The development of a formulation for the commercialization of entomopathogenic nematodes

by

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Summary

Entomopathogenic nematodes (EPNs) of the genera *Steinernema* and *Heterorhabditis*, and their associated symbiotic bacteria *Xenorhabdus* and *Photorhabdus*, are efficient biological control agents, due to their ease of culture, high rate of fatality caused against key pests, and safety. However, the large-scale commercial utilisation of EPNs as biological control agent, in integrated pest management (IPM) programmes, is limited by their finite shelf life, both in storage and formulations. Thus, efficient formulation of EPNs is essential in IPM strategies. To achieve this, nematode survival mechanisms, in terms of heat and cold tolerance, desiccation, osmotic stress / water activity (a_w), hypoxia, and energy reserves, or in formulation, and their influence on the formulation of EPNs, as well as in maintaining the quality of EPN products, should be investigated.

In this case, South African EPN species, including *Steinernema yirgalemense*, *S. jeffreyense* and *Heterorhabditis bacteriophora*, were investigated regarding their role in formulations according to various formulation techniques. These included the encapsulation of the infective juveniles (IJs) in alginate beads, as well as the use of diatomaceous earth (DE) at 6°C, 14°C and 25°C, for 4 weeks. The beads successfully retained most of the IJs with a longer storage capacity, while the survival rate for DE was still high (80%) by the fourth week.

The three EPN species researched revealed poor survival and loss of virulence at low temperatures, for both formulations. The optimisation process involved testing for the viability of *S. yirgalemense* at room temperature, and at a higher density in DE after 4 weeks, in addition to the direct effect of antifungal agents on its efficacy. Microbial contamination unequivocally lowers the quality and shelf life of EPNs in formulations. Peroxyacetic acid (PAA), trans-cinnamic acid (TCA) and nipagin were measured as antifungal agents in the study. A decline in the survival rate and pathogenicity of *S. yirgalemense*, due to PAA, was reported. In contrast, TCA and nipagin did not affect the survival rate and pathogenicity of *S. yirgalemense*. The shelf life of IJs stored in DE formulation at room temperature improved, when measured against the 80% mean survival rate of *S. yirgalemense* in week 4 at 25°C. There is lack of information on the respiratory physiology of the nematode/bacterium complex of EPNs during production, storage, and formulation. Equally important, low oxygen supply jeopardises their survival. The present study determined, by means of basal measurement, the specific oxygen consumption rate (OCR) of the IJs of *S. yirgalemense*, *S. jeffreyense*, and *H. bacteriophora*, using fibre-optic sensors. The results showed that nematode size inversely influences its OCR, with smaller nematodes, with a higher surface-area-to-volume ratio than larger nematodes, having a higher OCR. *Steinernema*

jeffreyense and *S. yirgalemense* did not significantly differ from each other in terms of the results obtained, probably due to their proximity in size, with the former being slightly larger than the latter, but they differed significantly from *H. bacteriophora*.

Water activity (a_w), as a determinant of microbial contamination, as well as desiccation, was investigated in relation to the quality and shelf life of EPNs in formulation. In the current study, the concept of determining moisture content at the corresponding a_w-values, using the Guggenheim-Anderson-Boer (GAB) isotherm model, has been studied concerning DE, as well as the survival of *S. yirgalemense*. Scanning electron microscopy was employed to determine the effect of DE on *S. jeffreyense* during storage in formulation. A decline in the survival rate of *S. yirgalemense* at high a_w-values, due to bacterial sporulation and toxin production, was reported. Scanning micrographs depicted a strong desiccative effect of DE on *S. jeffreyense*, exceeding rejuvenation on the addition of water. Desiccation was random and limited in terms of distribution throughout the sample.

Lastly, but of equal importance, because virulence remains the key standard for the measurement of nematode quality and is often determined through using either one-on-one or sand-well bioassays, which are costly in terms of laboratory consumables and time, new alternatives have been investigated. The potential for quality control of formulated *S. jeffreyense* and *S. yirgalemense* in DE, and the characterisation of different species using attenuated total reflectance (ATR), in conjunction with Fourier-transform infrared spectroscopy (FTIR) and hyperspectral imaging (HSI) tools, have been investigated. Such tools have a proven wide application in other fields of research, due to their quick, non-destructive and effective quality control techniques. Results report, for the first time, the use of ATR-FTIR spectral analysis in detecting chemometric changes in the formulated EPN product, and changes occurring over time, during storage. Such changes are mainly for purposes of nematode survival, due to environmental stresses. HSI tools were able to differentiate between variables, in terms of differences in nematode densities in the formulated sample. For EPN characterisation, the study reports close similarities among the species, as detected by the ATR-FTIR.

The above findings provide a much-required working formulation for the commercial application of EPN. However, much research still needs to be done, especially in areas such as the use of fibre-optic sensors for oxygen measurement, ATR-FTIR and HSI in quality control to draw realistic and meaningful conclusions.

Opsomming

Entomopatogeniese nematodes (EPNs) van die genera *Steinernema* en *Heterorhabditis*, tesame met hul geassosieerde simbiotiese bakterieë, is welbekende effektiewe biologiese beheer agente. Dit word toegeskryf aan die gemak waarmee hul aangeteel kan word, hul sukses in die beheer van belangrike pes insekte, asook hul veiligheid. Die kommersiële gebruik van EPNs as ’n biologiese beheermiddel in geïntegreerde pes beheer (GPB) programme word egter gekniehalter deur hul beperkte rakleeftyd na formulering en opberging. Die effektiewe formulasie van EPNs is dus noodsaaklik vir GPB strategieë. Om die formulasie van EPNs te optimaliseer, moet die oorlewingsmeganismes van nematodes ondersoek word in terme van hitte en koue toleransie, uitdroging, osmotiese stres / water aktiwiteit (a_w), hipoksie en energie reserwes, asook die invloed daarvan op die formulasie van EPNs en die handhaaf van die kwaliteit van EPN produkte.

In hierdie studie was drie Suid-Afrikaanse EPN spesies, *Steinernema yirgalemense*, *S. jeffreyense* en *Heterorhabditis bacteriophora* ondersoek vir faktore wat hul formulering sal beïnvloed, volgens verskillende formulasie tegnieke. Die twee tegnieke wat ondersoek was, was die formulering van infektiewe larwes (ILs) in alginaat korrels, asook die gebruik van diatomiet, by 6°C, 14°C en 25°C, vir 4 weke. Die korrels het die meeste van die ILs suksesvol binne gehou en ook ’n langer bergingskapasiteit gehad, terwyl die oorlewings van die nematodes in diatomiet steeds hoog was (80%) teen die vierde week.

Al drie EPN spesies het lae oorlewingsgetalle en verminderde infektiwiteit getoon by laer temperature, in beide formulasies. Die optimaliseringsproses was gefokus daarop om die lewensvatbaarheid te toets van *S. yirgalemense* by kamertemperatuur, asook in diatomiet by ‘n hoër temperatuur na 4 weke, tesame met die direkte effek van teen-swam middels op die effektiwiteit van die nematode. Mikrobiese kontaminasie verlaag die kwaliteit en rakleeftyd van EPNs in formulasie. Peracetic suur (PAS), trans-kaneel suur (TKS) en Nipagin was die teen-swam middels wat getoets was in hierdie studie. Resultate het getoon dat PAS gelei het tot ’n afname in die oorlewing en infektiwiteit van *S. yirgalemense*. TKS en Nipagin het egter nie oorlewing of infektiwiteit beïnvloed nie. Die rakleeftyd van ILs wat gestoor was in diatomiet by kamertemperatuur het verbeter, teenoor die 80% gemiddelde oorlewingskoers van *S. yirgalemense* in week 4 by 25°C. Daar is ’n tekort aan informasie oor die respiratoriese fisiologie van die nematode/bakterieë kompleks van EPNs gedurende produksie, berging en formulering. Dit is wel bekend dat lae suurstof vlakke in die formulasie die oorlewing van die nematodes benadeel. Met gebruik van veseloptiese sensors en basale metings, is die spesifieke koers waarteen suurstof verbruik word deur die ILs van *S. yirgalemense*, *S. jeffreyense* en *H.*

bacteriophora, bepaal. Resultate het getoon dat nematode grootte 'n omgekeerde effek het op suurstofverbruik, wat beteken dat kleiner nematodes wat 'n hoër oppervlak-tot-volume verhouding gehad het, hoër suurstofverbruik getoon het. Daar was nie 'n beduidende verskil in die suurstofverbruik resultate van *S. jeffreyense* en *S. yirgalemense* nie, moontlik omdat hulle bykans dieselfde grootte is, met *S. jeffreyense* wat effens groter is as *S. yirgalemense*. Albei het wel beduidende verskille getoon in vergelyking met die suurstofverbruik van *H. bacteriophora*.

Water aktiwiteit (a_w) as 'n bepaler van mikrobiese kontaminasie, sowel as uitdroging, was ondersoek met betrekking tot die kwaliteit en rakleeftyd van EPNs in formulasie. In die huidige studie, is die konsep van die bepaling van vog inhoud by ooreenstemmende a_w waardes ondersoek in diatomiet, deur gebruik te maak van die Guggenheim-Anderson-Boer (GAB), sowel as die oorlewing van *S. yirgalemense*. Skandeer elektron mikroskopie is gebruik om die effek van diatomiet op *S. jeffreyense* te ondersoek in berging, sowel as in formulasie. Die resultate het 'n afname getoon in die oorlewing van *S. yirgalemense* by hoë a_w-waardes, as gevolg van die groei van bakterieë en toksien produksie. Skandeer mikrograwe het getoon dat diatomiet 'n sterk uitdrogingseffek het op *S. jeffreyense*, sterker as die herstelproses van die nematode wat plaasgevind het wanner water bygevoeg was. Uitdroging was lukraak en beperk in terme van verspreiding deur die monster.

Die laaste deel van die studie was gefokus op die infektiwiteit van die nematode. Infektiwiteit is een van die belangrikste faktore in die bepaling van die kwaliteit van nematodes en word gewoonlik getoets met een-tot-een of sand biotoetse. Hierdie metodes verg egter baie tyd in terme van laboratorium verbruiksgoedere, wat gelei het tot die ontwikkeling van nuwe metodes. Die potensiaal vir die kwaliteitsbeheer *S. jeffreyense* en *S. yirgalemense,* geformuleer in diatomiet, asook die karakterisering van verskillende spesies is ondersoek, met gebruik van verswakte totale refleksie (VTR) tesame met Fourier-transformasie infrarooi (FTIR) spektroskopie asook hiperspektrale beeldanalise (HSB) tegnieke. Hierdie metodes word algemeen gebruik in ander navorsingsvelde, as gevolg van hul vinnige, nie-afbrekende en effektiewe kwaliteitsbeheer tegnieke. Die resultate toon dat VTR-FTIR spektrale analise vir die eerste keer gebruik was om chemometriese veranderinge op te spoor in die geformuleerde EPN produk, sowel as veranderinge oor tyd gedurende berging. Die funksie van sulke veranderinge is meestal vir die oorlewing van nematodes, as gevolg van omgewingstres. HSB tegnieke was suksesvol gebruik om te onderskei tussen veranderlikes, in terme van verskille in nematode digthede in die geformuleerde voorbeeld. In terme van die karakterisering van EPNs soos ondersoek deur die VTR-FTIR, het die resultate nabye ooreenkomste tussen spesies getoon.

Die bevindinge van die studie verskaf 'n belangrike werkende formulasie vir die kommersiële gebruik van EPNs. Baie navorsing moet egter steeds gedoen word, veral in areas soos die gebruik van veseloptiese sensors vir die meet van suurstofvlakke, VTR-FTIR en HSB in kwaliteitsbeheer, om betekenisvolle gevolgtrekkings te kan maak.

This dissertation is dedicated in the loving memory of my late mom Florence Nalwoga and the power of her broken cup that has been a driving force in my academics.

Biographical sketch

My name is Nicholas Kagimu and I'm a Ugandan. I hold a BSc (Honours) in Horticulture, Makerere University 2009, and MSc in Nematology, Ghent University, 2015. With a PhD completed, I look forward to postdoctoral research leading to full lectureship and University academic career as well as industry.

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Preface

This dissertation is presented as a compilation of seven chapters. Each chapter is introduced separately and is written according to the style of the Journal Nematology.

Chapter 1 **General Introduction, Literature review and project aims**

The attributes of survival in the formulation of entomopathogenic nematodes utilised as insect biocontrol agents

Chapter 2 **Research results**

Formulation of *Steinernema yirgalemense, S. jeffreyense* and *Heterorhabditis bacteriophora* in alginate beads and diatomaceous earth

Chapter 3 **Research results**

Effect of antifungal agents on the efficacy of *Steinernema yirgalemense* and room temperature storability in diatomaceous earth

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Chapter 1

Literature review

[1]The attributes of survival in the formulation of entomopathogenic nematodes utilised as insect biocontrol agents

Abstract

Entomopathogenic nematodes (EPNs) of the genera *Steinernema* and *Heterorhabditis* and their associated symbiotic bacteria of the genera *Xenorhabdus* and *Photorhabdus*, are efficient biological control agents, due to their ease of culture, their high caused fatality against key insect pests and their safety in use. However, their commercial utilisation is limited by their finite shelf life, both in storage and in formulations. Thus, efficient storage in the formulation of EPNs is essential so as to attain success in integrated insect pest management strategies. This paper reviews the latest information that is available on EPN storage, formulation, quality and application methods, coupled with improvement strategies for the effective control of insects. Nematode survival mechanisms investigated were heat and cold tolerance, desiccation, osmotic stress, hypoxia and energy reserves, among others, in storage, field or formulations. Their influence on the formulation of EPNs is also discussed.

Key words: above-ground application, formulation, *Galleria mellonella, Heterorhabditis, Steinernema,* shelf life, survival, virulence.

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Introduction

Entomopathogenic nematodes (EPNs) from the *Steinernema* Travassos, 1927 (Rhabditida: Steinernematidae) and *Heterorhabditis* Poinar, 1976 (Rhabditida: Heterorhabditidae) and their associated symbiotic bacteria *Xenorhabdus* (Enterobacteriales: Enterobacteriaceae) Thomas & Poinar 1983 and *Photorhabdus* (Enterobacteriales: Enterobacteriaceae) Boemare, Akhurst & Mourant, 1993 (Boemare *et al.* 1993; Akhurst *et al.* 1996; Ehlers, 2007) are efficient biological control agents, due to their ease of culture, their high fatality caused against key pest insects, and their safety in use (Grewal, 2002). Species mainly commercially investigated include *Steinernema carpocapsae* (Weiser, 1955) Wouts, Mráček, Gerdin & Bedding, 1982, *S. feltiae* (Filipjev, 1934) Wouts, Mráček, Gerdin & Bedding, 1982 and *Heterorhabditis bacteriophora* Poinar, 1976. They are utilised in controlling a wide range of insect pests (Peters, 1996) that occur in soil and cryptic environments (Grewal & Peters, 2005) worldwide (Hominick, 2002; Campos Herrera *et al.,* 2012). Nevertheless, up-to-date information on their geographic distribution is obscured and it is unrealistic, since it is either prejudiced/biased, or it is influenced by the researchers' interests in nematology research and where the latter are based, with more sampling taking place in Europe and North America, and considerably less in Africa (Stock, 2005; Campos Herrera *et al.,* 2012; San-Blas, 2013).

Equally important, EPNs do not add to biological pollution and are environmentally safe; they are also specific to the intended insect pest, with no detrimental outcomes, unlike chemical insecticides (Ehlers, 2003). They can be mass-produced on an industrial scale in liquid culture, due to the scale up and the downstream processing of large culture volumes, which can be mechanised, thus reducing production costs (Ehlers, 2001; Shapiro-Ilan & Gaugler, 2002; Ehlers & Shapiro-Ilan, 2005). Many countries have exempted EPNs from registration obligations (Akhurst & Smith, 2002). In addition, EPNs can be incorporated into integrated pest management (IPM) programmes (Grewal, 2002); which has enabled both small and medium-sized enterprises to develop nematode-based plant protection products (Ehlers, 2003).

For both the steinernematid and heterorhabditid nematodes, the dauer or infective juvenile (IJ) stage is the only free-living stage. Their mutualistic bacteria are the actual killing agents on entry into the target pest insect, where they multiply in the insect, and cause septicaemia, resulting in the death of the insect (Ciche *et al.,* 2006). Although the IJs are non-feeding since their mouth and anus are closed (Grewal *et al.,* 2002); they can persist for a long time in the soil, until a potential host turns up. They enter mostly the soil-inhabiting insect stages, through the anus, mouth, and trachea, or even through the cuticle (Ehlers, 2001). The released bacteria, once they are within the host insect, change phase from a dormant stage

to a metabolically active stage. They then digest the haemolymph, propagating and producing toxins, enzymes and other metabolites that suppress the host's defence mechanism. The insect dies within 24 to 48 hours after invasion (Dunphy & Webster, 1988; Burnell & Stock, 2000; Dowds & Peters, 2002; Webster *et al.,* 2002). The nematodes develop into adults, feed on the bacteria, and reproduce. After approximately three generations, when all the nutrients of the insect carcass are depleted, the development of IJs is induced, as a result of the food depletion and the high nematode density. The IJs then leave the cadaver, so as to find a new host (Han & Ehlers, 2000, 2001; Ehlers, 2001).

Moreover, EPNs are so immensely competent in the soil that on some occasions, they exceed the control results obtained with chemical compounds, probably due to their mobility (Ehlers, 2003). EPNs can be stored for a few months, which aids in the marketing of nematode-based products (Grewal & Peters, 2005). They have extended prospects of large-scale outdoor use, with production estimates of roughly 35,000 ha per year, chiefly against soil-dwelling insect pests in high value crops and in turf (Shapiro-Ilan *et al.,* 2002; Shapiro-Ilan *et al.,* 2012). Presently, EPNs are chiefly used on occasions where no other control procedures against a specific insect are available. Examples of such use are against insects that were once thought impossible to control like Coleoptera, or in habitats where chemical compounds are found wanting/failing/lacking, primarily being in the soil, in galleries of boring insects, and on occasions of resistance to insecticides (Ehlers, 2003; Grewal & Peters, 2005).

More so, EPNs are competent biological control agents against insect pests, but their commercial use is limited by their finite shelf life, although some species have already become commercially available (Gaugler *et al.,* 2000). However, their short shelf life is a major drawback to their large-scale commercial use (Grewal, 2000a,b). The poor survival rate at room- temperature storage is a major hindrance for their potential use as bio-insecticides (Grewal, 2002). Their ability to survive is poor in terms of desiccation (Womersley, 1990; Surrey & Wharton, 1995).

Factors affecting nematode survival

Research is currently under way to understand the underlying factors influencing the survival and the longevity of EPNs in storage, with recent emphasis on the in-progress genome sequence of the *H. bacteriophora* TTO1 strain. More so, the prolonged survival of IJs without food at temperatures that are favourable for the normal growth and reproduction of EPNs has led to the asking of interesting questions about the latent mechanisms and the genetic factors controlling the metabolism and the survival of the IJs concerned (Grewal *et al.,* 2011). Several studies have investigated the following factors:

HEAT AND COLD TOLERANCE

The capability of EPNs to tolerate such environmental stressors as desiccating or freezing conditions, can add significantly to their insecticide efficacy. Thus, when selecting nematodes for use in specific biocontrol programmes, it is essential to be able to predict which strain or species to utilise in target areas where environmental stress is anticipated (Shapiro-Ilan *et al.,* 2014). Temperature is a major environmental factor influencing the life processes of all organisms. Certain EPN species are known to be able to adapt to both cold and warm environments (Grewal *et al.,* 2006). Temperature has a resounding influence on IJ longevity, with the extent of the influence differing with the EPN species involved (Grewal, 2000a). For example, Hill *et al.* (2015) reported that acclimation has been recorded as having both negative and positive effects on the temperature stress survival of *Steinernema yirgalemense* Nguyen, Tesfamariam, Gozel, Gaugler & Adams, 2004 and on that of *Heterorhabditis zealandica* Poinar 1990, despite it being with non-significant overall variation. Mass-produced EPNs are often stored for variable spans of time preceding their application in the field. Heterorhabditid nematodes like *H. bacteriophora*, however, have poor storage capability (Grewal, 2002), as compared with such steinernematids as *S. carpocapsae, S. feltiae,* and *Steinernema riobrave* Cabanillas, Poinar & Raulton, 1994 (Grewal, 2000a; Grewal *et al.,* 2002; Ebssa & Koppenhöfer, 2012).

Some nematodes are able to survive temperatures as low as -80°C (Glazer 2002), which is a temperature at which the metabolism is liable to have ceased. For example, it has been established that *S. feltiae, S. arenarium* (Artyukhovsky, 1967) Wouts, Mráček, Gerdin & Bedding) (syn. *S. anomali* (Kodzodoi, 1984) Curran, 1989) and *H. bacteriophora* are all freezing tolerant with the lower lethal temperature (LLT) of -22, -14 and -19°C, respectively (Brown & Gaugler, 1995). Cryopreservation studies (Popiel & Vasquez, 1989; Curran *et al.,* 1992) have shown that EPNs can be stored indefinitely in liquid nitrogen. Ali & Wharton (2013) observed that the cold survival of *H. bacteriophora* IJs, after being subjected to freezing conditions overnight at -1°C, was similar to that of *S. feltiae* IJs, with a LLT of -13°C. The above-mentioned researchers also detected that the ability of *S. feltiae* and their infectivity of *Galleria mellonella* L. (Lepidoptera: Pyralidae) larvae were not affected in IJs that survived freezing at -13°C. However, no meaningful increase in survival occurred after acclimation, or rapid cold-hardening in *H. bacteriophora*. On the contrary, (Jagdale & Grewal, 2003) reported that the level of improved thermal stress tolerance due to accumulation of trehalose varied with nematode species. Nevertheless, high amounts of trehalose are accumulated by EPNs during freezing (Jagdale & Grewal, 2007; Jagdale & Grewal, 2003).

OSMOTIC STRESS

The existing shipping system for EPNs requires low temperature conditions. If IJs in a physiological state induced by a salt solution can be shipped at room temperature, and under elevated temperatures, without losing their viability, their intensive application in the field stands to improve substantially (Feng *et al.,* 2006). Also, the IJs in their heat-tolerance state are very suitable for foliar application, in cases where cumulative osmotic stress occurs as water evaporates, and when high temperatures occur as a result from the sun (Feng *et al.,* 2006). Approaches of partial anhydrobiosis or quiescence (Womersley, 1990) initiation include absorption, desiccation and osmotic dehydration (Feng *et al.,* 2006). Osmotic dehydration offers the following advantages over evaporative dehydration: (1) large numbers of nematodes can be treated under dehydration pressure, for example, if their acclimatisation is necessary, with their rate of increase being simply and precisely controlled; (2) the nematodes can be separated from the solution without difficulty; and (3) many IJs can be equally exposed to levels of dehydration pressure, so as to facilitate more reproducible results than might otherwise be obtained (Qiu *et al.,* 2000; Yan *et al.,* 2010). Nematodes in anhydrobiotic state are extremely resilient to extreme environment conditions, including those that are subject to hypoxia, radiation and metabolic poisons that are lethal to active organisms (Glazer & Salame, 2000; Grewal, 2000b). Efforts to induce EPNs into partial anhydrobiosis through imposing such conditions on them to increase their shelf life have been successful (Chen & Glazer, 2005; Feng *et al.,* 2006). Osmotic dehydration usually uses two types of osmotic solutions: ionic solutions, like different salts, and non-ionic solutions, such as glycerol, sucrose and polyethylene gels (Glazer & Salame, 2000). In the formulation of stable nematode products by means of the partial dehydration of IJs, osmotic dehydration has benefits over desiccative dehydration in terms of standardisation, the effective processing of large quantities of nematodes under dehydration pressure (Qiu *et al.,* 2000), and ease of application in the field (Feng *et al.,* 2006).

Various species of EPN and strains within a species have been reported to show variation in their tolerance to dehydration, either by means of disclosure to relative humidity (RH), or to osmotic solutions (Grewal *et al.,* 2002; Yan *et al.,* 2010). An investigation by Yan *et al.* (2011) indicated that a range of different strains of *S. carpocapsae* are tolerant to osmotic treatment, and that the treatment impressively increases the heat tolerance of such strains.

Desiccation

Strauch *et al.* (2004); in their work on genetic improvement of *H. bacteriophora* using selective breeding technique observed its low tolerance to desiccation. Certainly, this is also true of other EPNs. Desiccation can have a robust effect on the longevity of EPN IJ (Grewal

et al., 2011). EPNs can endure only a limited amount of desiccation or of partial anhydrobiosis in a quiescent state (Womersley, 1990). Simons & Poinar (1973) were the first to validate that *S. carpocapsae,* when air-dried slowly at 97% RH at room temperature, could endure subsequent exposure to much lower RH. It has been proven that desiccation tolerance varies noticeably among species and strains of EPNs using glycerol as an osmolyte. For example, *H. bacteriophora* IJs could only survive exposure to 25% glycerol for 72 h at 25°C, with their survival varying between 25% and 90% among 15 *H. bacteriophora* strains (Grewal *et al.,* 2002). Also, Mukuka *et al.* (2010) reported that desiccation tolerance, with or without previous adaptation varied among *H. bacteriophora* strains from diverse geographic zones.

Although the physiological mechanisms involved in the initiation of anhydrobiosis are not fully understood (Grewal *et al.,* 2011), a relationship between the accumulation of polyols and sugars, and their function in protecting intracellular proteins in addition to biological membranes, in the course of dehydration has been documented in various anhydrobiotic nematodes (Womersley, 1990). For example, a correlation between glycerol or trehalose accumulation and increased desiccation tolerance has been noted in *Heterorhabditis megidis* Poinar, Jackson & Klein, 1987*, Heterorhabditis indica* Poinar, Karunakar & David, 1992 and *S. carpocapsae* by O'Leary *et al.* (2001). The preconditioning of *S. feltiae, S. carpocapsae* and *H. bacteriophora* at 97% RH for 3 days improved their survival at 85% and 75% RH (Womersley, 1990; Solomon *et al.,* 1999), which has been correlated with the synthesis of trehalose, glycerol or water stress-related proteins (Solomon *et al.,* 1999; Grewal *et al.,* 2006).

As reviewed by Grewal *et al.* (2011), the ability of anhydrobiotic organisms to tolerate desiccation is largely associated with the accumulation of carbohydrates, including trehalose and water stress-related proteins. Trehalose protects membranes and proteins from desiccation and freezing injuries by substituting the structural water that is associated with the phospholipid bilayer, upholding membrane fluidity, in addition to retaining the duo layer in the liquid crystalline state and by forming glass (vitrification) to stabilise the cell content. During desiccation, trehalose equally safeguards proteins by replacing ‘bound water' in addition to decreasing the 'browning' or Maillard reaction. Further reviewed by Grewal *et al.* (2011), over 13,000 protein-coding sequences have been predicted from the in-progress genome sequence of *H. bacteriophora* TTO1 strain (Bai *et al.* unpubl.).

Hypoxia

In view of the fact that nematodes are aerobic organisms, hypoxic environments can decrease their survival and longevity. Details of the effect of soil oxygen on EPNs are further restricted. Burman & Pye (1980) reported that S. *carpocapsae* IJs could survive oxygen tensions of as low as 0.5% saturation at 20°C for 43 days. In sandy soil, in contrast, the survival

of S. *carpocapsae* and of *S. glaseri* lessened significantly after 8 weeks, as tested oxygen intensities declined from 20% to 1%, with no nematode survival being noted after 16 weeks (Kung *et al.,* 1990). Significant differences exist in the capability of EPN species and strains to survive hypoxic conditions (Grewal *et al.,* 2011). For instance, survival at about 0% dissolved O_2 at 25°C for 96 hours, differing significantly among *H. bacteriophora* populations collected from various zones (Grewal, 2002). In addition, a great genetic variability for tolerance to hypoxia, heat, and UV, as well as to desiccation, in addition to IJ longevity, was noted in the inbred lines of two strains (GPS11 and HP88) of *H. bacteriophora* (Sandhu *et al.* unpubl., as cited in Grewal *et al.* (2006). A positive correlation was found between IJ longevity, heat, and UV, as well as was hypoxia tolerance, in the inbred lines of both GPS11 and HP88 strains. In spite of this, the relationship between IJ longevity and desiccation tolerance differed in the inbred lines of the two strains. Nevertheless, S. *carpocapsae* can tolerate similar conditions (Grewal *et al.,* 2002) for up to 10 days (Somasekhar *et al.,* 2002). Similarly, there is more variation in hypoxia tolerance among strains of *H. bacteriophora* (Grewal *et al.,* 2002) than there is among strains of S. *carpocapsae* (Somasekhar *et al.,* 2002).

BIOCHEMICAL ENERGY RESERVES AND SURVIVAL

The presence of lipids is undoubtedly, imperative for survival as a chief energy reserve for non-feeding IJs, and infectivity with regard to lipid reserves has been studied (Perry *et al.,* 2012). Equally important, a number of reports have shown (Patel *et al.,* 1997a,b; Patel & Wright, 1997a,b,c) that nematode infectivity drops as energy reserves are depleted in storage. Such climatic influences as temperature, oxygen and nematode activity in the course of storage (Grewal & Georgis ,1999) tend greatly to affect the rate of lipid utilisation, which further differs between EPN species (Grewal, 2000b), as well as among individuals within a species. The quantity of lipids that are present in the IJs differs with the nematode species, and with different batches (Grewal & Georgis, 1999). The amount of stored energy reserves is a major factor in determining IJ longevity, with lipids constituting approximately 34-60% of the dry weight of IJs (Selvan *et al.,* 1993; Fitters *et al.,* 1999).

