 PROTOCOL 3: CALCULATING THE NUMBER OF NEMATODES IN THE BASE MATERIAL

Protocol ID	
SOP version	
Title	Calculating the number of nematodes per gram of nematode paste
Author	Luuk Jungerling

Background:
(Include time planning)

Materials and solutions:
- Paste
- Stemi 2000 C Stereo Microscope
- Tap water
- Stirring rod
- Magnetic stirrer
- object slides with 3 holes (5x)
- HandyStep repeating pipette
- Plastic packages for the product
- Measuring can 2L
- Tube 120 ml with cap

Experimental procedure:
1 Weigh 20g nematode paste and fill this up to 2000 grams with tap water (100x suspended)
3 Place a stirring rod in the suspension and let it stir for 5 minutes on a magnetic stirrer
4 Take 5 grams of suspension while it is still being stirred, add 95g tap water (20x suspended) put it in a tube of 120 ml with a cap
5 Fill 5 object slides each containing 3 holes with 10µl of the suspension
6 Count the living and dead nematodes using a microscope



Living nematodes move while dead ones are rigid

Calculation:

1 drop of nematode suspension (10µl) = amount of counted nematodes/15

Number of nematodes per ml = *100x

Number of nematodes per gram = *100*20

For a package of 50 million, the amount needed is calculated for 55 million nematodes per package

55E+6/6/number of nematodes per gram = amount of paste needed

Note:

This calculation is used to calculate the number of ingredients needed to make EPN formulations of 50 million nematodes per product but can be used to check the number of living nematodes as quality control in the formulations.

7.5 PROTOCOL 4: BIOASSAY MEALWORMS PROTOCOL TO TEST NEMATODE EFFICACY

Protocol ID	
SOP version	
Title	Bioassay of nematode efficacy using mealworms
Author	Luuk Jungerling

Background:

To test the efficacy of the nematode formulations a bioassay can be carried out using mealworms as test species

Materials and solutions:

- Formulation
- Stemi 2000 C Stereo Microscope
- Tap water
- Stirring rod
- Magnetic stirrer
- Microscope slide with 3 holes (5x)
- Pipette what type
- 25*3 mealworms (for every formulation tested)
- 3 bioassay cups with lids containing air holes
- Potting soil

Experimental procedure:

1 Weigh 20g of formulation and fill this up to 2000 grams with tap water (100x suspended)

3 Place a stirring rod in the suspension and let it stir for 5 minutes on a magnetic stirrer

4 Take 5 grams of suspension while it is still being stirred, add 95g tap water (20x suspended) put it in a tube of 120 ml with a cap

5 Fill 5 object slides each containing 3 holes with 10µl of the suspension

6 Count the living and dead nematodes using a microscope
7 Calculate the amount of nematode suspension needed to have 3125 nematodes per cup:

Total amount of nematodes	236	3125 (needed for mealworms bioassay)
Volume in ml	0,150	X

X= 0,150 * 3125 / 236 = 1,99mL => 2,0mL nematode suspension.

8 Put 25 mealworms in every cup and fill up with potting soil
9 Add 5ml liquid to every cup = amount of nematodes suspension needed +tap water
10 For the control group add an extra 3 cups with mealworms, soil and 5 ml tap water
11 Place the cups at 23°C for 4 days and count the amount of alive and dead mealworms

Note:

7.6 PROTOCOL 5: MAKING THE NEMATODE FORMULATION

Protocol ID	
SOP version	
Title	Making the nematode formulation in the lab
Author	Luuk Jungerling

Background:
This protocol is used when making nematode formulations in the lab.

Materials and solutions:
- H2O buffer
- Anticake organic
- Anticake mineral
- Blixer robot coupe 4 V.V.
- Paste

Experimental procedure:
1 Fill the calculated amount of paste in a pre-made excel file, for calculating the right amounts of ingredients.
2 Add the ingredients and paste to a 2L measuring can.
3 Mix the ingredients using the Blixer robot coupe 4 V.V. for 30 seconds at speed setting 2
4 Fill three packages, 2 yellow ones and 1 clear one with the amount of formulation for 50 million nematodes
5 Store the packages of the formulation at 4°C.

Note

7.7 RESULTS 2-WAY ANOVA QUALITY CONTROL TESTS

Tests of Between-Subjects Effects

Dependent Variable: Survival

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	53.450[a]	11	4.859	.232	.995
Intercept	1018872.332	1	1018872.332	48570.062	.849
Species	9.008	1	9.008	.429	.514
Treatment	23.260	5	4.652	.222	.952
Species * Treatment	21.182	5	4.236	.202	.961
Error	2013.828	96	20.977		
Total	1020939.609	108			
Corrected Total	2067.278	107			

a. R Squared = .026 (Adjusted R Squared = -.086)

Tests of Between-Subjects Effects

Dependent Variable: Efficacy

Dependent Variable: Efficacy

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	128.991[a]	11	11.726	.374	.963
Intercept	1013651.565	1	1013651.565	32302.853	.675
Species	15.565	1	15.565	.496	.483
Treatment	83.602	5	16.720	.533	.751
Species * Treatment	29.824	5	5.965	.190	.966
Error	3012.444	96	31.380		
Total	1016793.000	108			
Corrected Total	3141.435	107			

a. R Squared = .041 (Adjusted R Squared = -.069)