In commercial production systems, the lipid content of IJs is predisposed by such factors as the amount and type of media components, in addition to antifoam utilised, the temperature, as well as the amount of liquefied oxygen that is available during fermentation (Grewal, 2002). What is more, it is more cost-effective to produce steinernematid nematodes that are capable of surviving long-term storage better than to produce heterorhabditid nematodes that are less so (Georgis & Gaugler, 1991; Kaya & Gaugler, 1993). In spite of this, the former's efficacy against specific target insects is not as good as is that of *Heterorhabditis*. Selvan *et al.* (1993) contended that the poor storage stability of *H. bacteriophora* is possibly due to the existence

of large quantities of unsaturated fatty acids in the freshly emerged IJs. Such was inferred upon the analysis of lipid content, and upon the realisation of a positive correlation between the fatty acids composition of the total lipid and the surge in water content. Furthermore, Fitters *et al.* (1999) reported that the fatty acid patterns were similar between the three isolates of *Heterorhabditis* from diverse geographical backgrounds. Oleic (C18:1n−9), palmitic (C16:0), and linoleic (C18:2n−6) acid prevailed with 51, 13 and 12%, respectively, in the total lipid (TL) of fresh nematodes (in terms of the average for the three isolates). The levels of unsaturation, in respect of the Unsaturation Index (UI), of fresh nematodes were on average 110, 112, 113 and 152 for the TL, neutral lipid (NL), phospholipid, and free fatty acid fractions, correspondingly (Fitters *et al.,* 1999).

Additionally, high lipid levels supply non-feeding IJs with the necessary energy for host-finding or persistence in the soil, in the unavailability of hosts. The shelf life of commercially produced nematodes is similarly reliant on the quantity of energy reserves stored, and on the rate of utilisation throughout storage (Selvan *et al.,* 1993). Next, the UIs of TLs and phospholipids increased in *S. feltiae* and *S. carpocapsae* as the culture, or storage temperature, diminished from 25 to 5°C (Jagdale & Gordon, 1997a,b). Equally important, Fitters *et al.* (1997) reported that UIs of the phospholipids of two strains of *H. megidis* increased throughout a 5-week storage period, during which time they were kept at 5°C. Again, the IJs of EPNs comprise high levels of NLs that are utilised as energy substrates (Selvan *et al.,* 1993). Jagdale & Gordon (1997b) also affirm their belief that the increased unsaturation of TLs at cold temperatures was due to an increase in the proportion of polyunsaturated fatty acids present, with an associated amount of reduction in the proportion of saturated fatty acids, mainly consisting of palmitic (16:0) and stearic (18:0) acids. A modification in the action of metabolic enzymes, and in the proportion of saturated and unsaturated fatty acids, as well as in the synthesis of novel isozymes, sugars, and polyols, together with trehalose and glucose, in addition to the heat-shocking proteins are among the physiological mechanisms that are undertaken by EPN IJs in the course of survival under cold or overly warm conditions (Grewal *et al.,* 2006).

The further exposure of IJs to reasonable stress circumstances facilitates the synthesis of such molecules as trehalose, which is vital for membrane protection in the course of water loss (Womersley, 1990; Perry *et al.,* 2012). Desiccation survival, as well as trehalose, have long been implicated in the above; for instance, some nematode anhydrobiotes, such as the second-stage juveniles of *Anguina tritici* (Steinbuch, 1799) Chitwood, 1935 and *Ditylenchus dipsaci* (Kühn, 1857) Filipjev, 1936 J4, segregate trehalose. The latter is often recommended as a protectant against desiccation, due to the importance of membrane stability preservation, thus it helps to prevent protein denaturation, as well as acting as a free-radical scavenging

agent (Glazer, 2002; Perry *et al.,* 2012). Trehalose accumulation was noted among *S. feltiae* that had been slow dried at high RH (Solomon *et al.,* 1999; Perry *et al.,* 2012). However, conflicting reports exist concerning the significance of trehalose (Burnell & Tunnacliffe, 2011). Although the synthesising of trehalose in the course of dehydration might show initial preparation in dehydrated/arid situations, it does not, essentially, provide assurance of survival throughout subsequent severe desiccation (Perry *et al.,* 2012). For one thing, during initial reviews, a surge in the trehalose level in *Steinernema feltiae* IS-6 strain, in the course of pre-conditioning treatment, was established (Solomon & Glazer, unpubl.) cited in Solomon *et al.* (1999).

Patel & Wright (1997a) also reported that EPN IJs also have considerable amounts of glycogen at their disposal, in addition to lipids and trehalose. They reported that glycogen levels differed from 8% dry weight in *S. riobrave* to 18% in *Steinernema glaseri.* IJs of both *S. carpocapsae* and *S. riobrave* survived for 120 to 135 days and utilised 90% of their glycogen reserves at a nearly continuous rate in the course of a 112-day storage period. The IJs of *S. feltiae* and *S. glaseri* lived for much longer (>450 days) than did the above, but their glycogen content diminished by 27 and 40%, respectively, in the course of a 250-day storage period. In contrast to other species, the degree of lipid decline surpassed that of glycogen in *S. carpocapsae.*

Furthermore, although glycogen which is an important energy reserve, occurs in considerable quantities in some EPNs (Selvan *et al.,* 1993; Boemare, 2002), its significance has not yet been determined. Subsequent results established that *S. feltiae, S. carpocapsae, S. riobrave,* and *S. glaseri*'s glycogen content dropped in the course of storage. Patel *et al.* (1997b) proposed that glycogen might play a noteworthy, perhaps even a superior, role to NLs in the conservation of the infectivity of the species mentioned.

The longevity of IJs of EPNs is a function (proportionally) of their metabolic rate, in addition to their primary energy reserve concentrations (Boemare, 2002). The accessibility of energy reserves is indispensable to supporting the physiological and behavioural processes that are associated with adaptation to environmental stress (Glazer, 2002). Finally, expounding on the metabolic and physiological processes that are involved in IJ host-finding and survival should offer data that might enable the preloading of IJs, in the course of the production process, with adequate storage material to support their activities and persistence (Grewal *et al.,* 2006).

CULTURE METHOD

Nematodes produced *in vivo* have been cited in Grewal (2002) as being relatively steady in both the laboratory and the field environments in contrast to those that are produced *in vitro*. For instance, the endurance of *S. riobrave* in water at 9°C was greater when they were

cultured in larvae of *G. mellonella*, as compared to their culturing in liquid media. Evidently, this is because the nutritional status of *G. mellonella* larvae; and thus, the quality of IJs, varies from that produced in the much-simplified fermenter environment; which further varies between EPN species and strains (Ebssa & Koppenhöfer, 2012; Griffin, 2015). Nonetheless, the procedures for these disparities, which have not yet been investigated, might offer hints as to the physiological factors upsetting storage stability. The exposure of nematodes, in the course of culturing, to such factors as temperature stress, oxygen deficiency, shear stress, and kind and amount of antifoam, in addition to microbial adulteration, can impact on nematode quality, leading to decreased shelf life. Nematodes are extremely shear sensitive, so that their use in stirred fermenters can impact on their reproduction. Shear stress during fermentation can, correspondingly, lessen the survival rate of nematodes in formulations. In stirred fermenters, batches of *S. carpocapsae* were found to have extreme negative correlation with impeller tip speed, as well as with nematode endurance at 25°C, as reviewed by Friedman (1990).

ANTIMICROBIAL AGENTS

Microbial contamination is an important setback in the case of nematode formulations with elevated moisture content (Grewal, 2002). Contamination can exhaust the existing amount of oxygen, decrease the disposability of formulations, instigate the clogging of spray nozzles, and decrease the adequacy of the product. Even though antimicrobial agents can be used to subdue microbial growth, care must be taken with their use, as they can decrease the nematode survival rate in the formulations concerned. What is more, nematode species vary in their vulnerability to antimicrobial agents. For instance, both *S. feltiae* and *S. riobrave* are more susceptible to Proxel, a commonly used antimicrobial agent, than is *S. carpocapsae* (Grewal, 2002). *Heterorhabditis bacteriophora* is also predisposed to bacterial contamination in the course of storage (Grewal & Georgis, 1999).

Storage, formulations and application

The term ‘formulation’ relates to the preparation of a product from a constituent by means of combining precise functional (active) coupled with inert (non-active) ingredients. It is imperative to note that formulation, as well as quality control attributes, are very useful in the commercialisation of nematodes as biocontrol agents. Mass-produced nematodes are formulated for the conservation of quality and simplicity. They are also formulated for purposes of the enhancement of storage stability, shelf life and field efficacy, as well as for the reduction of transport costs and application (Georgis, 1990; Georgis *et al.,* 1995; Georgis & Kaya, 1998; Jones & Burges, 1998; Grewal, 2002; Shapiro-Ilan & Gaugler, 2002; Grewal & Peters, 2005; Hiltpold, 2015).

In spite of the fact that EPN IJs can be stored in water for several months in refrigerated aerated tanks, the high cost and the complications of maintaining quality prohibit the frequent use of this method. Furthermore, the sensitivity of various species to low temperatures, the settling of nematodes, their high oxygen requirements, their vulnerability to microbial adulteration, and the effect of antimicrobial agents on nematode survival are some of the major aspects influencing nematode quality in the course of their storage in water (Georgis, 1990; Georgis *et al.,* 1995; Grewal, 2002; Shapiro-Ilan & Gaugler, 2002; Grewal & Peters, 2005). Equally important, their formulation is generally intended to enhance their activity, absorption, and delivery, as well as their simplicity of usage or the storage stability of a functional component. Besides the above, the constituents of nematode formulations are comparable to those of pesticide formulations, with the ingredients, or additives, comprising antimicrobial agents, antioxidants, absorbents, adsorbents, binders, anticaking agents, carriers, dispersants, humectants, surfactants, preservatives, thickeners, solvents, and ultraviolet (UV) absorbers. Nematodes present unique challenges. For instance, the high oxygen and moisture requirements of concentrated nematodes, their sensitivity to temperature extremes, and the behaviour of IJs constrains the choice of mode of formulation, in addition to the ingredients (Georgis *et al.,* 1995; Georgis & Kaya, 1998; Jones & Burges, 1998; Grewal, 2002; Grewal & Peters, 2005).

According to Ehlers (2007), the successful introduction of EPNs into biocontrol practice decisively entails the immediate formulation of nematodes after production, so as to reduce the resulting number of dead nematodes, as many nematodes die in storage. For example, Matadamas-Ortiz *et al.* (2014) reported that the age of the IJs, among other factors experienced during storage at room temperature, decreased the EPN survival of encapsulation, and the recommended use of fresh IJs. Also, research is still required into the species and strain of EPNs in terms of their proper storage and formulation (Strauch *et al.,* 2000), since their physiology, ecology and behaviour vary (Ravensberg, 2011).

Nematode formulations for storage and transport are generally used in one of two forms. The one method entails the consignment of nematodes to inert carriers that allow them free gas exchange and movement. Such inert carriers as polyether–polyurethane sponge and vermiculite are extensively utilised for the storage and transport of small numbers of nematodes. The formulations are easy and relatively expensive to produce, despite requiring continuous refrigeration, as the nematodes remain active, freely moving in, or on the substrates. The shelf life of the formulations under refrigeration (2–10°C) varies from 1 to 3, or 4 months, subject to the nematode species concerned. A major drawback to the use of this method is the strict refrigeration requirement, even during transportation, which renders the formulations involved extremely costly for the customer (Georgis *et al.,* 1995; Grewal, 2002;

Shapiro-Ilan & Gaugler, 2002; Grewal & Peters, 2005). For one thing, the positioning of nematodes in such inert carriers as sponge or vermiculite, offers a suitable means of shipping small quantities of nematodes, despite the fact that the high activity of the nematodes concerned rapidly depletes their stored energy reserves. Occasionally, the nematodes emerge from the inert carriers and dry out. Therefore, in the second method, formulations have been developed in which the mobility/metabolism of nematodes is reduced by physical trapping, by the addition of metabolic inhibitors, or by using the induction of partial anhydrobiosis (Grewal & Georgis, 1999; Grewal & Peters, 2005).

The most recent and comprehensive reviews on formulation are those of Grewal (2002) and Grewal & Peters (2005), with all later research on formulation being mere modifications of the already existing formulations. Examples of the above include nematodes that are physically confined in alginate and flowable gel formulations that hold sufficient moisture to avert the induction of nematode anhydrobiosis. In a particular formulation, sheets of calcium alginate stretched over plastic screens have been employed to trap nematodes (Georgis, 1990). The entrapping of nematodes in alginate gels permits storage at room temperature. For example, in one alginate gel formulation, *S. carpocapsae* can be kept for 3 to 4 months at 25°C, while *S. feltiae* can be kept for 2 to 4 weeks (Grewal, 2002). In addition, Chen & Glazer (2005) disclosed that *S. feltiae* IJs could progressively enter a quiescent state in the calcium alginate granules in the course of 6 months of storage. They further affirmed that their study was the first time that osmotically treated nematodes had been stored in calcium alginate granules at room temperature for such a prolonged period of time.

The use of alginate capsules, or beads has of late become a highly sought-after formulation technique. Other microorganisms and/or structures, like the hyphae of the endoparasitic nematophagous fungi *Hirsutella rhossiliensis* Minter & Brady 1980 (Ascomycota: Hypocreomycetidae), feeding stimulants, or plumes of plant roots are combined with EPNs, with the sole purpose of baiting insects, to reduce the mobility of EPNs, and/or to control plant-parasitic nematodes (Hiltpold, 2015). This technique, though still requiring improvement, streamlines the transportation, storage, handling, and application of EPNs using seed planters (Hiltpold, 2015). In the research of Hiltpold *et al.* (2012), *H. bacteriophora* was encapsulated in alginate capsules, from which the IJ could easily escape. Later improvements by Kim *et al.* (2015) produced hard capsules that were intended to increase the time of release of the nematodes.

As reviewed by Grewal & Peters (2005), nematodes have also been formulated in various heteropolysaccharides (agarose, Carbopol®, carrageenan, dextran, or guar/gellan gum), surrounded by a paste of hydrogenated oil. Up to 35 days' storage of *S. carpocapsae* at room temperature has been reported for this hydrogenated oil formulation. Grewal (1998) reported

a liquid concentrate, including a proprietary metabolic inhibitor to decrease nematode oxygen demand that was developed for the transporting of nematodes in bulk tanks.

As already seen, the induction of anhydrobiosis not only decreases the nematode metabolism, but it also provides extra tolerance to warm and cold temperatures (Glazer & Salame, 2000; Grewal & Jagdale, 2002). A state of partial anhydrobiosis can be induced in steinernematid and heterorhabditid nematodes by means of regulating the water activity (a_w) of the substrate, via the composition of formulation ingredients (Grewal, 2000b). The a_w is a degree of exactly how firmly water is bound, chemically or structurally, to the substrate. Unlike water content, a_w is predisposed by way of water molecules bonded to the surface, in addition to the osmotic effect achieved. A_w equals the RH of air, which is in equilibrium with a sample of nematodes, in a fastened vessel. The formulations that are capable of sustaining moderate numbers of anhydrobiotic nematodes comprise powders, granules and gels (Grewal & Peters, 2005). Bedding & Butler (1994) established a formulation in which nematode slurry was blended in anhydrous polyacrylamide, so as to facilitate the subsequent gel achieving an A_w-value of between 0.800 and 0.995. Despite the nematodes being moderately desiccated, their endurance at room temperature was low. A composition of 2 to 3 g of polyacrylate with branded additives (Nemagel2) to 250 ml of nematode slurry containing 40 million *S. feltiae* resulted in the survival of the nematodes concerned for a period of 2 years at 4°C (Hokkanen & Menzler-Hokkanen, 2002). Furthermore, at room temperature, survival for a year was recorded in 25-ml bags containing 2 million *S. feltiae*. The A_w in this formulation was considerably greater than it had been in the case of the previous formulation (>0.995). Another formulation wherein nematodes were blended in clay to eradicate surplus surface moisture, in addition to inducing a state of partial anhydrobiosis, was described by Bedding (1988). The formulation, termed a ‘sandwich’, entailed using a film of nematodes sandwiched between two sheets of clay.

A report by Strauch *et al.* (2000) on formulation, using mixed attapulgite, or bentonite clay together with concentrated nematodes, or nematode slurry, revealed that *H. bacteriophora* (hybrid strain) and *H. indica* (LN2 strain) survived for only 2 weeks and 1 week respectively, at 25°C. At 5°C, the survival of *H. bacteriophora* was higher in sponge than it was in clay, but that of *H. indica* was superior in clay to what it was in sponge, at 15°C. Granular formulations, likewise, have been established for storage and transport of nematodes, as cited in Grewal & Peters (2005) and Grewal (2002). Capinera & Hibbard (1987) described a formulation wherein nematodes were, to some extent, encapsulated in lucerne meal, as well as in wheat flour. Connick *et al.* (1993) designed granules in which nematodes were spread all over a wheat gluten matrix. This ‘Pesta’ formulation comprised a filler and a humectant to improve the nematode survival. The process entailed the drying of granules to a low moisture content, so

as to avoid nematode migration and risk of contamination. However, the granules promptly dried out during storage, thus resulting in subsequent poor nematode survival rates (Grewal & Georgis, 1999; Grewal & Peters, 2005).

Furthermore, as cited in Grewal & Peters (2005), water-dispersible granules (WGs) have been developed, in which IJs are encased in 10–20-mm diameter granules entailing mixtures of multiple quantities of silica, clays, cellulose, lignin, and starches. The granular matrix enables the nematodes to access oxygen supplies in the course of storage and transport, with them entering a partial anhydrobiotic state at optimum temperature, due to the slow removal of body water by the substrate. The induction of a state of partial anhydrobiosis is often obvious within 4 to 7 days, by means of a three- to fourfold decrease in the oxygen intake of the nematodes, subsequent to an initial increase (Grewal, 2000a,b). What is more, WG formulation offers several advantages over other formulations, with the primarily commercial formulation permitting storage of *S. carpocapsae* for a period of over six months at 25°C, at a nematode concentration of over 300,000/g (Grewal, 2000a). The shelf life involved signified an extension of IJ longevity by 3 months, as compared to that of the nematodes stored in water (Grewal, 2000a,b). The WG also improved nematode tolerance to temperature stress, thus allowing easier and less expensive transportation, the enhanced ease of usage of nematodes, resulting from the eradication of labour-intensive preparation steps, a decreased container size and coverage ratio, and the condensing of disposal material. In contrast, the WG formulation is prone to microbial contamination at room temperature. Therefore, antimicrobial and antifungal agents are often supplemented, so as to overpower the growth of contaminating microbes (Grewal, 2002). Nonetheless, such agents are toxic. However, WGs have, as yet, not been successfully used with several steinernematid and heterorhabditid nematodes, and thus are no longer available on the market (Georgis, 2002).

Noteworthy, nematodes can also be applied, small-scale, in the form of infected insect cadavers (Shapiro-Ilan *et al.,* 2001, 2003; Ansari *et al.,* 2009; Deol *et al.,* 2011; Wang *et al.,* 2014). Cadavers can be coated with a protective formulation (e.g. a starch and clay mixture) to avert rupturing in the course of storage and shipping (Shapiro-Ilan *et al.,* 2001). As cited in Grewal (2002), they can also be applied in the form of capsules and baits. For detailed EPN formulation examples, with temperatures and shelf life period refer to Grewal (2002).

Again, recent works on the formulation of EPNs have focused on above-ground applications (Ravensberg, 2011; Hiltpold, 2015), in terms of which surfactants and absorbents are used to shield off the effect of desiccation. However, the results of such applications have been found to vary (Georgis *et al.,* 2006). For several examples on the use of EPNs against above-ground insect pests, refer to Arthurs *et al*. (2004) where the authors compiled a comprehensive list of insect pests and crops.

As with nematode formulation technologies that have been developed on the basis of existing chemical pesticide techniques, the application of nematodes in the field relies on the modification of the existing farm equipment, such as pressurised sprayers and mist blowers (Shapiro-Ilan & Dolinski, 2015). In the case of the use of such equipment, the swirl plates, filters, and screens are always removed to avoid any blockage. Furthermore, research is under way to apply several nematodes in combination, or together with other microorganisms, like nematophagous fungi and bacteria. Research is also ongoing into the immediate release of other insect parasitoids, after nematode application, or together with other chemical pesticides, which is intended to render an additive, or synergistic, effect to some pest management techniques (Koppenhöfer & Kaya, 1997; Ansari *et al.,* 2008; Dillon *et al.,* 2008; Koppenhöfer & Fuzy, 2008; Mbata & Shapiro-Ilan, 2010). For a comprehensive view of application technologies and future prospects in the application of EPNs, refer to works by Shapiro-Ilan *et al.* (2006), Ravensberg (2011), Hiltpold (2015), and Shapiro-Ilan & Dolinski (2015).

Quality Assessment

Quality assessment calls for the training of employees, and for robust managerial dedication. When nematodes are mass-produced by small companies, their resources are often restricted in terms of the promotion of quality control methods and the routine assessment of quality, despite the fact that there are no registration obligations for EPNs in many countries (Grewal & Peters, 2005), like France and Germany, unlike Belgium and Sweden, among others (De Luca *et al.,* 2015). Quality is the degree of excellence of a product, and quality control is a system of upholding standards in manufactured products, which is accomplished by means of testing a sample of the product in terms of particular specifications. EPN quality demands the verification of species identity, the total number of live nematodes, the ratio between live and dead nematodes, the matching of host-finding behaviour to the target pest, the pathogenicity and reproduction (recycling) capability in the target insect pest, and the age of the nematodes concerned, as well as their storability, heat tolerance, and cold- or warm-temperature activity (Grewal & Peters, 2005). In addition, effective quality control strategising relies on the conservation of extensive viability and virulence during production, formulation and storage (Grewal, 2002).

Over-packing is a method of guaranteeing the presence of the least total number of viable nematodes in a product (Grewal, 2002). Grewal & Peters (2005), further, report that nematode viability and virulence, and their associated bacteria (Bilgrami *et al.,* 2006), can be predisposed by various factors during mass production, formulation and storage. Such factors include the source and the genetic diversity of the master stock, the quality of the host, or of the media

used, exposure to environmental extremes (temperature, aeration, shear), contamination, and the toxicity of antifoaming and antimicrobial agents. Furthermore, such factors as moisture content and the rate of water loss from formulations, thermal cycling during storage, and RH might influence the quality of the nematodes concerned. The toxicity of detergents during harvesting, and the length of storage period of both bulk nematode and product storage, are among other factors that can affect the viability and virulence of nematodes (Grewal & Peters, 2005).

Of even greater importance than the above is the fact that the optimum levels of various factors might vary with nematode species, so that close consideration should be paid to monitoring all factors concerned (Grewal & Peters, 2005). For instance, the optimum storage temperature varies with nematode species. Although low temperatures (2–5°C) normally decrease nematode metabolic activity, and can, thus, improve their shelf life, some species, such as *H. indica* and *S. riobrave*, that are adapted to warm temperatures can also store well at temperatures below 10°C (Strauch *et al.,* 2000; Grewal, 2002). This is rather true in temperate climate with developed countries. However, in the tropics, its rather expensive and consequently nematodes die. Immediate formulation of nematodes and avoidance of low temperatures is thus required. The length of time of production, time from formulation to packaging, and time from packaging to shipping, is typically controlled, because, as the product matures, the exhaustion of stored energy reserves might degrade the degree of virulence that is obtainable with the use of the product (Patel *et al.,* 1997b). Such degradation can also affect the nictation capability of the EPN involved (Lewis *et al.,* 1995; Bilgrami *et al.,* 2006; Bal *et al.,* 2014), and the environmental tolerance of the IJs (Selvan *et al.,* 1993; Patel *et al.,* 1997a). Estimating the level of microbial contamination is an essential component of nematode product quality assessment. The use of batch codes and of expiration dating helps to facilitate the tracking and controlling of the products in terms of the refrigerated storage time before application. Such physical characteristics as product temperature and packaging, colour and weight, granule size distribution, and formulation ability in respect of scattering have also been observed to lessen batch−to−batch variability and the maintenance of product consistency (Grewal, 2002).

Noteworthy, nematode production batches can also vary in quality (Grewal, 2002). According to Grewal & Peters (2005), there is an equal risk of genetic deterioration via genetic drift or accidental selection during the recurrent sub-culturing of nematodes, with variation in the virulence of nematode batches being quite common. Incidentally, some nematode species might be more disposed to rapid deterioration than are others. For example, Wang & Grewal (2002) witnessed a drop in the environmental stress tolerance of *H. bacteriophora* within three to six cycles through *G. mellonella* in the laboratory. They also validated that the best method

of averting such genetic deterioration is the complete storage of the master stock in liquid nitrogen.

An assessment of nematode quality should offer information on whether or not the produced nematode batch will be able to control the target insect in the field. On the contrary, a recent review by San-Blas (2013) has depicted a paucity of recent literature on quality control of EPNs, compared to other aspects of EPN mass production yet it's of utmost importance in the improvement of shelf life. Of equal importance is the fact, as cited in Grewal (2002) and Grewal & Peters (2005), that virulence is, undeniably, the most important element in nematode quality. Several different techniques can be taken to measure the virulence of nematodes, including one-on-one bioassay, LC_{50}, establishment efficiency, and invasion rate (Georgis, 1992; Glazer, 1992; Grewal *et al.,* 1999). Conversely, assays using multiple nematodes against single or multiple hosts are deemed unsuitable for quality control purposes, because of the host–parasite interactions involved, such as the recruitment and over-dispersion of natural parasite populations (Grewal *et al.,* 1999). The invasion of pre-infected hosts has been considered to be more likely than is the invasion of non-infected hosts, but some studies have shown the repellence of IJs from infected cadavers (Glazer, 1997; Grewal *et al.,* 1997). If they are grouped in a single arena, infected cadavers would be likely to distress the infection of further insects (Grewal & Peters, 2005).

For one thing, one-on-one assays can be used to compare the virulence of any nematode species with a predestined yardstick, compared to the virulence of such a susceptible host as *G. mellonella* larvae. This method processes the number of infective nematodes that are present in a population, as well as reflecting the presence of damaged nematodes. The use of such a technique is applicable to species that have a lethal level of one IJ per larva (Grewal, 2002; Grewal & Peters, 2005). Yet, the lowest nematode concentrations that produce about 50% larval mortality in *G. mellonella* have been verified for use with other species. *Galleria mellonella* has been shown to be a preferred bioassay host, because it is highly susceptible to EPNs, as well as being commercially available, thus assuring supply (Grewal & Peters, 2005). As further cited in Grewal & Peters (2005) and Grewal (2002), filter paper arenas are fit for ambushing nematodes like *S. carpocapsae,* whereas sand columns are ideal for cruisers like *H. bacteriophora*. This is despite the fact that both ambushers and cruisers do equally well in sand-well bioassays that aid both the ambushing and the cruising behaviours of IJ. The method is also easy to establish, and it is more comparable to field conditions than are the filter paper bioassays. Thus, it is suggested that the sand-well bioassay be adopted as a standard quality control tool for assessing the virulence of EPNs (Grewal, 2002).

Equally important, the use of *G. mellonella* in quality assessment has been criticised, due to the fact that it is overly susceptible to EPNs, and thus it might not be sensitive to unviable

nematodes (Grewal & Peters, 2005; Ulu & Susurluk, 2014). Another commercially available insect host, the mealworm *Tenebrio molitor* Linnaeus (Coleoptera: Tenebrionidae), is used in the quality assessment of EPNs, particularly in Europe (Peters, 2000; Shapiro-Ilan & Gaugler, 2002; Grewal & Peters, 2005). It goes without saying that IJs prefer *G. mellonella* when they are exposed to both *G. mellonella* and *T. molitor.* However, this technique seems to be problematic in terms of use in developing countries, where there are few breeding companies for the mass rearing of test insects like *G. mellonella* and *T. molitor*. It is for this reason that researchers in countries like Venezuela are employing infrared spectrometry/imaging tools as a relatively easy and indirect quality control measure (San-Blas *et al.,* 2015). With the use of this technique, energy reserves, proteins and differences in batches can, among others, be investigated using visual spectra (San-Blas *et al.,* 2011; Lau *et al.,* 2012; San-Blas *et al.,* 2012, 2015). To conclude the current discussion, a good assay must be able to detect dissimilarities between various nematode batches, and between the different age groups of a particular nematode species.

Conclusion

The most common underlying factor in the production, storage and formulation of EPNs is the cost implication involved, which invariably affects the final price of the product. Improvement in formulation and application technologies is likely to lower such costs. Many nematode formulations against a wide range of insect pests are available on the market, but they are largely suitable for small-scale niche markets, like those that are related to greenhouse and lawn turf production. Many aspects of EPN utilisation outdoors in fields and orchards still require investigation, with an eye to calling for improvements in the existing formulations, or to adopting formulations from other fields of research, like from those that have to do with entomopathogenic fungi and bacteria. Improving conditions in the field by means of the use of cover crops, mulches and proper tillage before the application of EPNs is likely to increase their survival rate, and thus to maintain their infectivity. An increase in the current amount of public awareness regarding the importance of EPNs in IPM is required, which should lead to an improvement in the existing legislative requirements for EPNs in the countries that have had to contend with outraged public opinion regarding the genetic modification of an organism. The raising of public awareness should also lead to an increase in the amount of research funding that is made available for EPN-related investigations. The issue is a serious one in Europe, where some governments emphasise the environmental effects of the mass release of EPNs in agrarian environments, where native or foreign, species of EPNs are applied. All in all, this field of research is promising, in terms of the recent above-ground EPN applications against key insect pests, and their corresponding use in the control of plant-parasitic nematodes.

Aim of the study

To develop a formulation for transport of entomopathogenic nematode (EPN) to the field for commercial application and to be fully cognisant of the survival mechanisms of the infective juveniles (IJ), with the intent of attaining an acceptable shelf life.

The objectives for the study include the following objectives:

- to evaluate *Steinernema yirgalemense*, *S. jeffreyense* and *Heterorhabditis bacteriophora* for their suitability in formulations and long-term storability at different temperatures;
- to optimise a selected formulation to improve its efficiency and productivity for long-term room temperature storability;
- to investigate desiccation, hypoxia, osmotic stress, and temperature storage effects of *S. yirgalemense*, *S. jeffreyense* and *H. bacteriophora*;
- to determine the water activity (a_W)of IJs of *Steinernema yirgalemense* at 25°C; and
- to assess the effect of formulation on nematode quality over time, as well as alternative methods for quality control.

The chapters of this study have been written as separate publishable papers, and for this reason, some repetition, in the different chapters, has been unavoidable. The format of the Journal Nematology is used as a guide.

References

Akhurst, R. & Smith, K. (2002). Regulation and safety. In: Gaugler, R. (Ed.). *Entomopathogenic nematology*. Wallingford, UK, CABI Publishing, pp. 311-332. https://doi.org/10.1079/9780851995670.0311.

Akhurst, R.J., Mourant, R.G., Baud, L. & Boemare, N.E. (1996). Phenotypic and DNA relatedness between nematode symbionts and clinical strains of the genus *Photorhabdus* (Enterobacteriaceae). *International Journal of Systematic Bacteriology* 46,1034-1041. https://doi.org/10.1099/00207713-46-4-1034.

Ali, F. & Wharton, D.A. (2013). Cold tolerance abilities of two entomopathogenic nematodes, *Steinernema feltiae* and *Heterorhabditis bacteriophora*. *Cryobiology* 66, 24-29. https://doi.org/10.1016/j.cryobiol.2012.10.004.

Ansari, M., Shah, F. & Butt, T. (2008). Combined use of entomopathogenic nematodes and *Metarhizium anisopliae* as a new approach for black vine weevil, *Otiorhynchus sulcatus*, control. *Entomologia Experimentalis et Applicata* 129, 340-347.

Ansari, M.A., Hussain, M.A. & Moens, M. (2009). Formulation and application of entomopathogenic nematode-infected cadavers for control of *Hoplia philanthus* in turfgrass. *Pest Management Science* 65, 367-374. https://doi.org/10.1002/ps.1699.

Arthurs, S., Heinz, K.M. & Prasifka, J.R., (2004). An analysis of using entomopathogenic nematodes against above-ground pests. Bulletin of entomological research, 94, pp.297–306. https://doi.org/10.1079/BER2003309.

Bal, H.K., Michel, A.P. & Grewal, P. S. (2014). Genetic selection of the ambush foraging entomopathogenic nematode, *Steinernema carpocapsae* for enhanced dispersal and its associated trade-offs. *Evolutionary Ecology* 28, 923-939. DOI: 10.1007/s10682-014-9706-y

Bedding, R. (1988). Storage of insecticidal nematodes. *World Patent No. WO 88/08668.*

Bedding, R. & Butler, K. (1994). Method for storage of insecticidal nematodes. *World patent No. WO* 94/05150.

Bilgrami, A.L., Gaugler, R., Shapiro-Ilan, D.I. & Adams, B.J. (2006). Source of trait deterioration in entomopathogenic nematodes *Heterorhabditis bacteriophora* and *Steinernema carpocapsae* during in vivo culture. *Nematology* 8, 397-409. DOI: 10.1163/156854106778493394.

Boemare, N. (2002). Biology, taxonomy, and systematics of, *Photorhabdus* and, *Xenorhabdus*. In: Gaugler, R. (Ed.) *Entomopathogenic nematology.* Wallingford, UK, CABI Publishing, pp. 35-56. DOI : 10.1079/9780851995670.0035.

Boemare, N., Akhurst, R. & Mourant, R. (1993). DNA relatedness between *Xenorhabdus* spp.(Enterobacteriaceae), symbiotic bacteria of entomopathogenic nematodes, and a proposal to transfer *Xenorhabdus luminescens* to a new genus, *Photorhabdus* gen. nov. *International Journal of Systematic Bacteriology* 43, 249-255. DOI: 10.1099/00207713-43-2-249.

Brown, I., & Gaugler, R. (1995). Cold tolerance of steinernematid and heterorhabditid nematodes. *Journal of Thermal Biology* 23, 75-80. DOI:10.1016/0306-4565(95)00033-X.

Burman, M. & Pye, A.E. (1980). *Neoaplectana carpocapsae*: respiration of infective juveniles. *Nematologica* 26, 214-219. DOI: 10.1163/187529280x00107.

Burnell, A. & Tunnacliffe, A. (2011). Gene induction and desiccation stress in nematodes. In: Perry, R. N. & Wharton, D. A. (Eds). *Molecular and physiological basis of nematode survival.* Wallingford, UK, CABI Press, pp. 126-156. DOI : 10.1079/9781845936877.0126.

Burnell, A.M. & Stock, S.P. (2000). *Heterorhabditis, Steinernema* and their bacterial symbionts—lethal pathogens of insects. *Nematology* 2, 31-42. DOI: 10.1163/156854100508872.

Campos Herrera, R., Barbercheck, M., How, C. & Stock, S.P. (2012). Entomopathogenic nematodes as a model system for advancing the frontiers of ecology. *Journal of Nematology* 44, 162–176. PMC3578465.

Capinera, J. & Hibbard, B. (1987). Bait formulations of chemical and microbial insecticides for suppression of crop-feeding grasshoppers. *Journal of Agricultural Entomology (USA)* 4, 337–344.

Chen, S. & Glazer, I. (2005). A novel method for long-term storage of the entomopathogenic nematode *Steinernema feltiae* at room temperature. *Biological Control* 32, 104-110. DOI: 10.1016/j.biocontrol.2004.08.006.

Ciche, T.A., Darby, C., Ehlers, R.-U., Forst, S. & Goodrich-Blair, H. (2006). Dangerous liaisons: the symbiosis of entomopathogenic nematodes and bacteria. *Biological Control* 38, 22-46. DOI: 10.1016/j.biocontrol.2005.11.016.

Connick, W.J., Nickle, W.R. & Vinyard, B.T. (1993). Pesta - new granular formulations for *Steinernema carpocapsae*. *Journal of Nematology* 25, 198-203. PMC2619378.

Curran, J., Gilbert, C. & Butler, K. (1992). Routine cryopreservation of isolates of *Steinernema* and *Heterorhabditis* spp. *Journal of Nematology* 24, 269-270. PMC2619270.

De Luca, F., Clausi, M., Troccoli, A., Curto, G., Rappazzo, G. & Tarasco, E. (2015). Entomopathogenic nematodes in Italy: occurrence and use in microbial control strategies. In: Campos-Herrera, R. (Ed.). *Nematode Pathogenesis of Insects and Other Pests*. Dordrecht, Netherlands, Springer International Publishing, pp. 431-449. DOI : 10.1007/978-3-319-18266-7_18.

Deol, Y.S., Jagdale, G.B., Cañas, L. & Grewal, P.S. (2011). Delivery of entomopathogenic nematodes directly through commercial growing media via the inclusion of infected host cadavers: a novel approach. *Biological Control* 58, 60-67. DOI : 10.1016/j.biocontrol.2011.03.002

Dillon, A.B., Moore, C.P., Downes, M.J. & Griffin, C.T. (2008). Evict or infect? Managing populations of the large pine weevil, *Hylobius abietis*, using a bottom–up and top–down approach. *Forest Ecology and Management* 255, 2634-2642. DOI : 10.1016/j.foreco.2008.01.021.

Dowds, B.C. & Peters, A. (2002). Virulence mechanisms. In: Gaugler, R. (Ed.). *Entomopathogenic nematology*. Wallingford, UK, CABI Publishing, pp. 79-98. DOI:10.1079/9780851995670.0079.

Dunphy, G.B. & Webster, J.M. (1988). Lipopolysaccharides of *Xenorhabdus nematophilus* (Enterobacteriaceae) and their haemocyte toxicity in non-immune *Galleria mellonella* (Insecta: Lepidoptera) larvae. *Journal of general microbiology* 134, 1017-1028. DOI: 10.1099/00221287-134-4-1017.

Ebssa, L. & Koppenhöfer, A.M. (2012). Entomopathogenic nematodes for the management of *Agrotis ipsilon*: effect of instar, nematode species and nematode production method. *Pest Management Science* 68, 947-957. DOI : 10.1002/ps.3259.

Ehlers, R.-U. (2001). Mass production of entomopathogenic nematodes for plant protection. *Applied Microbiology and Biotechnology* 56, 623-633. DOI: 10.1007/s002530100711.

Ehlers, R.-U. (2003). Biocontrol nematodes. In: Hokkanen, H.M.T. & Hajek, A.E. (Eds). *Environmental impacts of microbial insecticides.* Springer Netherlands, pp. 177-220. DOI: 10.1007/978-94-017-1441-9_10.

Ehlers, R.-U. (2007). Entomopathogenic nematodes: from science to commercial use. In: Vincent, C., Goettel, M.S. & Lazarovits, G. (eds.). *Biological Control: A Global Perspective: Case Studies from Around the World*. Wallingford, UK, CABI Publishing, pp. 136–151. DOI: 10.1079/9781845932657.0136.

Ehlers, R. & Shapiro-Ilan, D. (2005). Mass Production. In: Parwinder S. Grewal, Ralf-Udo Ehlers & Shapiro-Ilan, D. I. (Eds). *Nematodes as biocontrol agents.* Wallingford, UK, CABI Publishing, pp. 65-75. DOI: 10.1079/9780851990170.0065.

Feng, S.-P., Han, R.-C., Qiu, X.-H., Cao, L.I., Chen, J.-H. & Wang, G.-H. (2006). Storage of osmotically treated entomopathogenic nematode *Steinernema carpocapsae*. *Insect Science* 13, 263-269. DOI: 10.1111/j.1744-7917.2006.00093.x.

Fitters, P.F.L., Meijer, E.M.J., Wright, D.J. & Griffin, C.T. (1997). Estimation of lipid reserves in unstained living and dead nematodes by image analysis. *Journal of Nematology* 29, 160-167. PMC2619765.

Fitters, P.F.L., Patel, M.N., Griffin, C.T. & Wright, D. J. (1999). Fatty acid composition of *Heterorhabditis* sp during storage. *Comparative Biochemistry and Physiology B-Biochemistry & Molecular Biology* 124, 81-88. DOI : 10.1016/s0305-0491(99)00102-9.

Friedman, M.J. (1990). Commercial production and development. In: Gaugler, R. & Kaya, H.K. (Eds). *Entomopathogenic nematodes in biological control.* CRC Press, Boca Raton Florida USA, pp. 153-172.

Gaugler, R., Grewal, P., Kaya, H.K. & Smith-Fiola, D. (2000). Quality assessment of commercially produced entomopathogenic nematodes. *Biological Control* 17, 100-109. DOI : 10.1006/bcon.1999.0768.

Georgis, R. (1990). Formulation and application technology. In: Gaugler, R. & Kaya, H.K. (Eds). *Entomopathogenic nematodes in biological control.* Boca Raton, Florida, CRC Press, pp. 173-191.

Georgis, R. (1992). Present and future prospects for entomopathogenic nematode products. *Biocontrol Science and Technology* 2, 83-99. DOI : 10.1080/09583159209355222.

Georgis, R. (2002). The Biosys Experiment: an Insider's Perspective. In: Gaugler, R. (Ed.) *Entomopathogenic nematology.* Wallingford, UK, CABI Publishing, pp. 357-372. DOI : 10.1079/9780851995670.0357.

Georgis, R., Dunlop, D.B. & Grewal, P. S. (1995). Formulation of entomopathogenic nematodes. In: Fuxa, J., Hall, F. & Barry, J. (Eds). *Biorational Pest Control Agents: Formulation and Delivery.* Washington D.C., Am. Chem. Soc., pp. 197-205.

Georgis, R. & Gaugler, R. (1991). Predictability in biological control using entomopathogenic nematodes. *Journal of Economic Entomology* 84, 713-720. DOI : 10.1093/jee/84.3.713.

Georgis, R. & Kaya, H.K. (1998). Formulation of entomopathogenic nematodes. In: Burges, H. D. (Ed.) *Formulation of Microbial Biopesticides.* Dordrecht, Kluwer Academic Publishers, pp. 289-308. DOI : 10.1007/978-94-011-4926-6_9.

Georgis, R., Koppenhöfer, A., Lacey, L., Bélair, G., Duncan, L., Grewal, P., Samish, M., Tan, L., Torr, P. & Van Tol, R. (2006). Successes and failures in the use of parasitic nematodes for pest control. *Biological Control* 38, 103-123. DOI : 10.1016/j.biocontrol.2005.11.005.

Glazer, I. (1992). Invasion rate as a measure of infectivity of steinernematid and heterorhabditid nematodes to insects. *Journal of Invertebrate Pathology* 59, 90-94. DOI : 10.1016/0022-2011(92)90116-L.

Glazer, I. (1997). Effects of infected insects on secondary invasion of steinernematid entomopathogenic nematodes. *Parasitology* 114, 597-604. DOI : 10.1017/S0031182097008809.

Glazer, I. (2002). Survival biology. In: Gaugler, R. (Ed.) *Entomopathogenic nematology.* Wallingford, UK, CABI Publishing, pp. 169-187. DOI: 10.1079/9780851995670.0169.

Glazer, I., Klein, M., Navon, A., & Nakache, Y. (1992). Comparison of efficacy of entomopathogenic nematodes combined with antidesiccants applied by canopy sprays against three cotton pests (Lepidoptera: Noctuidae). *Journal of Economic Entomology*, 85,1636-1641. DOI:10.1093/jee/85.5.1636.

Glazer, I. & Salame, L. (2000). Osmotic survival of the entomopathogenic nematode *Steinernema carpocapsae. Biological Control* 18, 251-257. DOI : 10.1006/bcon.2000.0814.

Grewal, P. S. (1998). Formulations of Entomopathogenic Nematodes for Storage and Application. *Nematological Research (Japanese Journal of Nematology)* 28, 68-74. DOI : 10.1007/978-94-011-4926-6_9.

Grewal, P.S. (2000a). Anhydrobiotic potential and long-term storage of entomopathogenic nematodes (Rhabditida : Steinernematidae). *International Journal for Parasitology* 30, 995-1000. DOI: 10.1016/S0020-7519(00)00080-1.

Grewal, P.S. (2000b). Enhanced ambient storage stability of an entomopathogenic nematode through anhydrobiosis. *Pest Management Science* 56, 401-406. DOI: 10.1002/(SICI)1526-4998(200005)56:5<401::AID-PS137>3.0.CO;2-4.

Grewal, P.S. (2002). Formulation and Application Technology. In: Gaugler, R. (Ed.) *Entomopathogenic nematology.* Wallingford, UK, CABI Publishing, pp. 265 - 287. DOI: 10.1079/9780851995670.0265.

Grewal, P.S., Bai, X. & Jagdale, G. (2011). Longevity and stress tolerance of entomopathogenic nematodes. In: Perry, R. N. & Wharton, D. A. (Eds). *Molecular and Physiological Basis of Nematodes Survival.* Wallingford, UK, CABI Publishing, pp. 157-181. DOI: 10.1079/9781845936877.0157.

Grewal, P.S., Bornstein-Forst, S., Burnell, A.M., Glazer, I. & Jagdale, G.B. (2006). Physiological, genetic, and molecular mechanisms of chemoreception, thermobiosis, and anhydrobiosis in entomopathogenic nematodes. *Biological Control* 38, 54-65. DOI : 10.1016/j.biocontrol.2005.09.004.

Grewal, P., Converse, V. & Georgis, R. (1999). Influence of production and bioassay methods on infectivity of two ambush foragers (Nematoda: Steinernematidae). *Journal of Invertebrate Pathology* 73, 40-44. DOI : 10.1006/jipa.1998.4803.

Grewal, P. & Georgis, R. (1999). Entomopathogenic nematodes. In: Hall, F. R. & Menn, J. J. (Eds). *Biopesticides: use and delivery.* Totowa, New Jersey 075 I2, Humana Press Inc, pp. 271-299. DOI : 10.1385/0-89603-515-8:271.

Grewal, P.S. & Jagdale, G.B. (2002). Enhanced trehalose accumulation and desiccation survival of entomopathogenic nematodes through cold preacclimation. *Biocontrol Science and Technology* 12, 533-545. DOI: 10.1080/0958315021000016207.

Grewal, P., Lewis, E. & Gaugler, R. (1997). Response of infective stage parasites (Nematoda: Steinernematidae) to volatile cues from infected hosts. *Journal of Chemical Ecology* 23, 503-515. DOI : 10.1023/B:JOEC.0000006374.95624.7e.

Grewal, P. & Peters, A. (2005). Formulation and Quality. In: Parwinder S. Grewal, Ralf-Udo Ehlers & Shapiro-Ilan, D. I. (Eds). *Nematodes as biocontrol agents.* Wallingford, UK, CABI Publishing, pp. 79 - 89. DOI: 10.1079/9780851990170.0079.

Grewal, P.S., Wang, X. & Taylor, R. A. J. (2002). Dauer juvenile longevity and stress tolerance in natural populations of entomopathogenic nematodes: is there a relationship? *International Journal for Parasitology* 32, 717-725. DOI: 10.1016/S0020-7519(02)00029-2.

Griffin, C. (2015). Behaviour and population dynamics of entomopathogenic nematodes following application. In: Campos-Herrera, R. (Ed.) *Nematode Pathogenesis of Insects and Other Pests*. Dordrecht, Netherlands, Springer International Publishing, pp. 57−95. DOI:10.1007/978-3-319-18266-7_3.

Han, R.C. & Ehlers, R.U. (2000). Pathogenicity, development, and reproduction of *Heterorhabditis bacteriophora* and *Steinernema carpocapsae* under axenic in vivo conditions. *Journal of Invertebrate Pathology* 75, 55-58. DOI: 10.1006/jipa.1999.4900.

Han, R. & Ehlers, R. U. (2001). Effect of *Photorhabdus luminescens* phase variants on the in vivo and in vitro development and reproduction of the entomopathogenic nematodes *Heterorhabditis bacteriophora* and *Steinernema carpocapsae*. *FEMS Microbiology/Ecology* 35, 239-247. DOI: 10.1111/j.1574-6941.2001.tb00809.x.

Hill, M.P., Malan, A. P. & Terblanche, J. S. (2015). Divergent thermal specialisation of two South African entomopathogenic nematodes. *PeerJ* 3, e1023. DOI : 10.7717/peerj.1023.

Hiltpold, I. (2015). Prospects in the Application Technology and Formulation of Entomopathogenic Nematodes for Biological Control of Insect Pests. In: Campos-Herrera, R. (Ed.) *Nematode Pathogenesis of Insects and Other Pests.* Dordrecht, Netherlands, Springer International Publishing, pp. 187-205. DOI : 10.1007/978-3-319-18266-7_7.

Hiltpold, I., Hibbard, B.E., French, B.W. & Turlings, T.C. (2012). Capsules containing entomopathogenic nematodes as a Trojan horse approach to control the western corn rootworm. *Plant and Soil* 358, 11-25. DOI: 10.1007/s11104-012-1253-0.

Hokkanen, H. & Menzler-Hokkanen, I. (2002). Formulation of the nematodes *Steinernema feltiae* for a shelf-life up to one year. *VIII International Colloquim on Invertebrate Pathology and Microbial Control.* Foz do Iguassu, Brazil, Poster abstract MC25.

Hominick, W.M. (2002). Biogeography. In: Gaugler, R. (Ed.) *Entomopathogenic nematology.* Wallingford, UK, CABI Publishing, pp. 115-143. DOI : 10.1079/9780851995670.0115.

Jagdale, G.B. & Gordon, R. (1997a). Effect of temperature on the activities of glucose-6-phosphate dehydrogenase and hexokinase in entomopathogenic nematodes (Nematoda: Steinernematidae). *Comparative Biochemistry and Physiology Part A: Physiology* 118, 1151-1156. DOI : 10.1016/S0300-9629(97)00034-0.

Jagdale, G.B. & Gordon, R. (1997b). Effect of temperature on the composition of fatty acids in total lipids and phospholipids of entomopathogenic nematodes. *Journal of Thermal Biology* 22, 245-251. DOI : 10.1016/s0306-4565(97)00019-3.

Jagdale, G.B., & Grewal, P. (2003). Acclimation of entomopathogenic nematodes to novel temperatures: trehalose accumulation and the acquisition of thermotolerance. *International Journal for Parasitology* 33**,** 145-152. DOI:10.1016/S0020-7519(02)00257-6.

Jagdale, G.B., & Grewal, P. S. (2007). Storage temperature influences desiccation and ultra violet radiation tolerance of entomopathogenic nematodes. *Journal of Thermal Biology*, 32, 20-27. DOI:10.1016/j.jtherbio.2006.07.004.

Jones, K. A. & Burges, H. D. (1998). Technology of formulation and application. In: Burges, H. D. (Ed.) *Formulation of Microbial Biopesticides.* Dordrecht, Kluwer Academic Publishers, pp. 7-30. DOI : 10.1007/978-94-011-4926-6_2.

Kaya, H.K. & Gaugler, R. (1993). Entomopathogenic nematodes. *Annual Review of Entomology* 38, 181-206. DOI: 10.1146/annurev.en.38.010193.001145.

Kim, J., Jaffuel, G. & Turlings, T. C. (2015). Enhanced alginate capsule properties as a formulation of entomopathogenic nematodes. *BioControl* 60, 527-535. DOI: 10.1007/s10526-014-9638-z.

Koppenhöfer, A.M. & Fuzy, E.M. (2008). Early timing and new combinations to increase the efficacy of neonicotinoid–entomopathogenic nematode (Rhabditida: Heterorhabditidae) combinations

against white grubs (Coleoptera: Scarabaeidae). *Pest Management Science* 64, 725-735. DOI: 10.1002/ps.1550.

Koppenhöfer, A. & Kaya, H. (1997). Additive and Synergistic Interaction between Entomopathogenic Nematodes and *Bacillus thuringiensis* for Scarab Grub Control. *Biological Control* 8, 131-137.

Kung, S.-P., Gaugler, R. & Kaya, H. K. (1990). Influence of soil pH and oxygen on persistence of *Steinernema* spp. *Journal of Nematology* 22, 440. PMC2619085.

Lau, K., Hobro, A., Smith, T., Thurston, T. & Lendl, B. (2012). Label-free non-destructive in situ biochemical analysis of nematode *Steinernema kraussei* using FPA-FTIR and Raman spectroscopic imaging. *Vibrational Spectroscopy* 60, 34-42. DOI: 10.1016/j.vibspec.2012.01.009.

Lewis, E., Selvan, S., Campbell, J. & Gaugler, R. (1995). Changes in foraging behaviour during the infective stage of entomopathogenic nematodes. *Parasitology* 110, 583-590. DOI: 10.1017/S0031182000065306.

Matadamas-Ortiz, P.T., Ruiz-Vega, J., Vazquez-Feijoo, J. A., Cruz-Martinez, H. & Cortes-Martinez, C. I. (2014). Mechanical production of pellets for the application of entomopathogenic nematodes: factors that determine survival time of *Steinernema glaseri*. *Biocontrol Science and Technology* 24, 145-157. DOI: 10.1080/09583157.2013.852161.

Mbata, G.N. & Shapiro-Ilan, D. I. (2010). Compatibility of *Heterorhabditis indica* (Rhabditida: Heterorhabditidae) and *Habrobracon hebetor* (Hymenoptera: Braconidae) for biological control of *Plodia interpunctella* (Lepidoptera: Pyralidae). *Biological Control* 54, 75-82. DOI: 10.1016/j.biocontrol.2010.04.009.

Mukuka, J., Strauch, O. & Ehlers, R.-U. (2010). Variability in desiccation tolerance among different strains of the entomopathogenic nematode *Heterorhabditis bacteriophora*. *Nematology* 12, 711-720. DOI: 10.1163/138855409x12607871174454.

O'Leary, S.A., Power, A.P., Stack, C.M. & Burnell, A.M. (2001). Behavioural and physiological responses of infective juveniles of the entomopathogenic nematode Heterorhabditis to desiccation. *BioControl* 46, 345-362. DOI: 10.1023/A:1011470021857.

Patel, M. & Wright, D. (1997a). Glycogen: its importance in the infectivity of aged juveniles of *Steinernema carpocapsae*. *Parasitology* 114, 591-596. DOI: 10.1017/S0031182097008780.

Patel, M.N. & Wright, D.J. (1997b). Fatty acid composition of neutral lipid energy reserves in infective juveniles of entomopathogenic nematodes. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology* 118, 341-348. DOI:10.1016/S0305-0491(97)00057-6.

Patel, M.N. & Wright, D.J. (1997c). Phospholipids and their fatty acids in infective juveniles of entomopathogenic steinernematid nematodes. *Comparative Biochemistry and Physiology B-Biochemistry & Molecular Biology* 118, 649-657. DOI:10.1016/s0305-0491(97)00270-8.

Patel, M.N., Perry, R.N. & Wright, D.J. (1997a). Desiccation survival and water contents of entomopathogenic nematodes, *Steinernema* spp. (Rhabditida: Steinernematidae). *International Journal for Parasitology* 27, 61-70. DOI: 10.1016/S0020-7519(96)00154-3.

Patel, M.N., Stolinski, M. & Wright, D. J. (1997b). Neutral lipids and the assessment of infectivity in entomopathogenic nematodes: observations on four *Steinernema* species. *Parasitology* 114, 489-496. DOI:10.1017/s0031182096008748.

Perry, R.N., Ehlers, R.-U. & Glazer, I. (2012). A realistic appraisal of methods to enhance desiccation tolerance of entomopathogenic nematodes. *Journal of Nematology* 44, 185 - 190. PMC3578469.

Peters, A. (1996). The natural host range of *Steinernema* and *Heterorhabditis* spp and their impact on insect populations. *Biocontrol Science and Technology* 6, 389-402. DOI:10.1080/09583159631361.

Peters, A. (2000). Insect based assays for entomopathogenic nematode infectiousness: definitions, guidelines, problems. *IOBC WPRS Bulletin* 23, 109-114.

Popiel, I. & Vasquez, E. (1989). Cryoprotection of *Steinernema carpocapsae* and *Heterorhabditis bacteriophora*. *J. Nematol* 23, 423-437.

Qiu, L., Lacey, M.J. & Bedding, R. A. (2000). Permeability of the infective juveniles of *Steinernema carpocapsae* to glycerol during osmotic dehydration and its effect on biochemical adaptation and energy metabolism. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology* 125, 411-419. DOI:10.1016/S0305-0491(99)00178-9.

Ravensberg, W.J. (2011). Mass production and product development of a microbial pest control agent. In: Ravensberg, W. J. (Ed.) *A Roadmap to the Successful Development and Commercialization of Microbial Pest Control Products for Control of Arthropods.* Dordrecht, Springer, pp. 59-127. DOI:10.1007/978-94-007-0437-4_3.

San-Blas, E. (2013). Progress on entomopathogenic nematology research: A bibliometric study of the last three decades: 1980–2010. *Biological Control* 66, 102-124. DOI:10.1016/j.biocontrol.2013.04.002.

San-Blas, E., Cubillán, N., Guerra, M., Portillo, E. & Esteves, I. (2012). Characterization of *Xenorhabdus* and *Photorhabdus* bacteria by fourier transform mid-infrared spectroscopy with attenuated total reflection (FT-IR/ATR). *Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy* 93, 58-62. DOI: 10.1016/J.SAA.2012.03.006.

San-Blas, E., Guerra, M., Portillo, E., Esteves, I., Cubillán, N. & Alvarado, Y. (2011). ATR/FTIR characterization of *Steinernema glaseri* and *Heterorhabditis indica*. *Vibrational Spectroscopy* 57, 220-228. DOI: 10.1016/j.vibspec.2011.07.008.

San-Blas, E., Rosales, C. & Torres, Á. (2015). Entomopathogenic nematodes in tropical agriculture: current uses and their future in Venezuela. In: Campos-Herrera, R. (Ed.) *Nematode*

pathogenesis of insects and other pests. Springer, pp. 375-402. DOI:10.1007/978-3-319-18266-7_15.

Selvan, S., Gaugler, R. & Grewal, P.S. (1993). Water-content and fatty-acid composition of infective juvenile entomopathogenic nematodes during storage. *Journal of Parasitology* 79, 510-516. DOI:10.2307/3283375.

Shapiro-Ilan, D.I., Brown, I. & Lewis, E.E. (2014). Freezing and desiccation tolerance in entomopathogenic nematodes: diversity and correlation of traits. *Journal of Nematology* 46, 27-34. PMC3957569

Shapiro-Ilan, D. & Dolinski, C. (2015). Entomopathogenic nematode application technology. In: Campos-Herrera, R. (Ed.) *Nematode pathogenesis of insects and other pests.* Springer, pp. 231-254. DOI:10.1007/978-3-319-18266-7_9.

Shapiro-Ilan, D.I. & Gaugler, R. (2002). Production technology for entomopathogenic nematodes and their bacterial symbionts. *Journal of Industrial Microbiology & Biotechnology* 28, 137-146. DOI:10.1038/sj/jim/7000230.

Shapiro-Ilan, D.I., Gouge, D.H. & Koppenhofer, A.M. (2002). Factors affecting commercial success: case studies in cotton, turf and citrus. In: Gaugler, R. (Ed.) *Entomopathogenic nematology.* Wallingford, UK, CABI Publishing, pp. 333–355. DOI:10.1079/9780851995670.0333.

Shapiro-Ilan, D.I., Gouge, D.H., Piggott, S.J. & Fife, J.P. (2006). Application technology and environmental considerations for use of entomopathogenic nematodes in biological control. *Biological Control* 38, 124-133. DOI:10.1016/j.biocontrol.2005.09.005.

Shapiro-Ilan, D.I., Han, R. & Dolinksi, C. (2012). Entomopathogenic nematode production and application technology. *Journal of Nematology* 44, 206-217. PMCID: PMC3578468.

Shapiro-Ilan, D.I., Lewis, E.E., Behle, R.W. & Mcguire, M.R. (2001). Formulation of entomopathogenic nematode-infected cadavers. *Journal of Invertebrate Pathology* 78, 17-23. DOI:10.1006/jipa.2001.5030.

Shapiro-Ilan, D.I., Lewis, E.E. & Tedders, W.L. (2003). Superior efficacy observed in entomopathogenic nematodes applied in infected-host cadavers compared with application in aqueous suspension. *Journal of Invertebrate Pathology* 83, 270-272. DOI:10.1016/S0022-2011(03)00101-0.

Simons, W. & Poinar, G. (1973). The ability of *Neoaplectana carpocapsae* (Steinernematidae: Nematodea) to survive extended periods of desiccation. *Journal of Invertebrate Pathology* 22, 228-230. DOI:10.1016/0022-2011(73)90138-9.

Solomon, A., Paperna, I. & Glazer, I. (1999). Desiccation survival of the entomopathogenic nematode Steinernema feltiae: induction of anhydrobiosis. *Nematology* 1, 61-68. DOI:10.1163/156854199507983.

Somasekhar, N., Grewal, P.S. & Klein, M. G. (2002). Genetic variability in stress tolerance and fitness among natural populations of *Steinernema carpocapsae*. *Biological Control* 23, 303-310. DOI:10.1006/bcon.2001.1013.

Stock, S.P. (2005). Insect-parasitic nematodes: from lab curiosities to model organisms. *Journal of Invertebrate Pathology* 89, 57-66. DOI:10.1016/j.jip.2005.02.011.

Strauch, O., Niemann, I., Neumann, A., Schmidt, A.J., Peters, A. & Ehlers, R.U. (2000). Storage and formulation of the entomopathogenic nematodes *Heterorhabditis indica* and *H. bacteriophora*. *BioControl* 45, 483-500. DOI:10.1023/a:1026528727365.

Strauch, O., Oestergaard, J., Hollmer, S. & Ehlers, R.U. (2004). Genetic improvement of the desiccation tolerance of the entomopathogenic nematode *Heterorhabditis bacteriophora* through selective breeding. *Biological Control* 31, 218-226. DOI:10.1016/j.biocontrol.2004.03.009.

Surrey, M.R. & Wharton, D.A. (1995). Desiccation survival of the infective larvae of the insect parasitic nematode, *Heterorhabditis zealandica* Poinar. *International Journal for Parasitology* 25, 749-752. DOI:10.1016/0020-7519(94)00190-Y.

Ulu, T.C. & Susurluk, I.A. (2014). Heat and desiccation tolerances of *Heterorhabditis bacteriophora* strains and relationships between their tolerances and some bioecological characteristics. *Isj-Invertebrate Survival Journal* 11, 4-10.

Wang, X. & Grewal, P.S. (2002). Rapid genetic deterioration of environmental tolerance and reproductive potential of an entomopathogenic nematode during laboratory maintenance. *Biological Control* 23, 71-78. DOI:10.1006/bcon.2001.0986.

Wang, X., Wang, H., Feng, Q. Z., Cui, X.Y., Liu, R.Y., Sun, Y.B., Li, G.C., Tan, H., Song, D.M., Liu, W., Ruan, W.B. & Harvey, J.A. (2014). Desiccation and cold storage of Galleria mellonella cadavers and effects on in vivo production of *Steinernema carpocapsae*. *Pest Management Science* 70, 895-904. DOI:10.1002/ps.3685.

Webster, J.M., Chen, C. & Hu, K. (2002). Bacterial metabolites. In: Gaugler, R. (Ed.) *Entomopathogenic nematology.* Wallingford , UK, CABI Publishing, pp. 99-114. DOI:10.1079/9780851995670.0099.

Womersley, C. Z. (1990). Dehydration survival and anhydrobiotic potential. In: Gaugler, R. & Kaya, H.K. (Eds). *Entomopathogenic nematodes in biological control.* Boca Raton Florida USA, CRC Press, pp. 117-137.

Yan, X., De Clercq, P., Han, R.C., Jones, J., Chen, S. L. & Moens, M. (2011). Osmotic responses of different strains of *Steinernema carpocapsae*. *Nematology* 13, 845-851. DOI:10.1163/138855411X554181.

Yan, X., Liu, X., Han, R., Chen, S., Clercq, P. D. & Moens, M. (2010). Osmotic induction of anhydrobiosis in entomopathogenic nematodes of the genera *Heterorhabditis* and *Steinernema*. *Biological Control* 53, 325-330. DOI:10.1016/j.biocontrol.2010.01.009.

Chapter 2

Formulation of *Steinernema yirgalemense*, *S. jeffreyense* and *Heterorhabditis bacteriophora* in alginate beads and diatomaceous earth

Abstract

The desire to use entomopathogenic nematodes (EPNs) on a large scale as biological control agent, in integrated pest management programmes, is frustrated by their short shelf life. To investigate their role in formulations, three local South African EPN species were used, including *Steinernema yirgalemense*, *S. jeffreyense* and *Heterorhabditis bacteriophora*, employing different formulation techniques. Encapsulation of the infective juveniles (IJs) in alginate beads, as well as the use of diatomaceous earth (DE), with reduced water activity (a_w -value at 0.97) to induce quiescence, as well as to reduce the metabolism of the IJs, was investigated. Survival of the IJs in the formulations was determined at 6°C, 14°C and 25°C for 4 weeks. Of the IJs, 10% to 20% were observed to escape from the beads, depending on the prevailing temperature, and readily survived the encapsulation process. DE did not cause the desiccation of the nematodes, with there still being a high survival rate by the fourth week of the study. However, desiccation effect was noticeable at 6°C. In both formulations, the survival and virulence rates differed significantly at 6°C, as compared to at 14°C and 25°C, with a drastic decrease over time for *S. yirgalemense*. The EPN species revealed poor survival and loss of virulence at low temperatures in both formulations. Thus, future research should investigate the survival of the species between the higher temperatures of 8°C and 10°C. The beads successfully retained most of the IJs and can be stored for a longer time. Of the two methods studied, formulating EPNs in DE is regarded as being the best way forward, due to the relative ease of optimisation.

Key words: Encapsulation, temperature, quiescence, survival, virulence, desiccation, anhydrobiosis, *Galleria mellonella*, tixosol

Introduction

Entomopathogenic nematodes (EPN) have many advantages and are mass-produced on an industrial scale in liquid culture. This is due to the relatively easy scale-up and downstream processing of large culture volumes, which can be mechanised, thus reducing production costs (Ehlers, 2001; Ehlers & Shapiro-Ilan, 2005; Shapiro-Ilan & Gaugler, 2002). Although many countries have exempted EPNs from registration obligations (Akhurst & Smith, 2002), in South Africa such is not the case (Hatting *et al.*, 2018). In addition, EPNs can be incorporated into integrated pest management (IPM) programmes (Grewal, 2002), which has enabled both small and medium-sized enterprises to develop nematode-based plant protection products (Ehlers, 2003).

For both the steinernematid and heterorhabditid nematodes, the dauer or infective juvenile (IJ) stage is the only free-living stage. Their mutualistic bacteria, with the aid of the nematode, are the etiological agents, which, on entering the target pest insect, where they multiply, cause septicaemia, resulting in the death of the insect (Ciche *et al.,* 2006; Lu *et al*., 2017). Although the IJs are non-feeding (Grewal *et al.,* 2002), they can persist for a long time in the soil, until a potential host turns up. They enter mostly the soil-inhabiting insect stages, either through the anus, mouth and trachea, or even through the soft or thin parts of the cuticle (Ehlers, 2001). The insect dies within 24 to 48 h after nematode invasion (Burnell & Stock, 2000; Dowds & Peters, 2002; Dunphy & Webster, 1988; Webster *et al.,* 2002). When the food in the insect is depleted, the IJs then leave the cadaver to find a new host (Han & Ehlers, 2000, 2001). Moreover, EPNs are, in some cases, so competent when present in the soil that they can exceed the control results obtained with chemical compounds, probably due to their mobility (Ehlers, 2003).

The ability of EPNs to survive in storage for a few months aids in the marketing of nematode-based products (Grewal & Peters, 2005). Extended prospects of using EPNs in large-scale outdoor areas, chiefly against soil-dwelling insect pests in high-value crops, and in turf, have been investigated (Shapiro-Ilan *et al.,* 2002*,* 2012). Presently, EPNs are mainly used where no other control procedures against a specific insect are available. Examples of such use are against insects, like Coleoptera, that have proved themselves to be very difficult to control, or in habitats where chemical compounds are found wanting/ failing/lacking, primarily in the soil, or in galleries of boring insects with resistance to insecticides, and during the harvesting of edible produce (Ehlers, 2003; Grewal & Peters, 2005).

With the withdrawal from use of agrochemicals against pest insects for many horticultural crops in Europe, and with the existing Framework Directive 2009/128/EC on the sustainable use of pesticides, marketing opportunities for biopesticide products have increased, including

use of EPNs. The success of a biological control agent depends on the presence of certain key traits, including environmental tolerance, compatibility with the target pest, host-finding ability, reproductive potential, and the ability to mass culture (Bilgrami *et al*., 2006; Hopper *et al*., 1993).

Even though EPNs are competent biological control agents against insect pests, their commercial use is limited by their predetermined shelf life, which is a major drawback in terms of large-scale commercial use (Grewal, 2000a, b). Their poor survival rate in storage at room temperature is a major hindrance to their potential use as bioinsecticides (Grewal, 2002). In addition, their ability to survive under such conditions is also highly compromised in terms of desiccation (Surrey & Wharton, 1995; Womersley, 1990).

The successful commercialisation of EPNs after mass production requires the development of storage and formulation techniques that curtail nematode mortality, loss of virulence, and pathogenicity. In many commercial EPN-based biopesticide companies, the formulations that are manufactured result from the use of a variety of methods, ranging from simply saturating EPNs on artificial sponge, all the way through to highly advanced granular formulations. Major challenges have included obtaining room temperature shelf stability, ease of use, and contamination control.

Kagimu *et al*. (2017) recently reported that DE is mainly used for formulations, due to its many advantages, including its ease of application using available farm implements and irrigation systems, among others. The authors, further, draw attention to the shift in research trends regarding formulations devised, ranging from a focus on the soil to a focus on above-ground applications using adjuvants, and alginate beads and capsules (Kagimu *et al.,* 2017).

In South Africa, investigations to determine the commercial prospects of using a local isolate of *Steinernema yirgalemense* Nguyen, Tesfamariam, Gozel, Gaugler & Adams, 2004 for the control of codling moth (*Cydia pomonella* L.) (Lepidoptera, Tortricidae), vine mealybug (*Planococcus ficus*) (Hemiptera, Pseudococcidae), citrus mealybug (*Planococcus citri*), and maize stalk borer (*Busseola fusca*) (Lepidoptera, Noctuidae) is under way (Malan & Hatting, 2015; Odendaal *et al*., 2015, 2016). Similar investigations have been undertaken in considering the commercial possibilities of *H. bacteriophora* for the control of bollworm (*Helicoverpa armigera*) (Lepidoptera, Noctuidae), fruit fly (*Ceratitis capitata*; *C. rosa*), (Diptera: Tephritidae), maize stalk borer (*B. fusca*), and sugarcane borer (*Eldana saccharina*) (Lepidoptera: Pyralidae) (Malan & Hatting, 2015).

Nematodes encapsulated in sodium and calcium alginate gels by means of internal or external techniques (Kaya & Nelsen, 1985; Kaya *et al*., 1987), and in other hydrophilic colloids (Patel & Vorlop, 1994), have been used to protect the IJs from both desiccation and ultraviolet

light (Navon *et al*., 1998, 2002). The beads and capsules are produced by forming droplets from liquids, and by solidifying the liquid droplets to form particles. The process of gelation, or of membrane formation, is categorised by the way in which droplet formation (dripping and emulsification) occurs (Hiltpold *et al.,* 2012; Vemmer & Patel, 2013). Increasingly, beads and capsules are coated using such polyelectrolytes as xanthan gum, with altering charges (Hiltpold *et al*., 2012; Kim *et al*., 2015; Vemmer & Patel, 2013) being used to strengthen them, and a few drops of colouring being added to distinguish the solution during gelation. Presently, such EPN formulation is still undergoing development, with the process encountering many diverse challenges. For example, Hiltpold *et al*. (2012) and Kim *et al*. (2015) report that EPNs readily escaped from soft capsules within a few days, especially when they were unrefrigerated, and that the capsules did not retain EPNs over an extended period of time, therefore limiting the long-term storage of such a medium. Adjusting the capsule properties, like the formation of alginate capsules at 4°C, resulted in thinner shelled, yet harder, capsules than when the polymerisation was performed at 24°C. Post-treatment of the capsules with additional Ca^{2+} markedly improved the hardness of the capsules concerned. Although the hardened capsules retained EPNs significantly better than did the unhardened ones, surprisingly, post-treatment with Ca^{2+} exerted an adverse effect on EPN retention, with very few IJs being able to escape from the capsules concerned. Ideally, EPN beads should retain their EPNs inside the bead, until they are required, and they should maintain the EPN viability for a few months, at room temperature (Kim *et al*., 2015).

In contrast, DE, consisting of unicellular, or colonial, silicified skeletons of algae (Bacillariophyceae) (Buchholz *et al.*, 2009), and composed of 89% amorphous SiO_2, 4%, Al_2O_3 1.7% Fe_2O_3, 1.4% CaO, >1% MgO + K_2O and 3% H_2O, was tested equally (Wakil *et al*., 2011). Inert dusts have been reported to be effective for the control of various pests (Golob, 1997). Although several DE formulations have been tested and evaluated for their effectiveness against the insect pests of stored products, all DE formulations do not give the same level of effectiveness. Thus, the use of a proper tested grade of DE is encouraged for formulation (Wakil *et al*., 2011).

DE desiccates the IJs by causing partial anhydrobiosis, whereby the nematode enters a physiological state of quiescence, in which its metabolic activity and energy reserve consumption diminish, so that it can retain its survival capacities and infectivity until field application (Silver *et al*., 1995). Upon being reactivated by the moisture in the soil, it is liberated, and, if it locates a susceptible host, the effective biological control of the pest infestation may be achieved (Matadamas-Ortiz *et al*., 2014). The efficacy of EPN formulation can be influenced by some characteristics such as homogeneity (shape, size, quantity of

nematodes, and weight), structure, mechanical resistance, and the properties of the inert granular materials of DE formulation (Hiltpold *et al*., 2012; Silver *et al*., 1995).

The objectives of the current study were to formulate three South African EPN species and to test them for suitability and survival enhancement, using two different types of formulation. The types of formulation used consisted of alginate beads and DE. With the beads, the number of nematodes escaping from them was determined, after formulation and storage at different temperatures, as was the pathogenicity of the IJ. In the case of DE, the viability of the nematodes was determined after storage at different temperatures, up until a period of four weeks had elapsed.

Materials and methods

SOURCE OF NEMATODES AND HOST INSECTS

The three-nematode species used in the study were *Steinernema jeffreyense* Malan, Knoetze & Tiedt, 2015, *S. yirgalemense* and *Heterorhabditis bacteriophora* Poinar, 1976, all endemic to South Africa. The species, which were collected from previous surveys, were maintained in the EPN collection of the Nematology laboratory, at the Department of Conservation Ecology and Entomology, Stellenbosch University (Malan & Hatting, 2015). The details regarding the origin and size of the IJ are summarised in Table 2.1.

Table 2.1. The strain, origin, mean body length, width and mass of the infective juveniles of different *Steinernema* and *Heterorhabditis* species.

Species	Strain	Origin	Infective juvenile		
			Length (µm)	Width (µm)	Mass (µg)
S. jeffreyense	J194	Jeffreys Bay, Eastern Cape	924 (784-1043)	35 (23-43)	0.7089
S. yirgalemense	157-C	Friedenheim, Mpumalanga	635 (548-693)	29 (24-33)	0.3338
H. bacteriophora	SF351	Wellington, Western Cape	588 (512-671)	23 (18-31)	0.1944

Malan *et al*., 2016; Nguyen *et al*., 2004; Poinar, 1976

Galleria mellonella (Lepidoptera: Pyralidae) larvae were cultured according to Van Zyl & Malan (2015), on an artificial diet kept at 25°C in a growth chamber. The *Galleria* larvae were inoculated with IJs of the three-nematode species in 9-mm-diam Petri dishes, supplied with moist filter paper. Freshly harvested IJs were cultured *in vivo*, using last-instar larvae of *G. mellonella*, at 25°C in growth chambers. Modified White traps (Kaya & Stock, 1997) were used to harvest the emerged EPNs. The harvested IJs were stored in distilled water at 14°C, collected in 5-L Erlenmeyer flasks that were constantly stirred using a 70 × 10 mm cylindrical

magnetic stirring bar on an AGE magnetic stirrer (VELP® Scientifica) for approximately three weeks, until the desired concentration of IJs for each batch was achieved.

FORMULATION IN ALGINATE BEADS

The encapsulation of IJs in sodium alginate beads was done by means of modification of the methods of Chen & Glazer (2005) and of Kim *et al*. (2015) at room temperature (24°C), with two batches per species being studied. Two solutions of different composition were utilised. The infective juveniles (IJ) of 98 000 IJs/ml of each nematode species were suspended in 20 ml of solution containing 10% glycerol, 2% sodium alginate (FMC Biopolymer, Cape Town, South Africa), and 0.075% of formaldehyde and Moir's crimson red or apple green food colour dye in distilled water. A droplet of 10-µl alginate solution was dripped from a 1-ml disposable syringe into 20 ml of Ca^{2+}- solution, containing 0.5% $CaCl_2$ H_2O (Merck SA (Pty) Ltd), 10% glycerol and 0.075% formaldehyde in distilled water. The formation of the alginate beads was immediate. The Ca^{2+}- solution was shaken at 1200 rpm, using an orbital shaker (Benchmark's ORBI-SHAKER™ JR), which prevented the beads from sticking together. After 20 min of bead formation, the beads were removed from the reaction beaker with a spatula and dried on paper towels (SCOTT® KIMDRI*, Bedfordview, South Africa). Each bead finally contained approximately 490 ± 25 nematodes (5% error).

RATE OF NEMATODE ESCAPED FROM ALGINATE BEADS

The 22 beads used for storage and the 8 beads used for crushing at each temperature were placed individually in 2.0-ml microcentrifuge tubes (QSP®, USA). The tubes were stored in cardboard microtube freezer boxes (TrueNorth®) in the dark, at 6°C, 14°C and 25°C. The EPNs that escaped from each bead, after being retrieved from each tube with 500 µl of distilled water, were counted using a stereomicroscope (40 × magnification) weekly up to 4 weeks, to determine the survival percentage. Each bead was transferred to a new microcentrifuge tube. Two representative beads per sample were crushed in a 1.5 ml microcentrifuge tube, using a disposable tissue grinder pestle (Axygen®, Axygen Biosciences, Union City, USA) and observed, using a stereomicroscope, in an embryo dish, to determine the status of the IJs that remained inside the beads. The process was repeated on a different test date, using a fresh batch of nematodes.

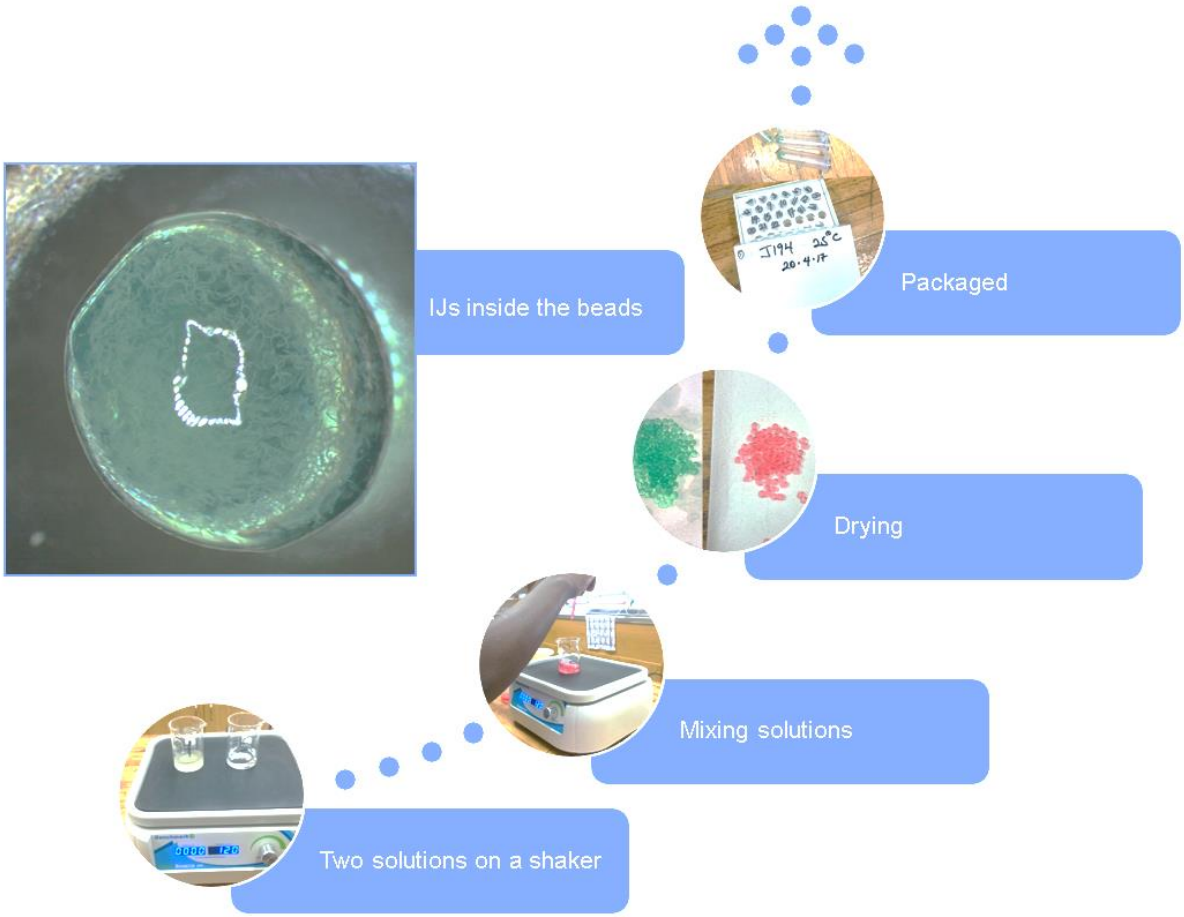


Fig.2. 1. Alginate bead external gelation and formulation process.

Pathogenicity of IJs stored in alginate beads

The three EPN species that were encapsulated in sodium alginate beads, as was previously described, and which were stored for four weeks at 6°C, 14°C and 25°C, were crushed in an embryo dish, following which dilutions of 100 IJs in 50 µl were made. The dilutions were tested for pathogenicity against the last-instar larvae of *G. mellonella,* using 24-well bioassay plates. Each bioassay plate contained 12 larvae, placed alternately in the wells, fitted with a piece of filter paper. Each well was inoculated with 100 IJs in 50 µl of distilled water, while water only was used for the controls. The lid of each well was fitted with a piece of glass of the same shape as the lid, to prevent the *G. mellonella* larvae from escaping. Five 24-well plates, each consisting of 12 wells (n = 60), were used for each treatment (nematode species), with the control receiving water only. The plates were placed in a plastic container, lined with wet paper towels, thus creating 100% humidity, and kept in a growth chamber at 25°C for a period of 48 h. Mortality was confirmed by means of the visual observation of the colour of the cadavers of the wax moth larvae, which were stained yellow, brick-red, and black, for *S. yirgalemense*, *H. bacteriophora,* and *S. jeffreyense*, respectively. The experiment was conducted twice with each nematode species on different test dates, using fresh batches of

nematodes encapsulated in sodium alginate beads, and stored in the same conditions described above.

FORMULATING USING DIATOMACEOUS EARTH

Nematodes were concentrated into a paste using a 32-µm sieve (Clear Edge Filtration SA (Pty) Ltd, South Africa). The three EPN species were formulated in DE (Celite 209 – Imerys Refractory Minerals SA (Pty) Ltd). The steps followed in the formulation process are depicted in Fig. 2.2. The proportions of the ingredients used in the formulation were followed according to Grewal & Jagdale (2002). The relative free water activity of the formulation was 0.970, which induced partial anhydrobiosis and slow desiccation in the IJs (Grewal 2000a, b).

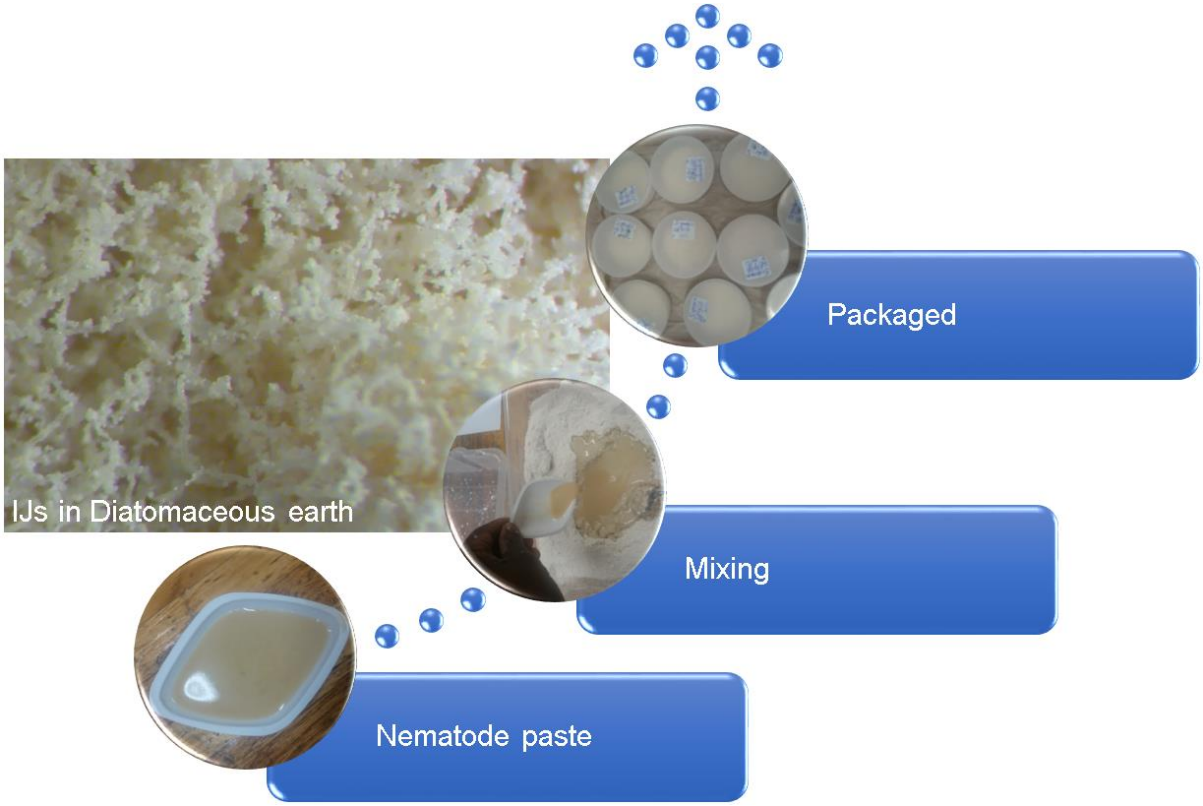


Fig. *2. 2.* Diatomaceous earth formulation process.

SURVIVAL OF NEMATODES IN DIATOMACEOUS EARTH

The formulated nematodes were weighed, with 10 g being added to the plastic containers with lids (Mambo's Plastics) (*n* = 30). The ten containers were placed in larger plastic containers, lined with moistened paper towels (SCOTT® KIMDRI*, Bedfordview, South Africa), and covered with a lid to maintain the humidity levels at 100%. The containers were stored for four weeks at 6°C, 14°C and 25°C. The experiment was conducted twice with each nematode

species on different test dates, using another batch of nematodes, and stored in similar conditions to those described above.

Formulated nematodes were counted according to the modification method of Peters (2004). One gram from a container of 10 g containing 500 000 IJs was dissolved in 10 ml of distilled water in a 50-ml beaker. Air was bubbled in from an aquarium air pump (Second Nature Whisper™ 1000), with its tube leading to the bottom of the beaker. One 100 µl sample was pipetted into 5 ml distilled water in a clean beaker. Mixing was undertaken by shaking, and 1 ml was further diluted with 2 ml of distilled water, with the number of IJs being counted, using a binocular microscope, weekly, for four weeks, to determine the survival percentage in each of the 10 containers, at the respective temperatures of 6°C, 14°C and 25°C.

Statistical analysis

Statistical analyses were conducted using STATISTICA 13.2 software (StatSoft. Inc). The two-way ANOVA and repeated-measure ANOVAs were calculated accordingly. Where the results were not normally distributed, bootstraps were performed on the data for multiple comparisons. In other instances, the means were accordingly separated by means of the Fisher's least significant difference or the Games-Howell post hoc test.

Results

Rate of nematode escaped from alginate beads

No significant differences ($p > 0.05$) were obtained between the main effects of the nematode treatments and the dates between the two batches, thus the data were pooled for analysis. The results indicated that temperature had a significant effect on the number of nematodes moving out of the beads. Fewer IJs moved out of the beads at 6°C, followed by an increase at 14°C and 25°C, respectively. At 6°C, the mean numbers of *S. jeffreyense* differed significantly from those of *H. bacteriophora* and *S. yirgalemense* ($p < 0.001$), which did not differ significantly ($p = 1.00$) from each other (Fig. 2.3).

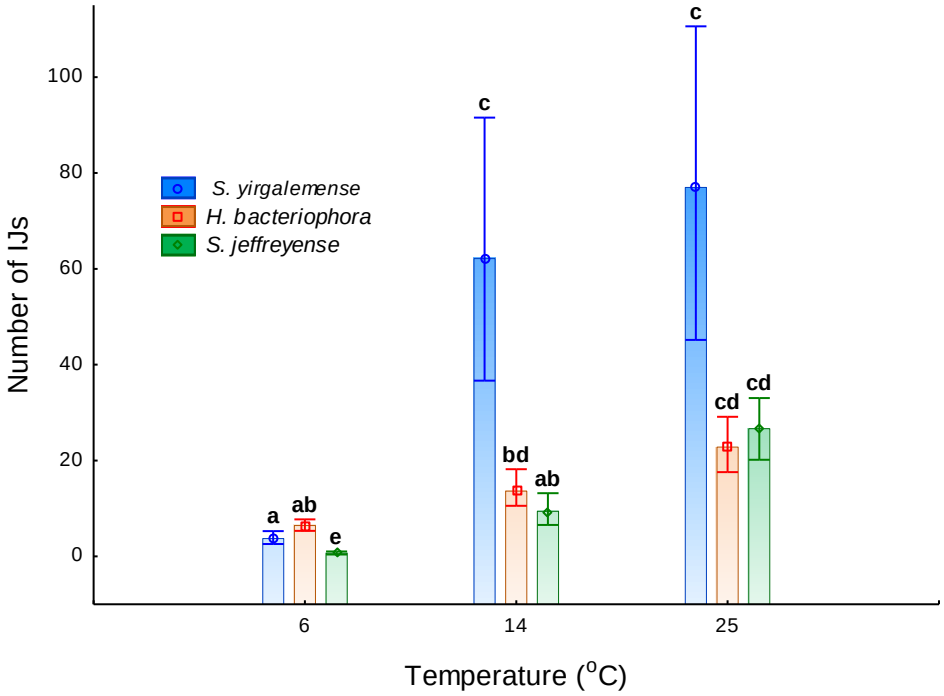


Fig. 2. 3. Mean number of infective juveniles (IJs) (95% confidence level) that moved out of the beads after 4 weeks in respect of *S. jeffreyense, H. bacteriophora* and *S. yirgalemense*. Different letters above the bars indicate significant differences ($p < 0.05$).

The highest mean number of 6.50 IJs was recorded for *H. bacteriophora*, followed by the mean numbers of 3.80 and 0.68 for *S. yirgalemense* and *S. jeffreyense* IJs, respectively, at 6°C. Equally, at 14°C, the mean number of IJs of *S. yirgalemense* differed significantly from those of *H. bacteriophora* ($p = 0.01$) and *S. jeffreyense* ($p < 0.001$), which did not differ significantly ($p = 0.13$) from each other. The highest mean number of 62.73 IJs was recorded for *S. yirgalemense,* followed by those of 13.75 IJs and 9.59 IJs for *H. bacteriophora* and *S. jeffreyense*, respectively. Additionally, at 25°C, the mean number of IJs of *S. yirgalemense* differed significantly from those of *H. bacteriophora* ($p < 0.001$) and *S. jeffreyense* ($p < 0.001$), which did not differ significantly ($p = 0.99$) from each other. The highest mean number of 77.39 IJs was recorded for *S. yirgalemense*, followed by those of 26.59 IJs and 23.11 IJs for *S. jeffreyense* and *H. bacteriophora*, respectively (Fig. 2.3). The mean number of IJs moving out of the beads for the nematode species at different temperatures could be observed to have increased. The mean number of *Steinernema yirgalemense* IJs, at 6°C, differed significantly from those at 14°C ($p = 0.001$) and 25°C ($p = 0.001$). At 14°C, the mean number did not differ significantly from that at 25°C ($p = 0.99$). For *S. yirgalemense*, the largest mean number of 77.39 IJs moving out of the beads occurred at 25°C, followed by the mean numbers of 62.73 and 3.80 occurring at 14°C and 6°C, respectively (Fig. 2.3).

The *H. bacteriophora* IJs, at 6°C, did not differ significantly from those at 14°C ($p = 0.99$), but significantly differed from those at 25°C ($p < 0.001$). For *H. bacteriophora*, the largest mean number of IJs moving out of the beads was 23.11 at 25°C, followed by 13.75 and 6.5 at 14°C and 6°C, respectively. The *S. jeffreyense*, at 6°C, differed significantly from those at 14°C ($p = 0.04$) and 25°C ($p = 0.013$). At 14°C, the mean number differed significantly from that at 25°C ($p = 0.01$). For the *S. jeffreyense* IJs, the largest mean number of 26.59 IJs moving out of the beads was observed at 25°C, followed by the 9.59 and 0.68 moving out at 14°C and 6°C, respectively (Fig. 2.3).

PATHOGENICITY OF IJ STORED IN ALGINATE BEADS

No significant differences ($p > 0.05$) were obtained between the two batches in terms of the main effects of nematode species and treatment, thus the data from the two batches were pooled before analysis. At 6°C, no significant difference was found in the *Galleria* mortality between *S. jeffreyense* and *S. yirgalemense* ($p = 0.38$) and *H. bacteriophora* ($p = 0.93$), which did not differ significantly ($p = 0.22$) from each other. The *H. bacteriophora* IJs caused the highest mean percentage mortality of *Galleria* larvae, of 27.50% ± 4.34%, followed by *S. jeffreyense* and *S. yirgalemense*, with 15.83% ± 4.34% and 4.17% ± 4.34%, respectively (Fig. 2.4).

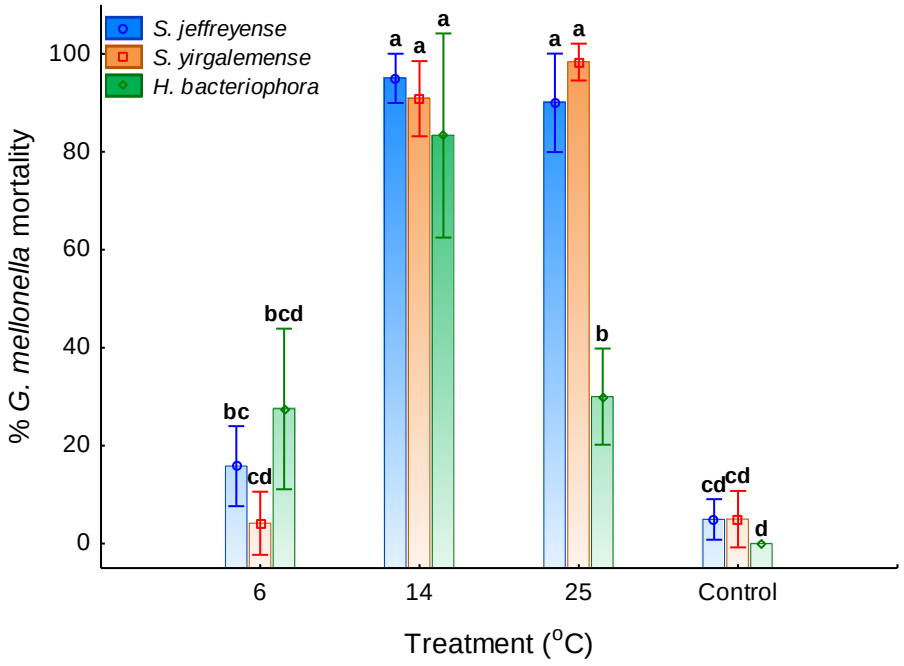


Fig. 2. 4. Mean percentage mortality (95% confidence level) of *Galleria mellonella* inoculated with the infective juveniles of *Steinernema jeffreyense, Heterorhabditis bacteriophora* and *S. yirgalemense*, formulated and stored in alginate beads at different temperatures for four weeks ($F_{(6, 108)} = 22.164$, $p < 0.001$). Mean separated by Games-Howell post hoc test; Error: Between MSE = 188.49, df = 108.00. Different letters above the bars indicate significant differences ($p < 0.05$).

At 14°C, no significant differences were found among the mean percentage *Galleria* mortality for *S. jeffreyense*, *S. yirgalemense* (p = 0.99), and *H. bacteriophora* (p = 0.97), which did not differ significantly (p = 0.99) from each other. The *S. jeffreyense* IJs caused the highest mean percentage *Galleria* mortality of 95.00% ± 4.34%, followed by *S. yirgalemense* and *H. bacteriophora*, with 90.83% ± 4.34% and 83.33% ± 4.34%, respectively (Fig. 2.4). At 25°C, no significant difference was found between the mean percentage *Galleria* mortality for *S. jeffreyense* and *S. yirgalemense* (p = 0.81), while a significant (p < 0.01) difference was recorded with *H. bacteriophora*, which also significantly (p < 0.01) differed from the mean percentage obtained for the *S. yirgalemense* IJs. The *S. yirgalemense* IJs had the highest mean percentage *Galleria* mortality of 98.33% ± 5.27%, followed by *S. jeffreyense*, and by *H. bacteriophora* with 90.00% ± 14.05% and 30.00% ± 13.72%, respectively (Fig. 2.4). No significant differences were found among the mean percentage *Galleria* mortality values for *S. jeffreyense* and *S. yirgalemense* (p = 1.00), and for *H. bacteriophora* (p = 0.35), which did not differ significantly (p = 0.71) from each other in the control (Fig. 2.4).

Within the nematode species, there were significant (ρ < 0.05) and non-significant (p > 0.05) differences per treatment. The *S. jeffreyense* IJs at 6°C differed significantly from those at 14°C (p < 0.0001) and at 25°C (p < 0.0001) but did not differ significantly (p = 0.32) from the control. At 14°C, they did not differ significantly from those at 25°C (p = 0.99). For the *S. jeffreyense* IJs, the highest mean percentage mortality value of 95.00% ± 4.34% was observed at 14°C, followed by 90.00% ± 4.34% and 15.83% ± 4.34%, at 25°C and 14°C respectively (Fig. 2.4).

The *S. yirgalemense* IJs, at 6°C, differed significantly from those at 14°C (p < 0.0001) and 25°C (p < 0.0001). At 14°C, they differed significantly from those at 25°C (p = 0.006). At 25°C, they did not differ significantly from those at 14°C (p = 0.70). For the *S. yirgalemense* IJs, the highest mean percentage *Galleria* mortality value of 98.33% ± 4.34% was observed at 25°C, followed by 90.83% ± 4.34% at 14°C, and by 5.00% ± 4.34% at 6°C, (Fig. 2.4).

The *H. bacteriophora* IJs, at 6°C, differed significantly from those at 14°C (p = 0.007), but did not differ significantly from those either at 25°C (p = 1.00), or from the control (p = 0.89). At 14°C, they differed significantly from those at 25°C (p = 0.006), and from the control (p = 0.0002). At 25°C, the IJs differed significantly from the control (p = 0.002). For *H. bacteriophora* IJs, the highest mean percentage *Galleria* mortality of 83.33% was observed at 14°C, followed by 30.00% ± 4.34% at 27°C, and the 27.50% ± 4.34% at 6°C (Fig. 2.4).

Effect of temperature on the survival of EPNs in diatomaceous earth

No significant differences ($p > 0.05$) were obtained between the two batches in terms of the main effects of temperature and date in the two-way ANOVA, thus enabling the data obtained from the two batches to be pooled and analysed. The analysis of data for weeks 1 to 4 showed significant differences (repeated-measures two-way ANOVA: $F_{(6, 171)} = 171.89$, $p < 0.0001$) between the temperatures regarding the survival of EPNs over time. A steep decline occurred in the mean percentage survival of the EPNs over time at 6°C, whereas a near-stable condition was maintained at 14°C and at 25°C, respectively. In week 1, the mean percentage survival of EPNs at 6°C differed significantly from that at 14°C ($p < 0.0001$) and at 25°C ($p < 0.0001$), with neither differing significantly from the other either at 14°C ($p = 0.94$), or at 25°C ($p = 0.94$) (Fig. 2.5).

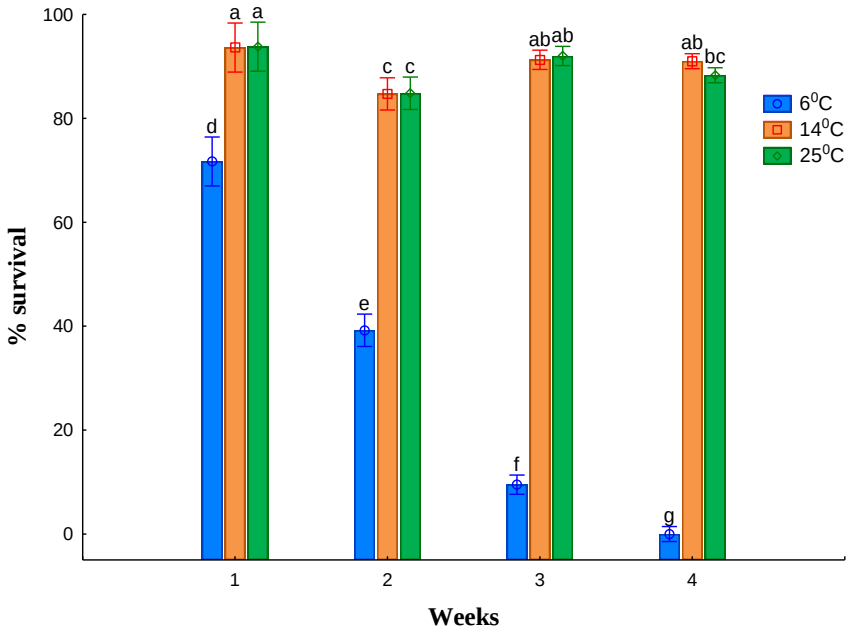


Fig. 2. 5. Mean percentage survival rate (95% confidence level) of EPNs in diatomaceous earth at different temperatures during the 4 weeks, with the repeated-measures two-way ANOVA: ($F_{(6, 171)} = 171.89$, $p < 0.0001$). Mean separated by Fisher's least significant difference (LSD) post hoc test; Error: Between; Within; Pooled MS = 46.642, df = 211.53. Different letters above the bars indicate significant differences.

In week 1, an almost equal mean percentage survival rate was obtained at 25°C (93.81% ± 2.36%), and at 14°C (93.63% ± 2.36%), followed by that which was obtained at 6°C (71.70% ± `2.36%). In week 2, the mean percentage survival rate of EPNs at 6°C significantly differed from that at 14°C ($p < 0.0001$) and at 25°C ($p < 0.0001$), of which neither differed significantly from each other at 14°C ($p = 0.96$), or at 25°C ($p = 0.96$). Similarly, a practically equal mean

percentage survival rate was obtained at 25°C (84.83% ± 1.55%), and at 14°C (84.72% ± 1.55%), followed by that which was obtained at 6°C (39.21% ± 1.55%), respectively (Fig. 2.5).

In week 3, the mean percentage survival rate of EPNs at 6°C significantly differed from that which was obtained at 14°C ($p < 0.0001$), and at 25°C ($p < 0.0001$), but neither differed significantly from the other (14°C ($p = 0.73$) and 25°C ($p = 0.73$)). Similarly, a practically equal mean percentage survival rate was obtained at 25°C (92.02% ± 0.92%), and at 14°C (91.28% ± 0.92%), followed by that which was obtained at 6°C (9.49% ± `0.92%). In week 4, the mean percentage survival rate of EPNs at 6°C significantly differed from that at 14°C ($p < 0.0001$) and at 25°C ($p < 0.0001$), of which neither differed significantly from the other (14°C ($p = 0.94$) and 25°C ($p = 0.94$)). An almost equal mean percentage survival rate was obtained at 14°C (91.01% ± 0.72%) and at 25°C (88.31% ± 0.72%), followed by that which was obtained at 6°C (0.00% ± `0.72%) (Fig. 2.5).

Desiccative effect of diatomaceous earth on the survival of EPNs

No significant differences ($p > 0.05$) were obtained between the two batches in relation to the main effects of nematode species and date in respect of the two-way ANOVA, thus enabling the data from the two batches to be pooled and analysed. The analysis of the data from weeks 1 to 4 showed significant differences (repeated-measures two-way ANOVA: ($F_{(6, 531)} = 8.4622$, $p < 0.01$)) between the different nematode populations regarding their survival over time. A general decline in the mean percentage survival rate of the nematodes occurred over time (Fig. 2.6).

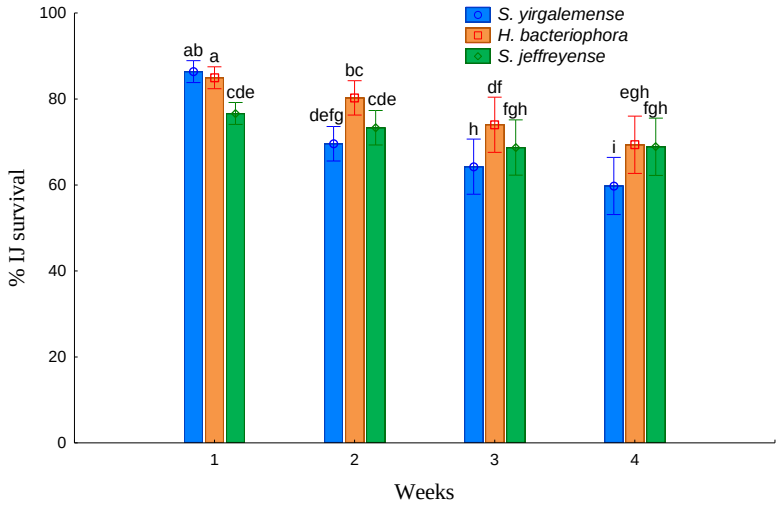


Fig. 2. 6 Mean percentage survival (95% confidence level) of infective juveniles of *Steinernema yirgalemense, Heterorhabditis bacteriophora,* and *S. jeffreyense* in diatomaceous earth (data for different temperatures pooled) during the 4 weeks, and repeated-measures two-way ANOVA: ($F_{(6, 531)} = 8.4622$, $p < 0.0001$). Mean separated by Fisher's least significant difference (LSD) post hoc test; Error: Between; Within; Pooled MS = 416.77, df = 293.30. Different letters above the bars indicate significant differences ($P < 0.05$).

In week 1, the mean percentage survival rate of *S. yirgalemense* did not differ significantly from that of *H. bacteriophora* (p = 0.70), although it did differ from *S. jeffreyense* (p = 0.009), which also differed significantly from each other (*H. bacteriophora* (p = 0.027); *S. jeffreyense* (p = 0.027)). The highest mean percentage survival was obtained with *S. yirgalemense* (86.38% ± 1.29%), followed by *H. bacteriophora* (84.95% ± 1.29%) and by *S. jeffreyense* (76.64% ± 1.29%) (Fig. 2.6).

In week 2, the mean percentage survival rate of *S. yirgalemense* differed significantly from *H. bacteriophora* (p = 0.004), but it did not differ significantly from *S. jeffreyense* (p = 0.059), with the difference from each other not being significant (*H. bacteriophora* (p = 0.06); *S. jeffreyense* (p = 0.06)). The highest mean percentage survival rate was obtained with *H. bacteriophora* (80.27% ± 2.03%), followed by *S. jeffreyense* (73.33% ± 2.03%), and *S. yirgalemense* (69.59% ± 2.03%). In week 3, the mean percentage survival rate of *S. yirgalemense* significantly differed from that for *H. bacteriophora* (p = 0.009), but it did not differ significantly from *S. jeffreyense* (p = 0.23), with the difference from each other not being significant (*H. bacteriophora* (p = 0.16); *S. jeffreyense* (p = 0.16)). The highest mean percentage survival rate was obtained with *H. bacteriophora* (74.03% ± 3.25%), followed by that which was obtained with *S. jeffreyense* (68.72% ± 3.25%), and with *S. yirgalemense* (64.26% ± 3.25%). In week 4, the mean percentage survival rate of *S. yirgalemense* differed significantly from that of *H. bacteriophora* (p = 0.011) and from that of *S. jeffreyense* (p = 0.015) which did not differ significantly from each other (*H. bacteriophora* (p = 0.90); *S. jeffreyense* (p = 0.90)). A slightly higher mean percentage survival rate was obtained with *H. bacteriophora* (69.35% ± 3.38%) than with *S. jeffreyense* (68.90% ± 3.38%), followed by that which was obtained with *S. yirgalemense* (59.77% ± 3.38%) (Fig. 2.6).

Overall survival rate in diatomaceous earth at different temperatures

A summary of the survival rate of the EPNs investigated, as affected by both temperature and DE, is depicted in Fig. 2.7.

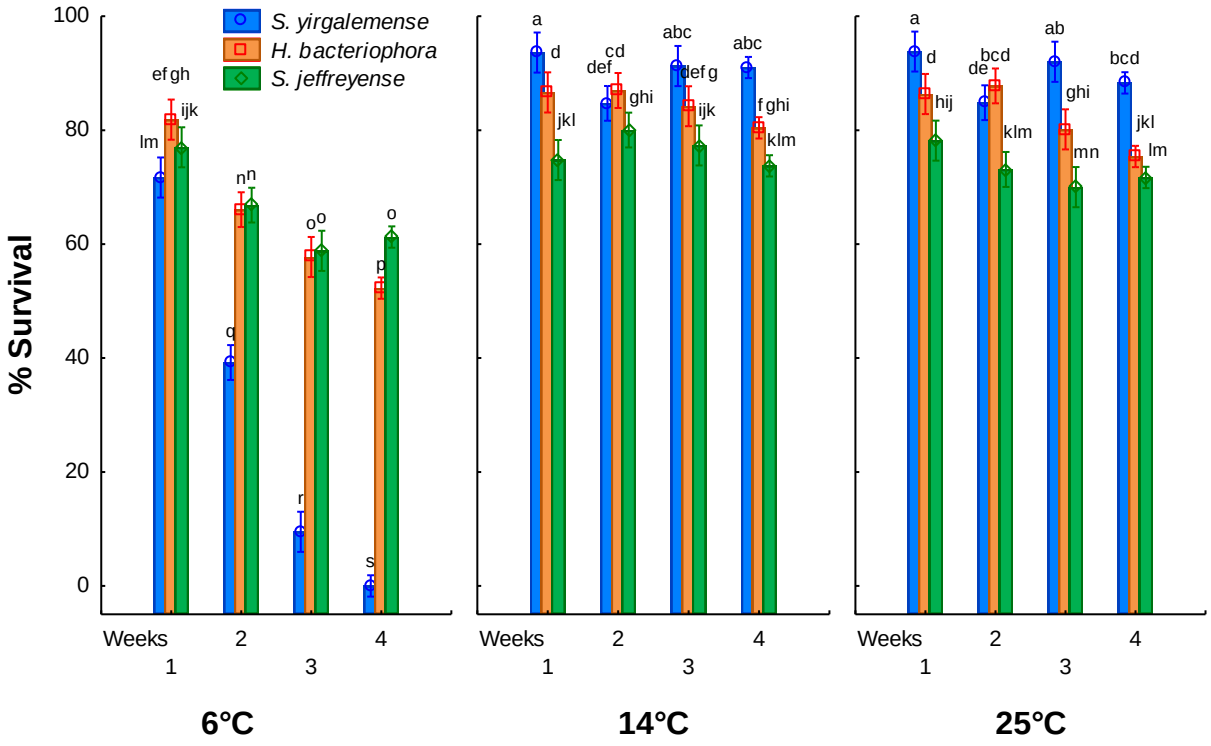


Fig. 2. 7. Mean percentage survival (95% confidence level) of *S. yirgalemense, H. bacteriophora,* and *S. jeffreyense* IJs in diatomaceous earth at different temperatures during the 4 weeks and repeated measures two-way ANOVA: ($F_{(12,\ 513)}$ = 32.860, $p < 0.0001$). Mean separated by Fisher's least significant difference (LSD) post hoc test; Error: Between; Within; Pooled MS = 48.306, df = 666.44. Different letters above the bars indicate significant differences ($p < 0.05$).

Discussion

The current study reports on the improvement in the alginate beads formulation of the IJs of *S. jeffreyense*, *H. bacteriophora* and *S. yirgalemense*. Currently, the formulation of IJs in alginate beads is being faced with several challenges, including that of the IJs being able to readily escape from the soft alginate beads (Hiltpold *et al*., 2012). The result is that the beads cannot retain the EPNs over an extended period, therefore limiting the long-term storage of alginate beads containing nematodes. Also, the adjustment of the bead properties, as with the post-treatment of the beads with additional Ca^{2+} to improve their hardness, could exert an adverse effect on EPN retention, thus preventing the IJs from escaping from the beads (Kim *et al*., 2015). Attaining a month-long room temperature shelf stability in the formulation of the three local EPN species was necessary, given the commercial interest that the EPNs hold in terms of protecting crops within the sphere of South African agricultural production. The mean number of IJs that escaped from the beads at 25°C during the 4 weeks was relatively low, indicating that alginate beads can release IJs at a relatively slow pace, and thus improve their long-term room temperature shelf stability.

Temperature had a significant effect on the different EPN species involved, regarding the number of nematodes moving out of the beads. Kagimu *et al*. (2017) and Stuart *et al*. (2015)

identified temperature, in terms of heat and cold tolerance, as one of the abiotic factors influencing the survival of IJs. Kagimu *et al*. (2017) further asserted that the prevailing temperature could add significantly to the insecticide efficacy of IJs, which could be seen at 6°C, at which temperature the IJs caused significantly lower mortality to the highly susceptible *G. mellonella* larvae than they did at the other temperatures at which the tests were conducted. Furthermore, Addis *et al*. (2016), in their study of the life history traits of *S. yirgalemense* using a hanging drop reported low survival rates, not exceeding 42 days, at a storage temperature of 4°C. In contrast, they found that, at 15°C and 25°C, more than 95% of the IJs survived for a period of up to 66 days. The *S. yirgalemense* strain 157-C used, was the same as that which was used in the current study. All species studied in the present research were local isolates, which are adapted to the relatively warm South African climate (Malan & Hatting, 2015), and which are, thus, less well adapted to surviving under conditions of low temperature, further explaining the observations made.

The survival of IJs is often improved in many formulations, by means of storing the nematodes concerned at lo w temperatures between 4°C and 15°C (Grewal & Peters, 2005). The present data do not support the making of such a supposition, however, given the low mean number of IJs observed to be moving out of the beads at 6°C. Furthermore, the results show loss of virulence at low temperatures. Therefore, the three formulated nematodes should be stored at temperatures ranging between 14°C and 25°C, leading to no refrigeration of the nematodes stored in formulation for the local species. Furthermore, the results clearly show that the temperature had a significant effect on the survival of the three species in DE, with the temperature of 6°C substantially reducing the survival of IJs over time, especially in the case of *S. yirgalemense*.

Diatomaceous earth is widely used as an insecticide, with control generally caused by desiccation, due to the hygroscopic nature of some of the grades available. In this study, relatively few of the IJs were expected to survive, due to the low density of IJs in the formulation. Coincidentally, the results showed a stable decrease, or slow lowering, of the shelf life of the IJs stored in DE formulation. The results also showed that the use of DE did not lead to the desiccation of the nematodes, with there being a considerably higher survival rate by the fourth week of the study. This finding can only be explained by such characteristics as homogeneity (shape, size, quantity of nematodes, and weight), structure, mechanical resistance, and the properties of the inert granular materials of DE formulation that could influence the efficacy of EPNs in the control of pest infestations (Hiltpold *et al*., 2012; Matadamas-Ortiz *et al*., 2014; Silver *et al*., 1995). For example, Ziaee *et al*. (2016), in their investigation of the possible use of Iranian DEs as grain protectants in stored-product pest management programmes, observed the increased mortality of adults of *Oryzaephilus*

surinamensis (L.), with increasing exposure intervals and concentration levels. The low density of IJs/g in the DE, as used in the current study, does not necessarily translate into the reduced shelf life of the IJs, as would be likely to be observed at higher densities. The above is due to the strong desiccative effect of the formulation on the IJs employed. In contrast, the results correspond to those of Matadamas-Ortiz *et al*. (2014), whose best results were obtained following 100DE:0AC proportions.

In conclusion, the properties of alginate beads were adjusted to enable them to release IJs on a regular basis, which has gone some way to solving the challenges encountered with the use of soft and hard beads. The loss of virulence and survival is reported for the first time as occurring at relatively low temperatures for the three EPNs stored at 6°C. The high survival and virulent abilities of the studied species at room temperature obtained showed potential for a reduction in the existing costs of refrigeration during both storage and transportation. In the present study, we were constrained from obtaining the much-desired number of IJs due to the *in vivo* culturing of IJs, so that we were only able to formulate IJs at a lower density (IJs/g) of DE formulation. We recommend, in terms of using *in vitro* cultures, striving to obtain a much higher density of IJs in formulation, to be able to obtain a more realistic and improved DE formulation.

References

Addis, T., Mijušković, N., Ehlers, R.-U. & Strauch, O. (2016). Life history traits, liquid culture production and storage temperatures of *Steinernema yirgalemense*. *Nematology* 18, 367-376. DOI: 10.1163/15685411-00002966.

Akhurst, R. & Smith, K. (2002). Regulation and safety. In: Gaugler, R. (Ed.). *Entomopathogenic nematology*. Wallingford, UK, CABI Publishing, pp. 311-332. DOI: 10.1079/9780851995670.0311.

Bilgrami, A.L., Gaugler, R., Shapiro-Ilan, D.I. & Adams, B.J. (2006). Source of trait deterioration in entomopathogenic nematodes *Heterorhabditis bacteriophora* and *Steinernema* carpocapsae during in vivo culture. *Nematology* 8, 397-409. DOI: 10.1163/156854106778493394.

Buchholz, S., Merkel, K., Spiewok, S., Pettis, J.S., Duncan, M., Spooner-Hart, R., Ulrichs, C., Ritter, W., Neumann, P. (2009). Alternative control of *Aethina tumida* Murray (Coleoptera: Nitidulidae) with lime and diatomaceous earth. *Apidologie* 40, 535-548. DOI: 10.1051/apido/2009020.

Burnell, A.M. & Stock, S.P. (2000). *Heterorhabditis*, *Steinernema* and their bacterial symbionts-lethal pathogens of insects. *Nematology* 2, 31-42. DOI: 10.1163/156854100508872.

Chen, S., & Glazer, I. (2005). A novel method for long-term storage of the entomopathogenic nematode *Steinernema feltiae* at room temperature. *Biological Control* 32, 104-110. DOI: 10.1016/j.biocontrol.2004.08.006.

Ciche, T.A., Darby, C., Ehlers, R.U., Forst, S. & Goodrich-Blair, H. (2006). Dangerous liaisons: the symbiosis of entomopathogenic nematodes and bacteria. *Biological Control* 38, 22-46. DOI: 10.1016/j.biocontrol.2005.11.016.

Dowds, B.C.A. & Peters, A. (2002). Virulence mechanisms. In: Gaugler, R. (Ed.). *Entomopathogenic nematology*. Wallingford, UK, CABI Publishing, pp. 79-98. DOI:10.1079/9780851995670.0079.

Dunphy, G.B. & Webster, J.M. (1988). Lipopolysaccharides of *Xenorhabdus nematophilus* (Enterobacteriaceae) and their haemocyte toxicity in non-immune *Galleria mellonella* (Insecta: Lepidoptera) larvae. *Journal of General Microbiology* 134, 1017-1028. DOI: 10.1099/00221287-134-4-1017.

Ehlers, R.-U. (2001). Mass production of entomopathogenic nematodes for plant protection. *Applied Microbiology and Biotechnology* 56, 623-633. DOI: 10.1007/s002530100711.

Ehlers, R.-U. (2003). Biocontrol nematodes. In: Hokkanen, H.M.T. & Hajek, A.E. (Eds). *Environmental impacts of microbial insecticides*. Dordrecht, Netherlands, Springer Netherlands, pp. 177-220. DOI: 10.1007/978-94-017-1441-9_10.

Ehlers, R.-U. & Shapiro-Ilan, D.I. (2005). Mass production. In: Grewal, P.S., Ehlers, R.-U. & Shapiro-Ilan, D.I. (Eds). *Nematodes as biocontrol agents.* Wallingford, UK, CABI Publishing, pp. 65-75. DOI: 10.1079/9780851990170.0065.

Golob, P. (1997). Current status and future perspectives for inert dusts for control of stored product insects. *Journal of Stored Products Research* 33, 69-79. DOI: 10.1016/S0022-474X(96)00031-8.

Grewal, P.S. (2000a). Enhanced ambient storage stability of an entomopathogenic nematode through anhydrobiosis. *Pest Management Science* 56, 401-406. DOI: 10.1002/(SICI)1526-4998(200005)56:5<401::AID-PS137>3.0.CO;2-4.

Grewal, P.S. (2000b). Anhydrobiotic potential and long-term storage of entomopathogenic nematodes (Rhabditida: Steinernematidae). *International Journal for Parasitology* 30, 995-1000. DOI: 10.1016/S0020-7519(00)00080-1.

Grewal, P.S. (2002). Formulation and application technology. In: Gaugler, R. (Ed.). *Entomopathogenic nematology*. Wallingford, UK, CABI Publishing, pp. 265-287. DOI: 10.1079/9780851995670.0265.

Grewal, P.S. & Jagdale, G.B. (2002). Enhanced trehalose accumulation and desiccation survival of entomopathogenic nematodes through cold preacclimation. *Biocontrol Science and Technology* 12, 533-545. DOI: 10.1080/0958315021000016207.

Grewal, P.S. & Peters, A. (2005). Formulation and quality. In: Grewal, P.S., Ehlers, R.-U. & Shapiro-Ilan, D.I. (Eds). *Nematodes as biocontrol agents.* Wallingford, UK, CABI Publishing, pp.79-89. DOI: 10.1079/9780851990170.0079.

Grewal, P.S., Wang, X. & Taylor, R.A.J. (2002). Dauer juvenile longevity and stress tolerance in natural populations of entomopathogenic nematodes: is there a relationship? *International Journal for Parasitology* 32, 717-725. DOI: 10.1016/S0020-7519(02)00029-2.

Han, R. & Ehlers, R.-U. (2000). Pathogenicity, development, and reproduction of *Heterorhabditis bacteriophora* and *Steinernema carpocapsae* under axenic in vivo conditions. *Journal of Invertebrate Pathology* 75, 55-58. DOI: 10.1006/jipa.1999.4900.

Han, R. & Ehlers, R. (2001). Effect of *Photorhabdus luminescens* phase variants on the in vivo and in vitro development and reproduction of the entomopathogenic nematodes *Heterorhabditis bacteriophora* and *Steinernema carpocapsae*. *FEMS Microbiology/Ecology* 35, 239-247. DOI: 10.1111/j.1574-6941.2001.tb00809.x.

Hatting, J.L., Moore, S.D. & Malan, A.P. (2018). Microbial control of phytophagous invertebrate pests in South Africa: current status and future prospects. *Journal of Invertebrate Pathology*, In press, Corrected proof. DOI: 10.1016/j.jip.2018.02.004.

Hiltpold, I., Hibbard, B.E., French, B.W. & Turlings, T.C.J. (2012). Capsules containing entomopathogenic nematodes as a Trojan horse approach to control the western corn rootworm. *Plant and Soil* 358, 11-25. DOI: 10.1007/s11104-012-1253-0.

Hopper, K.R., Roush, R.T. & Powell, W. (1993). Management of genetics of biological control Introductions. *Annual Review of Entomology* 38, 27-51. DOI: 10.1146/annurev.en.38.010193.000331.

Kagimu, N., Ferreira, T. & Malan, A.P. (2017). The attributes of survival in the formulation of entomopathogenic nematodes utilised as insect biocontrol agents. *African Entomology* 25, 275-291. DOI: 10.4001/003.025.0275.

Kaya, H.K. & Stock, S.P. (1997). Techniques in insect nematology. In: Lacey, L. (Ed.). *Manual of techniques in insect pathology*. San Diego, CA, Academic Press, pp. 281-324. DOI: 10.1016/B978-012432555-5/50016-6.

Kaya, H.K. & Nelsen, C.E. (1985). Encapsulation of Steinernematid and Heterorhabditid nematodes with calcium alginate: a new approach for insect control and other applications. *Environmental Entomology* 14, 572-574. DOI: 10.1093/ee/14.5.572.

Kaya, H.K., Mannion, C.M., Burlando, T.M. & Nelsen, C.E. (1987). Escape of *Steinernema feltiae* from alginate capsules containing tomato seeds. *Journal of Nematology* 19, 287-91. PMCID: PMC2618656.

Kim, J., Jaffuel, G. & Turlings, T.C.J. (2015). Enhanced alginate capsule properties as a formulation of entomopathogenic nematodes. *BioControl* 60, 527-535. DOI: 10.1007/s10526-014-9638-z.

Lu, D., Macchietto, M., Chang, D., Barros, M.M., Baldwin, J., Mortazavi, A. & Dillman, A.R. (2017). Activated entomopathogenic nematode infective juveniles release lethal venom proteins. *PLOS Pathogens* 13, e1006302. DOI: 10.1371/journal.ppat.1006302.

Malan, A.P., Knoetze, R. & Tiedt, L.R. (2016). *Steinernema jeffreyense* n. sp. (Rhabditida: Steinernematidae), a new entomopathogenic nematode from South Africa. *Journal of Helminthology* 90, 262-278. DOI: 10.1017/S0022149X15000097.

Malan, A.P. & Hatting, J.L. (2015). Entomopathogenic nematode exploitation: case studies in laboratory and field applications from South Africa. In: Campos-Herrera, R. (Ed.). *Nematode pathogenesis of insects and other pests*. Dordrecht, Netherlands, Springer International Publishing, pp. 477-508. DOI: 10.1007/978-3-319-18266-7_20.

Matadamas-Ortiz, P.T., Ruiz-Vega, J., Vazquez-Feijoo, J.A., Cruz-Martinez, H. & Cortes-Martinez, C.I. (2014). Mechanical production of pellets for the application of entomopathogenic nematodes: factors that determine survival time of *Steinernema glaseri*. *Biocontrol Science and Technology* 24, 145-157. DOI: 10.1080/09583157.2013.852161.

Navon, A., Nagalakshmi, V.K., Levski, S., Salame, L. & Glazer, I. (2002). Effectiveness of entomopathogenic nematodes in an alginate gel formulation against lepidopterous pests. *Biocontrol Science and Technology* 12, 737-746. DOI: 10.1080/0958315021000039914.

Navon, A., Keren, S., Salame, L. & Glazer, I. (1998). An edible-to-insects calcium alginate gel as a carrier for entomopathogenic nematodes. *Biocontrol Science and Technology* 8, 429-437. DOI: 10.1080/09583159830225.

Nguyen, K.B., Tesfamariam, M., Gozel, U., Gaugler, R & Adams, B.J. (2004). *Steinernema yirgalemense* n. sp. (Rhabditida: Steinernematidae) from Ethiopia. *Nematology* 6, 839-856. DOI: 10.1163/1568541044038605.

Odendaal, D., Addison, M.F. & Malan, A.P. (2015). Control of codling moth (*Cydia pomonella*) (Lepidoptera: Tortricidae) in South Africa with special emphasis on using entomopathogenic nematodes. *African Entomology* 23, 259-274. DOI: 10.4001/003.023.0224.

Odendaal, D., Addison, M.F. & Malan, A.P. (2016). Entomopathogenic nematodes for the control of the codling moth (*Cydia pomonella* L.) in field and laboratory trials. *Journal of Helminthology* 90, 615-623. DOI: 10.1017/S0022149X15000887.

Peters, A. (2004). Protocol quality assessment of entomopathogenic nematodes. In: *COST Action 850 meeting programme and mushroom protocols*. Wädenswil, Switzerland, Agroscope Faw, pp. 4-6.

Patel, A.V. & Vorlop, K.-D. (1994). Entrapment of biological control agents applied to entomopathogenic nematodes. *Biotechnology Techniques* 8, 569-574. DOI: 10.1007/BF00152148.

Poinar, G.O. (1976). Description and biology of a new insect parasite rhabditoid, *Heterorhabditis bacteriophora* n. gen. n. sp. (Rhabditida; Heterorhabditidae. fam.). *Nematologica* 21, 463-470. DOI: 10.1163/187529275x00239.

Shapiro-Ilan, D.I. & Gaugler, R. (2002). Production technology for entomopathogenic nematodes and their bacterial symbionts. *Journal of Industrial Microbiology & Biotechnology* 28, 137-146. DOI: 10.1038/sj/jim/7000230.

Shapiro-Ilan, D.I., Gouge, D.H. & Koppenhöfer, A.M. (2002). Factors affecting commercial success: case studies in cotton, turf and citrus. In: Gaugler, R. (Ed.). *Entomopathogenic nematology*. Wallingford, UK, CABI Publishing, pp. 333-355. DOI: 10.1079/9780851995670.0333.

Shapiro-Ilan, D.I., Han, R. & Dolinksi, C. (2012). Entomopathogenic nematode production and application technology. *Journal of Nematology* 44, 206-217. PMCID: PMC3578468.

Silver, S., Dunlop, D. & Grove, D. (1995). *WIPO Patent No. WO 95/0577*. Geneva, Switzerland, World Intellectual Property Organization.

Stuart, R.J., Barbercheck, M.E. & Grewal, P.S. (2015). Entomopathogenic nematodes in the soil environment: distributions, interactions and the influence of biotic and abiotic factors. In: Campos-Herrera, R. (Ed.). *Nematode pathogenesis of insects and other pests*. Dordrecht, Netherlands, Springer International Publishing, pp. 97-137. DOI: 10.1007/978-3-319-18266-7_4.

Surrey, M.R. & Wharton, D.A. (1995). Desiccation survival of the infective larvae of the insect parasitic nematode, *Heterorhabditis zealandica* Poinar. *International Journal for Parasitology* 25, 749-752. DOI: 10.1016/0020-7519(94)00190-Y.

Van Zyl, C. & Malan, A.P. (2015). Cost-effective culturing of *Galleria mellonella* and *Tenebrio Molitor* and entomopathogenic nematode production in various hosts. *African Entomology* 23, 361-375. DOI: 10.4001/003.023.0232.

Vemmer, M. & Patel, A.V. (2013). Review of encapsulation methods suitable for microbial biological control agents. *Biological Control* 67, 380-389. DOI: 10.1016/j.biocontrol.2013.09.003.

Wakil, W., Riasat, T.M., Ghazanfar, U., Kwon, YJ., Shaheen, F.A. (2011). Aptness of *Beauveria bassiana* and enhanced diatomaceous earth (DEBBM) for control of *Rhyzopertha dominica* F. *Entomological Research* 41, 233-241. DOI: 10.1111/j.1748-5967.2011.00342.x

Webster, J.M., Chen, G., Hu, K. & Li, J. (2002). Bacterial metabolites. In: Gaugler, R. (Ed.). *Entomopathogenic nematology*. Wallingford, UK, CABI Publishing, pp. 99-114. DOI:10.1079/9780851995670.0099.

Womersley, C.Z. (1990). Dehydration survival and anhydrobiotic potential. In: Gaugler, R. & Kaya, H.K. (Eds). *Entomopathogenic nematodes in biological control.* Boca Raton, FL, USA, CRC Press Inc, pp. 117-137.

Ziaee, M., Atapour, M. & Marouf, A. (2016). Insecticidal efficacy of Iranian diatomaceous earths on adults of *Oryzaephilus surinamensis*. *Journal of Agricultural Science and Technology* 18, 361-370.

Chapter 3

Effect of antifungal agents on the efficacy of *Steinernema yirgalemense* and room temperature storability in diatomaceous earth

Abstract

The quality of food systems is often deteriorated by microbial contamination. The formulation of entomopathogenic nematodes (EPNs) in diatomaceous earth (DE) for use as biological control agents, in integrated pest management programmes, is no exception. Microbial contamination unequivocally lowers the quality and shelf life of EPNs in formulation. For a feasible solution, the effect of such antifungal agents as peroxyacetic acid (PAA), trans-cinnamic acid (TCA) and nipagin on the efficacy of the infective juveniles of *Steinernema yirgalemense*, a local South African species of EPNs, was studied. The viability of the room temperature storability of *S. yirgalemense*, at a higher density in DE after 4 weeks, was equally considered. A decline in the survival rate and pathogenicity of *S. yirgalemense*, due to PAA, is reported upon. In contrast, TCA and nipagin had no effect on the survival rate and pathogenicity of *S. yirgalemense*. Furthermore, the shelf life of IJs stored in DE formulation at room temperature improved when measured against the 80% mean survival rate of *S. yirgalemense* in week 4 at 25°C.

Key words: formulation, nipagin, peroxyacetic acid, entomopathogenic, virulence, trans-cinnamic acid, microbial, toxicity

Introduction

The successful commercialisation of EPNs after mass production prerequisites the development of storage and formulation techniques that curtail nematode mortality, as well as their loss of virulence and pathogenicity. However, their meagre shelf life is a drawback especially in terms of large-scale commercial use (Grewal, 2000a,b). Yet, maintenance of high-quality EPNs at all levels from the bioreactor, liquid storage, and storage in formulation, until the product reaches the famers' field (Grewal & Peters, 2005; Kagimu *et al*., 2017), is of importance. Such maintenance is necessary, because EPNs are affected by both biotic and abiotic factors, especially when in formulation, in relation to which the temperature, aeration, moisture content and water loss, as well as the contamination and toxicity of antimicrobial agents in terms of IJs are of foremost importance (Grewal & Peters, 2005; Kagimu *et al*., 2017).

Microbial contamination, such as the frequent contamination of nematode formulations with high moisture content by microbial organisms, which is a significant hindrance, especially in relation to room temperature shelf life, is of significance (Grewal, 2002). The reason for the above is that microbials tend to compete for existing oxygen supplies, decrease the usability of formulations, and initiate the clogging of spray nozzles, thereby decreasing the suitability of the formulated product. Notably, some antimicrobial agents may also decrease the extent of nematode survival in the formulations (Grewal, 2002).

Peroxyacetic/ peracetic acid (PAA) is a strong oxidant and disinfectant, with a wide spectrum of antimicrobial activity, which is commercially available as a mixture containing acetic acid, hydrogen peroxide and water. Various industries have demonstrated its effectiveness as an antibacterial, antiviral and antifungal agent (Ayoub *et al*., 2017; Kitis, 2004). Peracetic acid (CH_3CO_3H) is registered by the United States Environmental Protection Agency (US EPA 2007) for its utilisation in agriculture and food processing, as well as in medical facilities, as an antimicrobial disinfectant. Furthermore, in Europe, PAA, which is approved for use in veterinary medicine, is one of the few compounds approved for use as a disinfectant in aquaculture (Straus *et al*., 2012).

Equally important, cinnamic acid, which is an organic acid that naturally occurs in plants, has low toxicity and a broad spectrum of biological activities. Cinnamic acid has antimicrobial activity and its derivatives, when isolated from plant material and synthesised, have antibacterial, antiviral and antifungal properties (Nascimento *et al*., 2000; Sova, 2012). Cinnamic acid mainly exists in cis and trans isomers. Besides, trans-cinnamic (TCA) has been isolated as a secondary metabolite of the EPN-associated bacteria *Photorhabdus*

luminescens and reported to be having an immense antifungal capability (Bock *et al*., 2014; Hazir *et al*., 2016, 2017, 2018).

Last but not least, nipagin M (methyl 4-hydroxybenzoate), or methyl paraben, methyl *p*-hydroxybenzoate, or methyl parahydroxybenzoate is the methyl ester of *p*-hydroxybenzoic acid. The compound, which has been used as an antimicrobial preservative in foods, drugs and cosmetics for over 50 years is stable and non-volatile (Błędzka *et al*., 2014; Mahuzier *et al*., 2001; Soni *et al*., 2002). Nipagin, which is biodegradable, since it is readily metabolised by common soil bacteria, is often used as an antimicrobial agent in many insect food diets (Garrido-Jurado *et al*., 2011; Quesada-Moraga *et al*., 2006; Rohlfs *et al*., 2005).

In Chapter 2, the storage of formulated local South African species of EPNs was recommended at temperatures ranging between 14°C and 25°C, due to the relatively high survival and virulence rates attained at such temperatures. In addition, the comparatively low density of the IJs/g of diatomaceous earth (DE), which was investigated in Chapter 2, coincidentally depicted the relatively long shelf life of IJs, as observed at relatively high densities. Thus, the need to provide a comparatively high density of IJs for a realistic and improved DE formulation was of interest to the current study.

The aim of the present study was to test *Steinernema yirgalemense* Nguyen, Tesfamariam, Gozel, Gaugler & Adams, 2004 at a relatively high density in DE, for the improvement of its shelf life. Furthermore, the study considered the likelihood of bacterial and fungal infections being present in the sample at a comparatively high-water activity, at which such microbials as fungi tend to thrive in formulation, due to the increased amount of water in the formulation. The study, hence, determined the survival and the efficacy of the IJs against three antifungal agents, namely PAA, TCA and nipagin.

Materials and methods

SOURCE OF NEMATODES AND HOST INSECTS

The nematode *S. yirgalemense,* which is endemic to South Africa, was used in the current study. *Galleria mellonella* (Lepidoptera: Phyralidae) larvae were cultured according to Van Zyl & Malan (2015), on an artificial diet at 25°C in a growth chamber. The *Galleria* larvae were inoculated with IJs in 9-mm-diameter Petri dishes, lined with moist filter paper. Freshly harvested IJ were cultured *in vivo*, using last-instar larvae of *Galleria*, kept at 25°C in growth chambers. Modified White traps (Kaya & Stock, 1997) were used to harvest the emerged EPNs. Harvested IJs were stored in distilled water at 14°C and collected in 5-L Erlenmeyer flasks that were constantly stirred using a 70 × 10 mm cylindrical magnetic stirring bar on an

AGE magnetic stirrer (VELP® Scientifica) for approximately three weeks, until the desired concentration of IJs for each batch was achieved.

FORMULATING USING DIATOMACEOUS EARTH

Nematodes were concentrated into a paste using a 32-µm sieve (Clear Edge Filtration SA (Pty) Ltd, - South Africa). The technique described in Chapter 2 was used to formulate 40 million IJs of *S. yirgalemense* in DE (Celite 209 - Imerys Refractory Minerals SA (Pty) Ltd). The proportions of the ingredients used in the formulation were employed according to Grewal & Jagdale (2002) as is indicated in Table 3.1 below. The water activity of 0.970 was used in the formulation and induced the IJs into a partial anhydrobiosis and a slow desiccation (Grewal 2000a, b). The nematode paste was hand-mixed with all the ingredients and the formulated nematodes weighing 10 placed in lidded containers (Mambo's Plastics) (n = 30). Ten containers were further placed into larger covered containers, lined with moistened paper towels (SCOTT® KIMDRI* – Bedfordview, South Africa) to maintain humidity at 100%. The containers were stored for four weeks at 14°C and 25°C. Fig. 3.1 below depicts the steps followed in the formulation process. The experiment was conducted twice on different test dates, using different batches of nematodes, and stored under the same conditions as those that are described above.

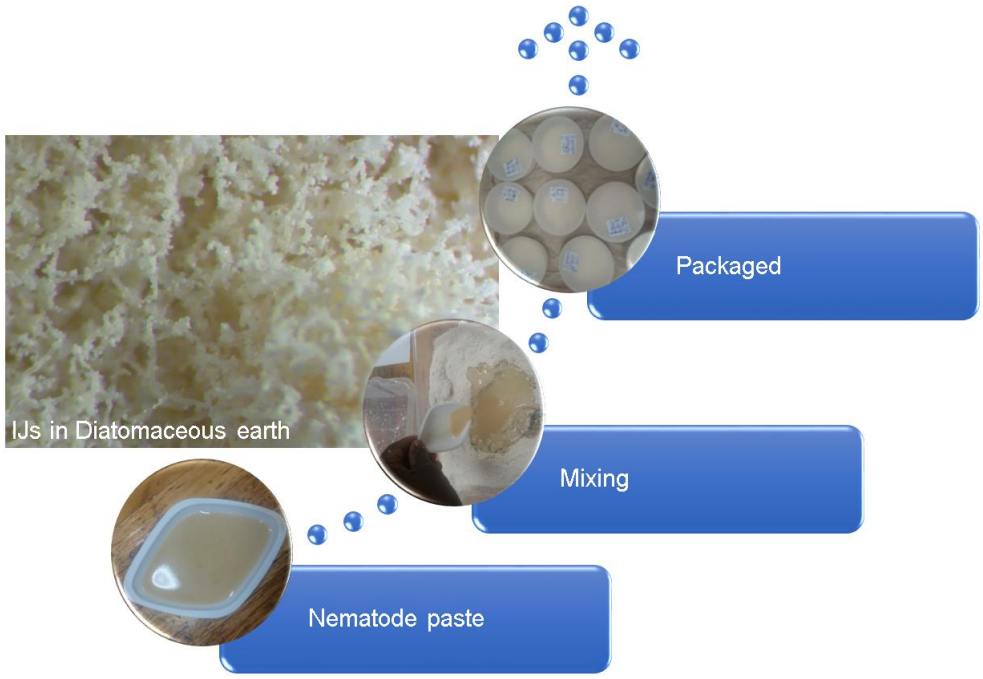


Fig. 3. 1. Diatomaceous earth formulation process.

SURVIVAL OF NEMATODES IN DIATOMACEOUS EARTH

Formulated nematodes were counted according to a modification method of Peters (2004). One g of the formulation from a container with 10 g with 2 000 000 IJs was dissolved in 10 ml of distilled water in a 50-ml beaker and the IJs suspended. Air was bubbled in from an aquarium air pump (Second Nature Whisper™ 1000), with its tube leading to the bottom of the beaker. One 100-µl sample was pipetted into 5 ml distilled water in a clean beaker, which was then brought into suspension, with 1 ml of the suspension being diluted with 2 ml of distilled water. The IJs were counted weekly, using a binocular microscope, for four weeks to determine the survival percentage in each of the 10 containers at the respective temperatures of 14°C and 25°C.

ANTIFUNGAL TOXICITY SCREENING

Three antifungal agents, PAA, TCA and nipagin, were tested for their toxic effects on *S. yirgalemense*. Approximately 250 IJs were suspended in 1 ml liquid, in 2-cm-diameter watch glasses containing either PAA solution (36-40 wt% in acetic acid, Sigma-Aldrich), TCA (Kosher; natural ≥ 99%, FG-Sigma-Aldrich), or nipagin (methylparaben). The concentrations used for each of the respective antifungal agents in the study were: 0.01%, 0.02%, 0.03%, 0.04%, 0.05%, 0.06%, 0.07%, 0.08%, 0.09%, 0.1%, 1%, 2%, 3%, 5%, and 10%. The concentrations were percentages of the corresponding antifungal agents in the 1 ml containing 250 IJs in the watch glass. The control involved the suspension of IJs in distilled water. Five watch glasses per concentration were placed in a large glass Petri dish, with a piece of moist tissue paper being placed between them to maintain 100% humidity. They were incubated at 25°C for 24 hours, upon the elapse of which all 250 IJs were counted out onto a Petri dish to determine the number of live and dead IJs. The experiment was conducted twice on different test dates, using different batches of nematodes.

EFFECT OF ANTIFUNGAL AGENT ON IJ PATHOGENICITY

After exposing the EPN to the antifungal agents for 24 h, they were tested for pathogenicity against the last-instar larvae of *G. mellonella,* using 24-well bioassay plates (flat-bottom, Nunc™, Cat. No. 144530). Each bioassay plate contained 10 larvae, placed alternately in the wells, fitted with a piece of filter paper. Each of the wells was inoculated with 50 IJs in 100 µl of the mixture of IJs/antifungal agents, while water only was used for the controls. The lid of each well was fitted with a piece of glass of the same shape as the lid, to prevent the *G. mellonella* larvae from escaping. Five 24-well-plates, with 10 wells in each (n = 50), were used for each treatment/concentration. The plates were placed in a plastic container that was lined with wet paper towels, thus creating 100% humidity. They were then kept in a growth chamber

at 25°C for a period of 48 h. Mortality was confirmed by means of the visual observation of the colour of the cadavers of the wax moth larvae, which turned yellowish for *S. yirgalemense*. The experiment was repeated once on a different test date, using another batch of nematodes.

Statistical analyses

All the experiments were conducted twice on different test dates, using different batches of nematodes, with the results being combined for analysis. Statistical analyses were conducted using STATISTICA 13.2 software (StatSoft. Inc). Data on the negative effect of antifungal agents on the survival percentage were arcsine transformed prior to analysis. All the results on antifungal agents were analysed using general linear models (GLM), with the mean being separated by means of the Games-Howell post hoc test. The results on the DE were analysed by means of a two-way repeated-measures ANOVA, and the means were separated by means of the LSD test.

Results

Antifungal agent toxicity screening

As no significant differences ($p > 0.05$) were obtained between the two batches in terms of the main effects of time and treatment of the nematodes with the different antifungal agents, the data from the two batches were pooled and analysed. A significant difference ($p < 0.001$) was found in the effect of the antifungal agents on the percentage survival of *S. yirgalemense* IJs after 24 h exposure. The nipagin differed significantly from the TCA ($p < 0.01$) and the PAA ($p < 0.01$), which also differed significantly ($p < 0.01$) from each other (Fig. 3.1). Nipagin caused the highest mean percentage survival value of 99.89% ± 0.87 %, followed by TCA acid, and then by PAA, with 98.78 % ± 0.87 % and 29.24 % ± 0.87%, respectively (Fig. 3.2).

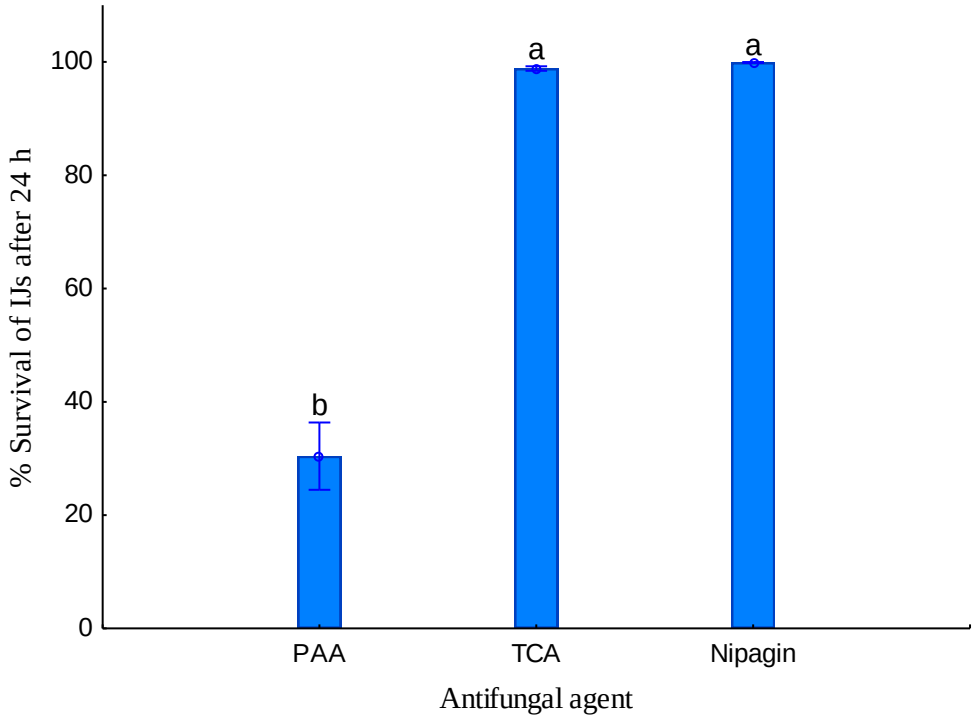


Fig. 3. 2. Mean percentage survival (95% confidence level) of *S. yirgalemense* infective juveniles (IJs) after 24 h in the antifungal agents peroxyacetic acid (PAA), trans-cinnamic acid (TCA) and nipagin ($F_{(2, 435)} = 2174.1$, $p < 0.001$). Mean separated by Games-Howell post hoc test; error: between MSE = 116.91, df = 435.00. Different letters above the bars indicate significant differences ($p < 0.05$).

Effect of antifungal agent on IJ pathogenicity

No significant differences ($p > 0.05$) were obtained between the two batches with the main effects of nematode and antifungal agents, thus the data from the two batches were pooled and analysed. A significant difference ($p < 0.001$) was found in the percentage mortality of *G. mellonella* larvae by *S. yirgalemense* IJs after 24 h exposure to the respective antifungal agents. The peracetic acid differed significantly from the TCA ($p < 0.0001$) and the nipagin ($p < 0.0001$), which did not differ significantly ($p = 0.58$) from each other. The *S. yirgalemense* IJs that were previously treated with TCA caused the highest mean percentage mortality value of *G. mellonella* larvae of 100% ± 0.91%, followed by nipagin, and then by PAA, with 99.97% ± 0.91% and 36.70% ± 0.91%, respectively (Fig. 3.3).

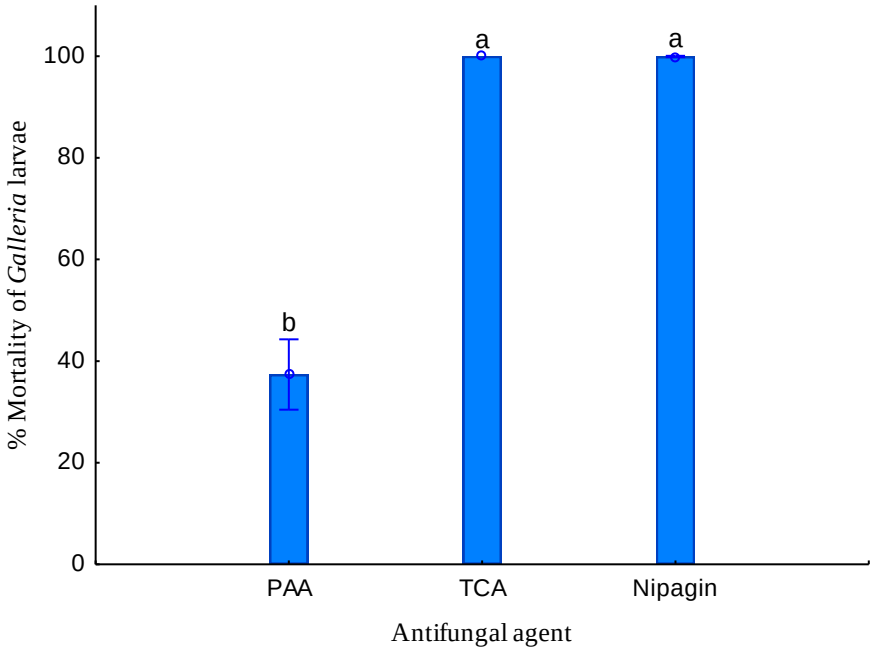


Fig. 3. 3. Mean percentage mortality (95% confidence level) of *Galleria mellonella* larvae inoculated with *Steinernema yirgalemense*, after 24 h exposure to the respective antifungal agents, peroxyacetic acid (PAA), trans-cinnamic acid (TCA) and nipagin ($F_{(2, 389)} = 1606.9$, $p < 0.001$). Mean separated by Games-Howell post hoc test; error: between MSE = 128.17, df = 389.00. Different letters above the bars indicate significant differences ($p < 0.05$).

SURVIVAL OF NEMATODES IN DIATOMACEOUS EARTH

No significant differences ($p > 0.05$) were obtained between the two batches with the main effects of treatment and date in the two-way ANOVA, thus the data from the two batches were pooled and analysed using a one-way ANOVA. The analysis of the data from weeks 2 and 4 showed significant differences (repeated measures two-way ANOVA: ($F_{(1, 38)} = 0.86115$, $p >$ 35927) between the treatments with regards to survival. A high survival rate was attained during the observation period. Besides the above, the mean percentage survival was significantly ($p < 0.05$) higher in week 2 than it was in week 4. At week 2, the survival of *S. yirgalemense* in DE did not significantly differ at both 14°C ($p = 0.69$) and 25°C ($p = 0.69$). The mean percentage survival rate obtained at 14°C (85.60% ± 0.84%) was slightly higher than was that at 25°C (85.16% ± 0.84%). Furthermore, at week 4, the survival of *S. yirgalemense* in DE also did not differ significantly at both 14°C ($p = 0.85$) and 25°C ($p = 0.85$). Unlike at week 2, the mean percentage survival rate obtained at 25°C (80.67% ± 0.71%) was slightly higher than it was at 14°C (80.67% ± 0.71%) (Fig. 3.4).

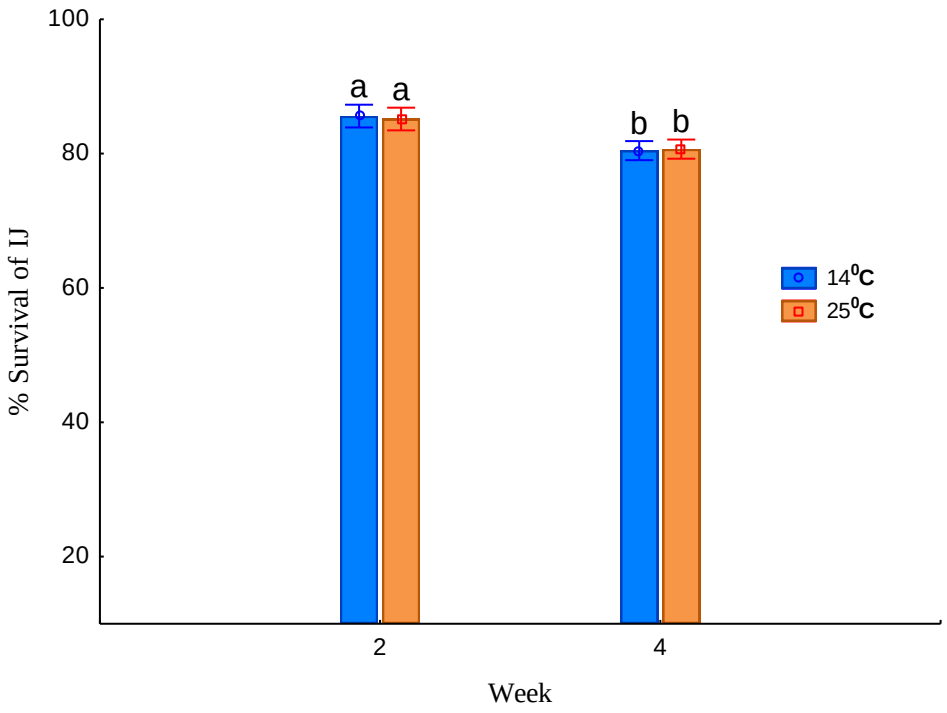


Fig. 3. 4. Mean percentage survival (95 % confidence level) of *Steinernema yirgalemense* infective juveniles (IJ) in diatomaceous earth at different temperatures during the 4 weeks (repeated measures ANOVA: ($F_{(1, 38)}$ = 0.86115, p > 35927). Mean separated by Fisher's least significant difference (LSD) post hoc test; error: between; within; pooled MS = 11.975, df = 46.626. Different letters above the bars indicate significant differences ($p < 0.05$).

Discussion

The study reports on the loss of survival and pathogenicity of *S. yirgalemense* due to PAA. Despite PAA being a strong disinfectant, with a wide spectrum of antimicrobial activity, it is not suited to protect EPN formulation against contamination. Peroxyacetic acid has previously been used as a nematicide (An *et al*., 2017; Jagdale & Grewal, 2002; Krishnayyaand & Grewal, 2002), with the previously obtained results agreeing with the findings made in the current study. For example, An *et al*. (2017) report that ZeroTol (BioSafe Systems) caused 100% mortality of *Aphelenchoides fragariae* (Ritzema Bos, 1891) Christie, 1932 (Aphelenchida: Aphelenchoididae) in aqueous suspension at 20-fold (low) dilution. ZeroTol further reduced, by over 85% and 75%, *A. fragariae* population in soil 7 and 42 days after treatment, respectively, in drench application. An *et al*. (2017) deduced that spray application of ZeroTol could reduce > 70% of the *A. fragariae* population in leaf discs, and that it, thus, has great potential to manage foliar nematodes in floriculture. The active ingredient of ZeroTol is PAA (270 g/l). The above-mentioned results clearly support the results obtained in the current study, in terms of which nematodes lost their survival and virulence in 24 hours. Similarly, Krishnayyaand & Grewal (2002) reported that the hydrogen dioxide/PAA mixture (ZeroTol) was incompatible with the IJs of *Steinernema feltiae* (Filipjev, 1934;) Wouts, Mráček,

Gerding & Bedding, 1982, as it caused 100% mortality after 120 h of incubation, and, hence, could not be tank-mixed during application. The current study confirms that PAA is not recommendable as an antimicrobial in formulations using EPNs.

Results from the study showed that TCA has no effect on the viability and virulence of the IJs of *S. yirgalemense* at all the tested concentrations, up to 24 h of exposure. Trans-cinnamic acid has been isolated as a secondary metabolite of the mutualistic bacteria, *Photorhabdus luminescens* (Bock *et al*., 2014; Hazir *et al.*, 2016, 2017, 2018). However, the acid's effect on EPN IJs, to the current researchers' knowledge, has not previously been tested, with this being the first report showing its compatibility with EPNs. Overall, several other antimicrobials have been isolated from the metabolites of the *Photorhabdus* and *Xenorhabdus* species. Such antimicrobials include numerous antimicrobial compounds, such as xenorhabdins, xenocoumacins, nematophin, cabanillasin, and xenofuranones A and B, isolated from *Xenorhabdus* species (Brachmann *et al.*, 2006; Houard *et al.*, 2013; Li *et al*., 1997; Sharma *et al*., 2016; Webster *et al*., 2002). Likewise, antifungal and/or antibacterial products, like hydroxystilbenes, *trans*-stilbenes, TCA, anthraquinone pigments, and the toxin complex (Tc), have been isolated from *Photorhabdus* (Bock *et al.*, 2014; Bode, 2009; Boemare & Akhurst, 2006; Shapiro-Ilan *et al*., 2009). The toxicity against important fungal pathogens from the already examined crude bacterial exudates and metabolites of *Photorhabdus* and *Xenorhabdus* species in the different studies is offering encouraging results (Bock *et al*., 2014; Chen *et al*., 1996; Fang *et al.*, 2014; Hazir *et al.*, 2016; McInerney *et al.*, 1991a,b; San-Blas *et al.*, 2012; Shapiro-Ilan *et al*., 2009, 2014). Though the bioactive metabolite of *Xenorhabdus indica Somvanshi, Lang, Ganguly, Swiderski, Saxena & Stackebrandt, 2009* associated with *S. yirgalemense* (Ferreira *et al*., 2016) is unknown and has not yet been isolated, the notion that it is compatible with TCA probably holds true. Incidentally, the results of the current study have revealed the bioactive secondary metabolites of the bacteria associated with EPN as being potent antimicrobials in formulation.

Furthermore, results of the present study also show that nipagin, likewise, has no effect on the viability and virulence of the IJs of *S. yirgalemense* at all the tested concentrations, up to 24 h of exposure. By contrast, Kermarrec & Mauléon (1989) reported that nipagin evidently lowered the pathogenicity of *Steinernema carpocapsae* (Weiser, 1955) Wouts, Mráček, Gerdin & Bedding, 1982 to the caterpillars of two Pyralid moths *(Diatraea saccharalis* and *Galleria mellonella*) by 200 times (all instars), ranging from 50 to 250 times with larval aging from the third to the fifth instar.

Research showed an improvement in the shelf life of IJs stored in DE formulation, in relation to a previous study in the formulation of *S. yirgalemense* (Chapter 2). A notable result in this case is the high survival rate (80%) of *S. yirgalemense* in week 4 at 25°C. The above

finding is reassuring in terms of the implications that it holds for the much-desired room temperature storage of IJs in DE formulation. The improvement in the number of nematodes in DE formulation has undoubtedly influenced the survival of EPNs in terms of the current research (Hiltpold *et al*., 2012; Matadamas-Ortiz *et al*., 2014; Silver *et al*., 1995). The above is in accordance with the findings of Matadamas-Ortiz *et al*. (2014), who obtained best results following 100DE:0AC proportions. The present results obtained are also similar to those of Ziaee *et al*. (2016), who reported the increased mortality of the adults of *Oryzaephilus surinamensis* (L.) (Coleoptera: Sylvanidae), with the increasing exposure intervals and concentration levels of DE proving to be key to the function of serving as the grain protectants of stored product pests. Preliminary results (not shown here) of *S. jeffreyense* in DE at a density of 4 000 000 IJs/g of formulation have given 100% survival and pathogenicity for three months at 14°C. Equally important, the study revealed an inverse relationship between the number of IJs in the DE formulation and the presence of water. The higher the number of IJs, the lower is the amount of DE and water that is required in the formulation. The use of such a formula leads to improved DE formulation. At the point where the DE becomes saturated with IJs, so much so that it forms a paste, no additional water is required, as adding more at this stage would serve to expedite microbial growth.

In conclusion, PAA is not recommended for use as an antimicrobial in the formulation of EPNs. The present study reports on the high survival and virulent abilities of *S. yirgalemense* in relation to both TCA and nipagin exposure. Future studies are recommended to include the bioactive metabolites of the mutualistically associated *Photorhabdus* and *Xenorhabdus* as antimicrobials in the EPN formulations concerned. Although good results were obtained during the study using the DE formulation described, still higher densities of nematodes are recommended for use in improving the survival rate and virulence of IJs in DE formulations. In addition, the current study has revealed an inverse relationship between the number of IJs in the DE formulation and the presence of water. In short, the higher the number of IJs present, the lower is the amount of DE and water that is required in the formulation. Following such a guideline should lead to the creation of an improved DE formulation. At a certain point, when the DE is saturated with IJs in the form of a paste, no further water should be added to the formulation, as doing so would serve to encourage microbial growth.

References

An, R., Karthik, N.K. & Grewal, P. (2017). Evaluation of botanical and chemical products for the control of foliar nematodes *Aphelenchoides fragariae*. *Crop Protection* 92, 107-113. DOI:10.1016/j.cropro.2016.10.021.

Ayoub, F., Ben Oujji, N., Chebli, B., Ayoub, M., Hafidi, A., Salghi, R. & Jodeh, S. (2017). Antifungal

effectiveness of fungicide and peroxyacetic acid mixture on the growth of *Botrytis cinerea*. *Microbial Pathogenesis* 105, 74-80. DOI: 10.1016/j.micpath.2017.02.014.

Błędzka, D., Gromadzińska, J. & Wąsowicz, W. (2014). Parabens. From environmental studies to human health. *Environment International* 67, 27-42. DOI: 10.1016/j.envint.2014.02.007.

Bock, C.H., Shapiro-Ilan, D.I., Wedge, D.E. & Cantrell, C.L. (2014). Identification of the antifungal compound, trans-cinnamic acid, produced by *Photorhabdus luminescens*, a potential biopesticide against pecan scab. *Journal of Pest Science* 87, 155-162. DOI: 10.1007/s10340-013-0519-5.

Bode, H.B. (2009). Entomopathogenic bacteria as a source of secondary metabolites. *Current Opinion in Chemical Biology* 13, 224-230. DOI: 10.1016/j.cbpa.2009.02.037.

Boemare, N. & Akhurst, R. (2006). The genera *Photorhabdus* and *Xenorhabdus*. In: Dworkin, M., Falkow, S., Rosenberg, E., Schleifer, K.H. & Stackebrandt E. (Eds). *The Prokaryotes.* New York, Springer Science+Business Media, pp. 451-494. DOI: 10.1007/0-387-30746-X_16.

Brachmann, A.O., Forst, S., Furgani, G.M., Fodor. A. & Bode, H.B. (2006). Xenofuranones A and B: Phenylpyruvate Dimers from *Xenorhabdus szentirmaii*. *Journal of Natural Products* 69, 1830-1832**.** DOI**:** 10.1021/np060409n.

Chen, G., Zhang, Y., Li, J., Dunphy, G. & Punja, Z. (1996). Chitinase activity of *Xenorhabdus* and *Photorhabdus* species, bacterial associates of entomopathogenic nematodes. *Journal of Invertebrate Pathology* 68, 101-108. DOI: 10.1006/jipa.1996.0066.

Fang, X., Zhang, M., Tang, Q., Wang, Y. & Zhang, X. (2014). Inhibitory effect of *Xenorhabdus nematophila* TB on plant pathogens *Phytophthora capsici* and *Botrytis cinerea* in vitro and in planta. *Scientific Reports*, pp. 1–7. 4:4300. DOI: 10.1038/srep04300Sci.

Ferreira, T., Van Reenen, C.A., Tailliez, P., Pagès, S., Malan, A.P. & Dicks, L.M.T. (2016). First report of the symbiotic bacterium, *Xenorhabdus indica*, associated with the entomopathogenic nematode *Steinernema yirgalemense*. *Journal of Helminthology* 90, 108-112. DOI: 10.1017/S0022149X14000583.

Garrido-Jurado, I., Valverde-García, P. & Quesada-Moraga, E. (2011). Use of a multiple logistic regression model to determine the effects of soil moisture and temperature on the virulence of entomopathogenic fungi against pre-imaginal mediterranean fruit fly *Ceratitis capitata*. *Biological Control* 59, 366-372. DOI: 10.1016/j.biocontrol.2011.09.011.

Grewal, P.S. (2000a). Enhanced ambient storage stability of an entomopathogenic nematode through anhydrobiosis. *Pest Management Science* 56, 401-406. DOI: 10.1002/(SICI)1526-4998(200005)56:5<401::AID-PS137>3.0.CO;2-4.

Grewal, P.S. (2000b). Anhydrobiotic potential and long-term storage of entomopathogenic nematodes (Rhabditida : Steinernematidae). *International Journal for Parasitology* 30, 995-1000. DOI: 10.1016/S0020-7519(00)00080-1.

Grewal, P.S. (2002). Formulation and application technology. In: Gaugler, R. (Ed.). *Entomopathogenic nematology*. Wallingford, UK, CABI Publishing, pp. 265-287. DOI: 10.1079/9780851995670.0265

Grewal, P.S. & Jagdale, G.B. (2002). Enhanced trehalose accumulation and desiccation survival of entomopathogenic nematodes through cold preacclimation. *Biocontrol Science and Technology* 12, 533-545. DOI: 10.1080/0958315021000016207.

Grewal, P.S. & Peters, A. (2005). Formulation and quality. In: Grewal, P.S., Ehlers, R.-U. & Shapiro-Ilan, D.I. (Eds). *Nematodes as biocontrol agents.* Wallingford, UK, CABI Publishing, pp. 79-89.

Hazir, S., Shapiro-Ilan, D.I., Bock, C.H., Hazir, C., Leite, L.G. & Hotchkiss, M.W. (2016). Relative potency of culture supernatants of *Xenorhabdus* and *Photorhabdus* spp. on growth of some fungal phytopathogens. *European Journal of Plant Pathology* 146, 369-381. DOI: 10.1007/s10658-016-0923-9.

Hazir, S., Shapiro-Ilan, D.I., Bock, C.H. & Leite, L.G. (2017). Trans-cinnamic acid and *Xenorhabdus szentirmaii* metabolites synergize the potency of some commercial fungicides. *Journal of Invertebrate Pathology* 145, 1-8. DOI: 10.1016/j.jip.2017.03.007.

Hazir, S., Shapiro-Ilan, D.I., Bock, C.H. & Leite, L.G. (2018). Thermo-stability, dose effects and shelf-life of antifungal metabolite-containing supernatants produced by *Xenorhabdus szentirmaii*. *European Journal of Plant Pathology* 150, 297-306. DOI: 10.1007/s10658-017-1277-7.

Hiltpold, I., Hibbard, B.E., French, B.W. & Turlings, T.C.J. (2012). Capsules containing entomopathogenic nematodes as a Trojan horse approach to control the western corn rootworm. *Plant and Soil* 358, 11-25. DOI: 10.1007/s11104-012-1253-0.

Houard, J., Aumelas, A., Noël, T., Pages, S., Givaudan, A., Fitton-Ouhabi, V., Villain-Guillot, P., Gualtieri, M., 2013. Cabanillasin, a new antifungal metabolite, produced by entomopathogenic Xenorhabdus cabanillasii JM26. *The Journal of Antibiotics* 66, pp.617–620. DOI: 10.1038/ja.2013.58.

Jagdale, G.B. & Grewal, P.S. (2002). Identification of alternatives for the management of foliar nematodes in floriculture. *Pest Management Science* 58, 451-458. DOI: 10.1002/ps.472.

Kagimu, N., Ferreira, T. & Malan, A.P. (2017). The attributes of survival in the formulation of entomopathogenic nematodes utilised as insect biocontrol agents. *African Entomology* 25, 275-291. DOI: 10.4001/003.025.0275.

Kaya, H.K. & Stock, S.P. (1997). Techniques in insect nematology. In: Lacey, L. (Ed.). *Manual of techniques in insect pathology*. San Diego, CA, Academic Press, pp. 281-324. DOI: 10.1016/B978-012432555-5/50016-6.

Kermarrec, A. & Mauléon, H. (1989). Laboratory production of *Neoaplectana carpocapsae* Weiser affected by nipagin and streptomycin. *Revue de Nématologie* 12, 103-104.

Kitis, M. (2004). Disinfection of wastewater with peracetic acid: A review. *Environment International* 30,

47-55. DOI: 10.1016/S0160-4120(03)00147-8.

Krishnayyaand, P.V. & Grewal, P.S. (2002). Effect of neem and selected fungicides on viability and virulence of the entomopathogenic nematode *Steinernema feltiae*. *Biocontrol Science and Technology* 12, 259-266. DOI: 10.1080/09583150210388.

Li, J., Chen, G. & Webster, J.M. (1997). Nematophin, a novel antimicrobial substance produced by *Xenorhabdus nematophilus* (Enterobactereaceae). *Canadian Journal of Microbiology* 43, 770-773. DOI: 10.1139/m97-110.

Mahuzier, P.-E., Altria, K.D. & Clark, B.J. (2001). Selective and quantitative analysis of 4-hydroxybenzoate preservatives by microemulsion electrokinetic chromatography. *Journal of Chromatography A* 924, 465-470. DOI: 10.1016/S0021-9673(01)00717-8.

Matadamas-Ortiz, P.T., Ruiz-Vega, J., Vazquez-Feijoo, J.A., Cruz-Martinez, H. & Cortes-Martinez, C.I. (2014). Mechanical production of pellets for the application of entomopathogenic nematodes: Factors that determine survival time of *Steinernema glaseri*. *Biocontrol Science and Technology* 24, 145-157. DOI: 10.1080/09583157.2013.852161.

McInerney, B.V., Gregson, R.P., Lacey, M.J., Akhurst, R.J., Lyons, G.R., Rhodes, S.H., Smith, D.R.J., Engelhardt, L.M., & White, A.H. (1991a). Biologically active metabolites from *Xenorhabdus* spp., part 1. Dithiolopyrrolone derivatives with antibiotic activity. *Journal of Natural Products* 54, 774-784. DOI: 10.1021/np50075a005.

McInerney, B.V., Taylor, W.C., Lacey, M.J., Akhurst, R.J. & Gregson, R.P. (1991b). Biologically active metabolites from *Xenorhabdus* spp., Part 2. Benzopyran-1-one derivatives with gastroprotective activity. *Journal of Natural Products* 54, 785-795. DOI: 10.1021/np50075a006.

Nascimento, G.G.F., Locatelli, J., Freitas, P.C. & Silva, G.L. (2000). Antibacterial activity of plant extracts and phytochemicals on antibiotic-resistant bacteria. *Brazilian Journal of Microbiology* 31, 247-256. DOI: 10.1590/S1517-83822000000400003.

Peters, A. (2004). Protocol quality assessment of entomopathogenic nematodes. In: *COST Action 850 meeting programme and mushroom protocols*. pp. 4-6.

Quesada-Moraga, E., Ruiz-García, A. & Santiago-Álvarez, C. (2006). Laboratory evaluation of entomopathogenic fungi *Beauveria bassiana* and *Metarhizium anisopliae* against puparia and adults of *Ceratitis capitata* (Diptera: Tephritidae). *Journal of Economic Entomology* 99, 1955-1966. DOI: 10.1093/jee/99.6.1955.

Rohlfs, M., Obmann, B. & Petersen, R. (2005). Competition with filamentous fungi and its implication for a gregarious lifestyle in insects living on ephemeral resources. *Ecological Entomology* 30, 556-563. DOI: 10.1111/j.0307-6946.2005.00722.x.

San-Blas, E., Carrillo, Z. & Parra, Y. (2012). Effect of *Xenorhabdus* and *Photorhabdus* bacteria and their exudates on *Moniliophthora roreri*. *Archives of Phytopathology and Plant Protection* 45, 1950-1967. DOI: 10.1080/03235408.2012.718688.

Shapiro-Ilan, D.I., Reilly, C.C. & Hotchkiss, M.W. (2009). Suppressive effects of metabolites from *Photorhabdus* and *Xenorhabdus* spp. on phytopathogens of peach and pecan. *Archives of Phytopathology and Plant Protection* 42, 715-728. DOI: 10.1080/03235400701390539.

Shapiro-Ilan, D.I., Bock, C.H. & Hotchkiss, M.W. (2014). Suppression of pecan and peach pathogens on different substrates using *Xenorhabdus bovienii* and *Photorhabdus luminescens. Biological Control* 77, 1-6. DOI: 10.1016/j.biocontrol.2014.05.010.

Sharma, K., Walia, S., Ganguli, S. & Kundu, A. (2016). Analytical characterization of secondary metabolites from Indian *Xenorhabdus* species the symbiotic bacteria of entomopatathogenic nematode (*Steinernema* spp.) as antifungal agent. *National Academy Science Letters* 39(3), 175-180. DOI: 10.1007/s40009-016-0453-1.

Silver, S., Dunlop, D. & Grove, D. (1995). *WIPO Patent No. WO 95/0577*.Geneva: World Intellectual Property Organization.

Soni, M.G., Taylor, S.L., Greenberg, N.A. & Burdock, G.A. (2002). Evaluation of the health aspects of methyl paraben: A review of the published literature. *Food and Chemical Toxicology* 40, 1335-1373. DOI: 10.1016/S0278-6915(02)00107-2.

Sova, M. (2012). Antioxidant and antimicrobial activities of cinnamic acid derivatives. *Mini-Reviews in Medicinal Chemistry* 12, 749-767. DOI: 10.2174/138955712801264792.

Straus, D.L., Meinelt, T., Farmer, B.D. & Mitchell, A.J. (2012). Peracetic acid is effective for controlling fungus on channel catfish eggs. *Journal of Fish Diseases* 35, 505-511. DOI: 10.1111/j.1365-2761.2012.01383.x.

Van Zyl, C. & Malan, A.P. (2015). Cost-effective culturing of *Galleria mellonella* and *Tenebrio Molitor* and entomopathogenic nematode production in various hosts. *African Entomology* 23, 361-375. DOI: 10.4001/003.023.0232.

Webster, J.M., Chen, G., Hu, K. & Li, J. (2002). Bacterial metabolites. In: Gaugler, R. (Ed.). *Entomopathogenic nematology*. Wallingford, UK, CABI Publishing, pp.99-114. DOI:10.1079/9780851995670.0099

Ziaee, M., Atapour, M. & Marouf, A. (2016). Insecticidal efficacy of Iranian diatomaceous earths on adults of *Oryzaephilus surinamensis*. *Journal of Agricultural Science and Technology* 18, 361-370.

Chapter 4

Basal metabolic oxygen consumption rate measurements for entomopathogenic nematodes, using fibre-optic sensors

Abstract

Entomopathogenic nematodes (EPNs) are aerobic organisms, whose infective juveniles (IJs) are constantly exposed to micro-aerobic, or anaerobic, conditions in the soil, yet low oxygen supply risks their survival. Unfortunately, there is limited information on the respiratory physiology of EPNs. Therefore, gathering information on the specific oxygen demands of the nematode/bacterium complex of EPNs during production, storage and formulation should maximise their utilisation. Nematode culturing in bioreactors has led to an awareness of their oxygen requirements, leading to a research focus on bioreactor designs and the nematode-bacterium complex. In the present study, fibre-optic sensors were used to determine, by means of basal measurement, the specific oxygen consumption rate (OCR) of the IJs of three locally isolated EPN species: *Steinernema yirgalemense*; *S. jeffreyense*; and *Heterorhabditis bacteriophora*. The results showed that nematode size inversely influences its OCR, with smaller nematodes having a higher surface-area-to-volume ratio than do larger nematodes with a higher OCR. *Steinernema jeffreyense* and *S. yirgalemense* did not significantly differ from each other in terms of the results obtained, probably due to their proximity in size, with the former being slightly larger than the latter, but they did significantly differ from *H. bacteriophora*. However, the results could not be reflected in all batches for *H. bacteriophora* and *S. yirgalemense*, due to the variation encountered. The results provide baseline screening for comparing respiratory and metabolic physiology among EPN species, using the latest available technology of fibre-optic sensors.

Key words - diatomaceous earth, polarographic*, Steinernema yirgalemense*; *S. jeffreyense*; *Heterorhabditis bacteriophora*.

Introduction

With the unending desire to consume safe food expressed among consumers in such markets as the European Union, coupled with the existing restrictions on the use of agrochemicals, challenges exist for farmers to seek alternative means of crop protection. The prevailing situation calls for the use of biocontrol organisms like entomopathogenic nematodes (EPNs) (Rhabditida: Steinernematidae). EPNs from the genera *Steinernema* (Travassos 1927) and *Heterorhabditis* (Poinar 1976), and their associated symbiotic bacteria *Xenorhabdus* (Thomas & Poinar 1983) and *Photorhabdus* Boemare, Akhurst & Mourant 1993 (Ehlers, 2007; Stock, 2015), are efficient biological control agents. The above is substantiated by their ease of culture, their high fatality caused against key pest insects, and their safety in use (Grewal, 2002; Piedra-Buena *et al*., 2015; Stuart *et al*., 2015). Research has commercially exploited the above advantages of EPNs by means of investigating several species, including *Steinernema carpocapsae* (Weiser 1955) Wouts, Mráček, Gerdin & Bedding 1982, *Steinernema feltiae* (Filipjev 1934) Wouts, Mráček, Gerdin & Bedding 1982 and *Heterorhabditis bacteriophora* Poinar 1976.

Formulation has been achieved by means of relying on the dauer, or the infective juvenile (IJ), stage (Kagimu *et al*., 2017), which is the only free-living nematode in the life cycle. The mutualistic bacteria of the IJs become the actual killing agents on entry into the target pest insect, where they multiply in the haemocoel, and cause septicaemia, resulting in the death of the insect (Ciche *et al*., 2006; Griffin, 2015; Karimi & Salari, 2015). Although the IJs are non-feeding, since their mouth and anus are closed (Grewal *et al*., 2002; Stock, 2015; Stuart *et al*., 2015), they can persist for a long time in the soil. The commercial use of EPNs is limited by their finite shelf life, even though some species have already become commercially available (Gaugler *et al*., 2000). The poor survival rate at room-temperature storage is a major hindrance for their potential use as bioinsecticides (Grewal, 2002). Their ability to survive is poor in terms of desiccation (Surrey & Wharton, 1995; Womersley, 1990).

Alternatively, since nematodes are aerobic organisms, availing them of low oxygen supply jeopardises their survival (Andaló *et al*., 2010; Evans & Perry, 1976; Glazer, 2002; Wharton, 1986), given that IJs are constantly exposed to micro-aerobic, or anaerobic, conditions in the soil (Atkinson, 1976; Wright, 1998). Grewal *et al*. (2011) also identified hypoxia as being one of the main abiotic factors affecting IJ survival in storage. The available information on oxygen consumption mainly pertains to terrestrial (Bair, 1955; Ferris *et al*., 1995; Fourie *et al*., 2014; Klekowski *et al*., 1972; Suda *et al*., 2005; Van Aardt *et al*., 2016), freshwater (Schiemer & Duncan, 1974), and marine (Atkinson, 1973) nematodes, with a limited amount of information being available about EPNs. Therefore, it is important to gather information about the specific

oxygen demands of the nematode/bacterium complex of EPNs, since an improved understanding of the oxygen balance during their production, storage and formulation maximises their utilisation.

The culturing of nematodes in bioreactors, in contrast, has led to the important realisation of the oxygen requirements of the nematodes, and, thus, the focus has shifted to bioreactor designs and the nematode-bacterium complex (Chavarría-Hernández *et al*., 2011, 2014; De la Torre, 2003; Friedman *et al*., 1989; Shapiro-Ilan *et al*., 2012; Sharma *et al*., 2011; Upadhyay *et al*., 2013). Some laboratories have investigated bacteria (Belur *et al*., 2013; Hodgson *et al*., 2003; Wang & Zhang, 2007), and formulation (Andaló *et al*., 2010; Silver, 1999), whereas others have investigated the effects of soil oxygen on EPNs (Kung *et al*., 1990). Few reports have been compiled about the specific oxygen consumption rate (OCR) of the IJ in general, with the existing examples including Burman & Pye (1980) and Qiu & Bedding (2000), who measured the respiration rate of *S. carpocapsae,* while Lindegren *et al*. (1986) reported that of *S. feltiae*.

In researching how oxygen levels tend to regulate gene expression in *Photorhabdus temperata* Fischer-Le Saux, Viallard, Brunel, Normand & Boemare 1999 (Enterobacteriales: Enterobacteriaceae), Hodgson *et al*. (2003) demonstrated the effect of oxygen on the two phases of the bacterium. They reported that the amount of oxygen present inhibited flagellum and antibiotic production by secondary-phase (phase II) cells, while the absence of oxygen down-regulated many primary-phase (phase I) traits. However, aeration is more urgently required to ensure enhanced production yields during phase I, in terms of nematode growth and development, than is the case in phase II. Also, Strauch & Ehlers (2000) doubled the yield production by increasing the aeration rate, while Jenkins & Goettel (1997), Friedman *et al*. (1989), and Wang & Zhang (2007) emphasised the need for oxygen by both nematode and bacteria during their mass production in a bioreactor.

In terms of behaviour, Rankin (2005) showed how *Caenorhabditis elegans* Maupas 1900 (Rhabditida: Rhabditidae) individuals migrate towards an oxygen level that is associated with food, by means of using taste, smell, and temperature cues. The observation could be in line with the behaviour of IJs of EPNs during their search for a possible host that could also be associated with the same oxygen gradient. Rankin (2005) further reports that *C. elegans* thrives within a threshold value of oxygen. Such a situation is also likely among EPNs, but an excess supply of oxygen relates to increased activity and metabolism, which leads to the overutilisation of the energy reserves of the IJ, and possibly further translates into a reduced shelf life for the IJs in storage and formulation.

The same observation by Rankin (2005) has been observed by Kagimu *et al*. (Unpubl.), in relation to work on the formulation of EPNs in diatomaceous earth, that the nematodes concerned tend to migrate to the surface, and to aggregate together, immediately after a homogeneous formulation is made and packaged in a container. Such behaviour by IJs is due to their search for enough oxygen, with them eventually dying off slowly, having lost the protection rendered by the formulation, due to their open exposure, which results in an overall decline in product shelf life. The same observations are discussed by Silver (1999).

Given the variability among EPN species and strains in almost all tested traits covered in the relevant literature, the notion that OCR is probably also variable betokens that it should be further investigated and confirmed prior to mass production and commercialisation. Intraspecific variability exists in the relationship between the OCR and the body size (Atkinson, 1976; Wright, 1998), temperature (Burman & Pye, 1980; Lindegren *et al*., 1986), and age of the nematodes concerned (Wright, 1998). For an explanation of such factors and their influence on the OCR, refer to Wright (1998) and Atkinson (1973).

Several methods are in place to measure the OCR of small aquatic animals, including nematodes (Zhdanov *et al*., 2012). The aforementioned methods include: the Winkler chemical analysis method (Bair, 1955); the Cartesian diver (Bhatt & Rohde, 1970; Van Aardt *et al*., 2016); and several manometric methods (Umbreit *et al*., cited in Van Aardt *et al*., 2016). However, the above-mentioned techniques require a considerable length of time for the running of a single experiment (Van Aardt *et al*., 2016). In contrast, extensive advances have been made in terms of the measurement of OCR, using polarographic oxygen sensors (POS) (Atkinson, 1973; Clark, 1956; Fourie *et al*., 2014; Van Aardt *et al*., 2016), infrared (IR) gas analysis (Ferris *et al*., 1995) and fibre-optic sensors (FOS) (Dancy *et al*., 2013; Suda *et al*., 2005), which require comparatively less time to use (Van Aardt *et al*., 2016). For example, Burman & Pye (1980) utilised POS in their studies, with, more recently, Van Aardt *et al*. (2016) having used the same technique, together with FOS, to measure the OCR of *Meloidogyne incognita* J2, thereby providing a baseline for the OCR measurement of EPNs, on a similar scale. Van Aardt *et al*. (2016) report substantial variation in J2 OCR measurements having been obtained by both analysers between the different J2 batches harvested over time, with no explanation being found for such discrepancy. For the aforementioned reasons, FOS measurements are regarded as the comparatively better option, due to: its sensitive nature, or high accuracy; the use of fewer nematodes per measurement; and the multiple measurement of the OCR of nematodes (Moodley *et al*., 2008; Van Aardt *et al*., 2016).

In the present study, FOS was used to determine the specific OCR of three locally isolated EPN species: *Steinernema yirgalemense* Nguyen, Tesfamariam, Gozel, Gaugler & Adams 2004; *Steinernema jeffreyense* Malan, Knoetze & Tiedt 2015; and *H. bacteriophora*.

Materials and methods

SOURCE OF ORGANISM

The three EPN species used in the current study were local isolates from South Africa, obtained from the EPN collection of the nematology laboratory at the Department of Conservation Ecology and Entomology, Stellenbosch University. Their details are summarised in Table **4.1** below.

Table 4. 1. *Steinernema and Heterorhabditis* species, their origin, mean body length and width of the IJs concerned.

			Infective juveniles		
Species	Strain	Origin	Length (µm)	Width (µm)	Mass (µg)
S. jeffreyense	J194	Jeffrey's Bay, Eastern Cape	924 (784-1043)	35 (23-43)	0.7089
S. yirgalemense	157-C	Friedenheim, Mpumalanga	635 (548-693)	29 (24-33)	0.3338
H. bacteriophora	SF351	Wellington, Western Cape	588 (512-671)	23 (18-31)	0.1944

Sources: Malan *et al*., 2011, 2016; Poinar, 1976.

Freshly harvested EPNs were cultured *in vivo*, using the last-instar larvae of *Galleria mellonella* (Lepidoptera: Phyralidae), at 25°C in growth chambers. The larvae of *G. mellonella* were cultured, according to the methodology of Van Zyl and Malan (2015), on an artificial diet at 25°C in a growth chamber. Modified White´s traps (Kaya & Stock, 1997) were used to harvest the emerged EPNs. Harvested IJs were stored in distilled water at 14°C, in cell culture flasks (CELL STAR®, Greiner Bio-One GmbH), for one to two days, before being transported in a 6-L CoolMate™ (cooler-box blue, ADDIS®) to the nematology laboratory of the Unit for Environmental Sciences and Management, North-West University, Potchefstroom. The fresh masses of *S. yirgalemense, S. jeffreyense* and *H. bacteriophora* were determined according to Andrássy (1956), with the body volume being calculated (Fourie *et al*., 2014) using published data regarding the average diameter and length of the IJs (Malan *et al*., 2011, 2016; Poinar, 1976).

OXYGEN CONSUMPTION RATE MEASUREMENTS

The OCR measurements, expressed per gram of live body mass (g live mass) $^{-1}$, are presented for the IJs of the three-nematode species concerned. The determination of the OCR, using FOS (Seahorse XFe96 analyser; Seahorse Bioscience), was carried out according to the methodology of Van Aardt *et al*. (2016), with their recommended use of 100 *M. incognita* J2 in future studies being applied to the study of the IJs of *S. yirgalemense, S. jeffreyense* and *H. bacteriophora* in the current study. The sensor made use of a cell culture microplate and a sensor cartridge, containing one probe, and four injection ports, for each well. The FOS analyser uses the fluorescence of a chemical complex in a sol-gel to measure the partial pressure of oxygen. Following on the probe calibration of the FOS, the utility plate was ejected, whereupon the cell culture microplate containing the IJs was inserted and analysed (Rogers *et al*., 2011; Van Aardt *et al*., 2016). The temperature of the FOS instrument, which was controlled by means of adjusting the room temperature, ranged between 24.8°C and 26.8°C. One drawback of using the Seahorse XFe96 analyser is that no specific temperature can be set for an analysis. Hence, a temperature range close to between 25°C and 26°C was used, since the EPN species used in the study were mass-reared under such a temperature regime. Six drops of 10-µl nematode suspension were counted out, using a stereomicroscope (40× magnification), with dilutions being made to ensure that 100 ± 5 IJs were transferred to the designated wells of a 96-well Seahorse cell culture microplate. Each well was then topped up with sterile tap water to a maximum volume of 220 *µ*l. Three replicates per trial were used for each species in the four trials performed. Sterile tap water was equally used as a control.

STATISTICAL ANALYSIS

Statistical analyses were conducted using STATISTICA 13.2 software (Statistica Version 13.3; http://www. statsoft.com). The data for each trial were first separately analysed, using the analysis of variance (ANOVA). The pooled data for the two batches were subjected to the application of general linear models, with the means being separated by means of the least significant difference (LSD) and Games-Howell post hoc tests.

Results

Significant differences ($\rho < 0.05$) were obtained between the two batches of the first trail (Trial 1), with the nematode species and the date concerned providing the main effects, preventing the possibility of pooling the data from the two batches, so that separate analysis was required. In Trial 1, batch 1, the OCR of *H. bacteriophora* was found to differ significantly

from that of *S. jeffreyense* and *S. yirgalemense* (ρ < 0.001), which did not differ significantly (ρ = 0.99) from each other. The *H. bacteriophora* IJs had the highest mean OCR value of 308.11 *µ*mol O_2 h^{-1} g^{-1}, followed by *S. jeffreyense*, and then by *S. yirgalemense*, with 198.79 and 198.66 *µ*mol O_2 h^{-1} g^{-1}, respectively (Fig. 4.1).

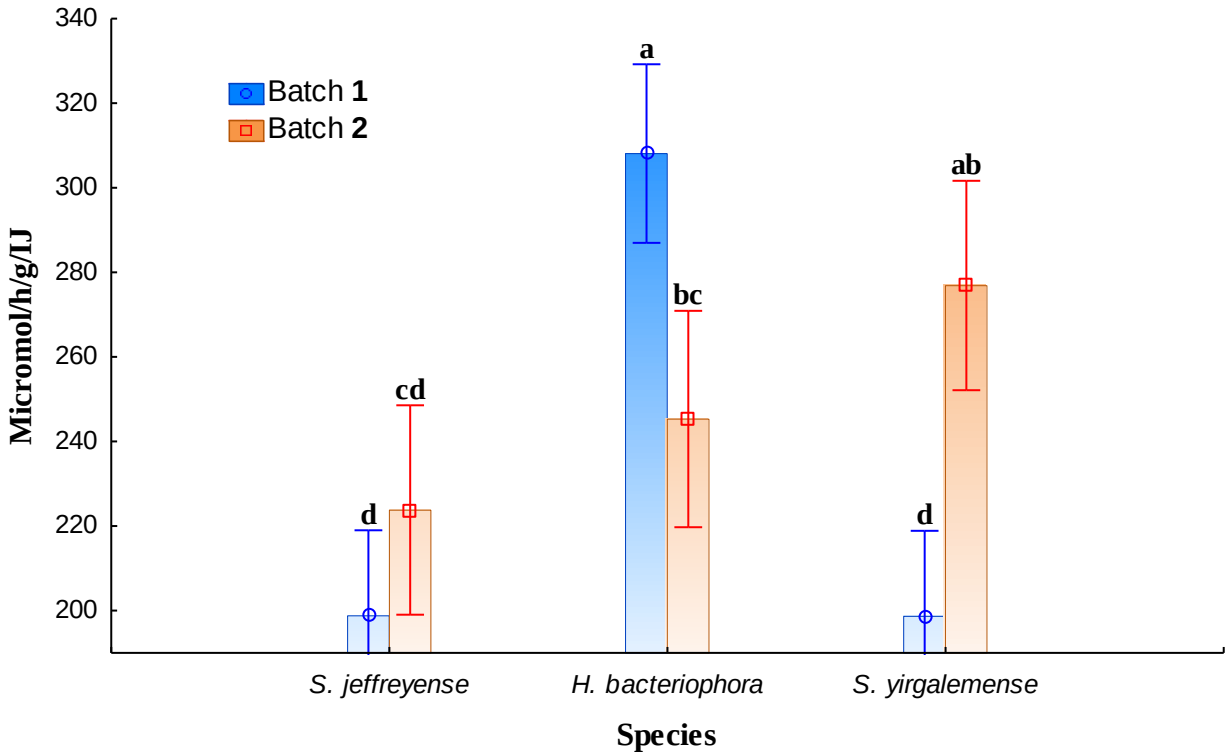


Fig. 4. 1. Trial 1: Mean basal measurement of oxygen consumption rate in micromole/h/g/IJ (95% confidence level) for infective juveniles of *Steinernema jeffreyense, Heterorhabditis bacteriophora,* and *S. yirgalemense* (two-way ANOVA: $F_{(2, 111)} = 18.670$, ρ < 0.001) for batches 1 and 2. Means were separated by applying the LSD test: ρ = 0.05; error: between MSE = 2499.2, df = 111.00. The same letter above the bars indicates the absence of significant difference.

In Trial 1, batch 2, a significant difference was found between *S. jeffreyense* and *S. yirgalemense* (ρ = 0.003), while no significant difference (ρ = 0.23) was found between *H. bacteriophora* and *S. jeffreyense*, and *S. yirgalemense* (ρ = 0.08) (Fig. 4.1). The *S. yirgalemense* IJs had the highest mean OCR value of 276.89 *µ*mol O_2 h^{-1} g^{-1}, followed by *H. bacteriophora*, with 245.31 *µ*mol O_2 $h^{-1}g^{-1}$, and *S. jeffreyense*, with 223.79 *µ*mol O_2 h^{-1} g^{-1} (Fig. 4.1). Furthermore, in view of the batch interaction in Trial 1, a significant increase in the OCR values of 198.79 to 223.79 *µ*mol O_2 h^{-1} g^{-1}, in the case of *S. jeffreyense*, a significant decrease from 308.11 to 245.31 *µ*mol O_2 h^{-1} g^{-1}, for *H. bacteriophora*, and a significant increase from 198.66 to 276.89 *µ*mol $O_2h^{-1}g^{-1}$, for *S. yirgalemense* (ρ < 0.001), was observed, respectively.

Even more markedly, significant differences were found between the two batches of the second trial (Trial 2), with the main effects relating to the date and nematode species

concerned. As the data from the two batches could not be pooled, they were presented separately. In batch 3, the OCR of *S. yirgalemense*, *S. jeffreyense*, and *H. bacteriophora* differed significantly from each other (ρ < 0.001). The *S. yirgalemense* IJs had the highest mean OCR value of 399.06 *μ*mol O_2 h^{-1} g^{-1}, followed by that of *S. jeffreyense* and *H. bacteriophora*, with 204.92 and 146.09 *μ*mol O_2 h^{-1} g^{-1}, respectively (Fig. 4.2).

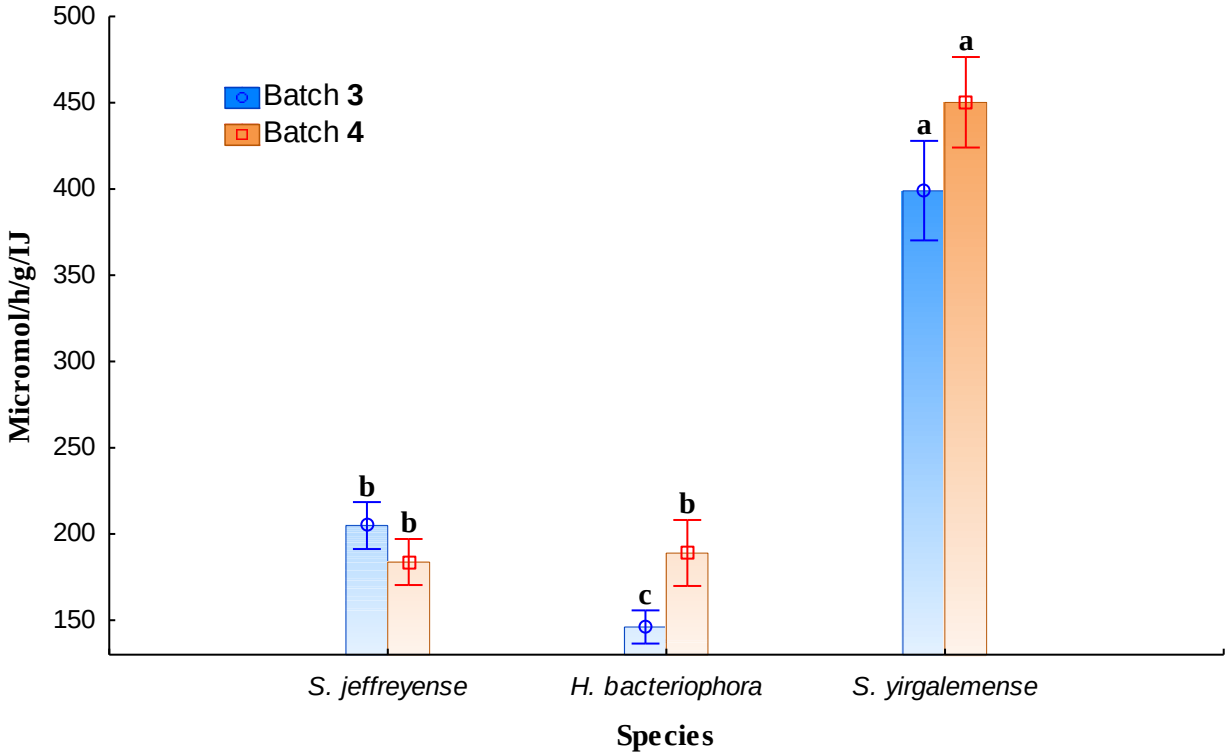


Fig. 4. 2. Trial 2: The mean basal measurement of the oxygen consumption rate in micromole/h/g/IJ (95% confidence level) for the IJs of *Steinernema jeffreyense, Heterorhabditis bacteriophora,* and *S. yirgalemense* ($F_{(2, 138)}$ = 8.5894, ρ < 0.005) for batches 3 and 4. Mean separated by Games-Howell post hoc test: error: between MSE = 2192.4, df = 138.00. The same letter above the bars indicates the absence of significant difference.

In Trial 2, batch 4, the mean OCR value for *S. yirgalemense* differed significantly from that for both *S. jeffreyense* and *H. bacteriophora* (ρ < 0.001), yet the latter two species did not differ significantly (ρ = 0.99) from each other (Fig. 4.2). The *S. yirgalemense* IJs had the highest mean OCR value of 450.29 *μ*mol O_2 h^{-1} g^{-1}, followed by that of *H. bacteriophora*, and of S. *jeffreyense*, with 188.98 and 183.74 *μ*mol O_2 h^{-1} g^{-1}, respectively (Fig. 4.2). Furthermore, for the batch interaction in Trial 2, the recorded mean OCR values for *S. jeffreyense* IJs lacked significance (ρ = 0.22), decreasing from 204.92 to 183.74 *μ*mol O_2 h^{-1} g^{-1} in batches 3 and 4, respectively. In contrast, the OCR in *H. bacteriophora* IJs significantly increased (ρ = 0.003) from 146.09 to 188.98 *μ*mol O_2 h^{-1} g^{-1} in batches 3 and 4, respectively. Also, there was a non-significant (ρ = 0.09) increase in the OCR values of *S. yirgalemense* IJs, from 399.06 to 450.29 *μ*mol O_2 h^{-1} g^{-1} in batches 3 and 4, respectively. Lastly, the mean OCR values of *S.*

yirgalemense IJs increased throughout the study, unlike was the case with both *S. jeffreyense* and *H. bacteriophora*, for which mixed trends were recorded in all batches.

Discussion

The study reports on the characterisation of the IJs of *S. jeffreyense*, *H. bacteriophora*, and *S. yirgalemense* by means of the basal measurement of their OCR, using fibre-optic oxygen sensors. The IJs were non-feeding, as their mouth and anus were closed (Grewal *et al*., 2002), and they only respired through the body wall. There is paucity in literature on the respiratory physiology of EPNs (Atkinson, 1976, 1980; Burman & Pye, 1980; Chitwood & Perry, 2009; Lindegren *et al*., 1986; Perry *et al*., 2013; Qiu & Bedding, 2000; Wright, 1998). Determination of the specific OCR of three EPN species, *S. yirgalemense*, *S. jeffreyense* and *H. bacteriophora*, was necessary, because neither the OCR of the IJs, nor the corresponding bacterium of either *S. yirgalemense* or *S. jeffreyense* was known. The lack of information has proved to be a hindrance in the past, given the commercial interest that the EPNs concerned hold in protecting crops in South African agricultural production.

The observations made in this study are heterogeneous. Unfortunately, there is scarcity of information on the matter for inference. Within the available literature search, the size of the nematode inversely influences its OCR, with smaller nematodes having a higher surface-area-to-volume ratio than do the larger nematodes, and thus a higher OCR, which tends to vary both within and between species (Atkinson, 1976; Wright, 1998). The results obtained in Trial 1, batch 1 (Fig. 4.1) represented a plausible scenario for all the nematodes involved, based on their size. The above explains why the smaller nematodes tend to sustain a higher rate of metabolism than do the larger nematodes, per unit of body weight, due to the increased surface-to-volume ratio of the smaller nematodes, and the subsequently increased efficiency of the surface-dependent processes involved, such as O_2 diffusion (Atkinson, 1980; Ferris *et al*., 1995). Yet, the metabolic rate of equal-sized nematodes may vary with such factors as life-history strategies, among others (Ferris *et al*., 1995). The above held true for the results obtained in Trial 1, batch 1, in terms of which *S. jeffreyense* and *S. yirgalemense* did not significantly differ from each other regarding their OCR. The above could be ascribed to their proximity in size, with *S. jeffreyense* being slightly larger than *S. yirgalemense*, but significantly different in OCR from *H. bacteriophora*. However, due to nature of heterogeneity the result obtained could neither be replicated in Trial 1, batch 2, nor in Trial 2, batches 3 and 4, for either *H. bacteriophora*, or for *S. yirgalemense*.

Although Burman & Pye (1980) and Lindegren *et al*. (1986) reported temperature-dependent results, the results in the current study contrasted to the previous researchers'

observations made in the above respect. The local strains that were used in the current study were isolated from the dry areas of South Africa. The strains were adapted to temperatures ranging between 24.8°C and 26.8°C on the FOS instrument, and they were also cultured *in vivo* at 25°C; thus, the temperature could not have had much influence on the observed variation in the measured OCR, within the batches of the different EPN species studied. Ferris *et al*. (1995) and Anderson & Coleman (1982), in contrast, suggest that nematode species in the same environment have different thermal optima. However, in concurrence with the observations made by Burman & Pye (1980), the observed respiration rate in the present study might have been due to the increased amount of activity in the nematodes experienced at a temperature of 25°C during their culturing and harvesting. The above might have led to the depletion of some energy reserves, leading to a lowering of their activity levels in the wells of the FOS, even though the growth temperature did not affect the respiration of *Caenorhabditis elegans*, as was found to occur in the research of Dusenbery *et al*. (1978).

Nordmeyer & Dickson (1989) report that antibiotics affected the oxygen uptake by *Meloidogyne* spp. J2 during the measurement of the OCR, leading to them not being used in the present study. The above was despite Burman & Pye (1980) obtaining the desired results, after having disinfected the IJs of *S. carpocapsae* with 0.1% quaternary ammonium compounds and M9 buffer. However, such treatment ought to have a negligible effect on such contaminants as mites and collembola (Epsky *et al*., 1988; Gilmore & Potter, 1993; Kaya & Koppenhöfer, 1996; Wilson & Gaugler, 2004), which are often antagonistic to EPNs.

Mites tend to have a high OCR (Block, 1977; Ellingsen, 1978; Kanungo, 1965; Karagoz *et al*., 2007), with mites of the genus *Sancassania* engaging in predatory behaviour towards EPNs (Cakmak *et al*., 2010; Ekmen *et al*., 2010a, b; Ulug *et al*., 2014). Contamination of the EPN culture with such mites, and the later determination of the OCR by means of the taking of highly sensitive measurements by way of the FOS analyser (Moodley *et al*., 2008; Van Aardt *et al*., 2016; Zhdanov *et al*., 2012) would be likely to result in the production of such large values as those that were observed for *S. yirgalemense* in trials 2, 3 and 4. However, no mites were observed during the counting of the number of IJs present in the current study.

Another plausible source of the variation found in the results was the pipetting that was used, which might have reflected a drop, or rise, in the OCR, due to the increase/decrease in the number of IJs employed per treatment. Any error in counting and/or pipetting could have influenced the total number of nematodes accessed in the wells by the highly sensitive FOS. Given that multiple treatments were investigated at any one given time, the error involved would also have been largely magnified, too. The nematodes were pipetted into the 96-well plates after counting, under conditions of constant stirring on a magnetic stirrer, with the probability of uneven distribution occurring at the subsequent sampling times. The result was,

then, the observed decreasing and increasing means of the OCR found in trials 2, 3 and 4 for *H. bacteriophora* and *S. yirgalemense*. *Steinernema yirgalemense* was the only nematode whose mean OCR values increased throughout the study in the corresponding batches and trials. Van Aardt *et al*. (2016) and Dancy *et al*. (2013) reported an increase in the mean OCR with and increasing number of J2 of *M. incognita*, and for bigger nematodes (*C. elegans*), respectively.

The current study cannot conclude on a discussion of the cause(s) of the variation in the different trials and batches involved. The portrayal of the results can, however, provide baseline screening for the comparing of respiratory and metabolic physiology among EPN species, using the latest available technology, like FOS. Therefore, the conclusions drawn as to the OCR measurements of the different EPNs require further research. There is need for improving the protocol to get homogeneous results.

References

Andaló, V., Cavalcanti, R.S., Molina, J.P. & Moino Jr., A. (2010). Substrates for storing entomopathogenic nematodes (Rhabditida: Steinernematidae, Heterorhabditidae). *Scientia Agricola* 67, 342-347. DOI : 10.1590/S0103-90162010000300013

Anderson, R.V. & Coleman, D.C. (1982). Nematode temperature responses: A niche dimension in populations of bacterial-feeding nematodes. *Journal of Nematology* 14, 69-76. PMC2618149

Andrássy, I. (1956). Rauminhalts- und Gewichtsbestimmung der Fadenwurmer (Nematoden). *Acta Zoologica Academica Scientiarum Hungaricae* 2, 1-15.

Atkinson, H.J. (1973). The respiratory physiology of the marine nematodes *Enoplus brevis* (Bastian) and *E. communis* (Bastian) I. The influence of oxygen tension and body size. *Journal of Experimental Biology* 59, 255-266.

Atkinson, H.J. (1976). Respiratory physiology of nematodes. In: Croll, N.A. (Ed.). *The Organization of Nematodes*. New York, Academic Press, pp. 243-272.

Atkinson, H. J. (1980). Respiration in nematodes. In: Zuckerman, B.M. (Ed.). *Nematodes as Biological Models, Volume 2.* New York, Academic Press, pp. 101–142.

Bair, T.D. (1955). The oxygen consumption of *Rhabditis strongyloides* and other nematodes related to oxygen tension. *Journal of Parasitology* 41, 613-623. DOI:10.2307/3274143

Belur, P., Inman III, F.L. & Holmes, L. (2013). Determination of specific oxygen uptake rate of *Photorhabdus luminescens* during submerged culture in lab scale bioreactor. *Biocontrol Science and Technology* 23, 1458-1468. DOI:10.1080/09583157.2013.840361

Bhatt, B.D. & Rohde, R.A. (1970). The influence of environmental factors on the respiration of plant-parasitic nematodes. *Journal of Nematology* 2, 277-285. PMC2618771

Block, W. (1977). Oxygen consumption of the terrestrial mite *Alaskozetes antarcticus* (Acari:

Cryptostigmata). *Journal of Experimental Biology* 68, 69-87.

Boemare, N.E., Akhurst, R.J. & Mourant, R.G. (1993). DNA relatedness between *Xenorhabdus* spp. (Enterobacteriaceae), symbiotic bacteria of entomopathogenic nematodes, and a proposal to transfer *Xenorhabdus luminescens* to a new genus, *Photorhabdus* gen. nov. *International Journal of Systematic Bacteriology* 43, 249-255. DOI: 10.1099/00207713-43-2-249.

Burman, M. & Pye, A.E. (1980). *Neoaplectana carpocapsae*: Respiration of infective juveniles. *Nematologica* 26, 214-219. DOI: 10.1163/187529280x00107

Cakmak, I., Ekmen, Z.I., Karagoza, M., Hazir, S. & Kaya, H.K. (2010). Development and reproduction of *Sancassania polyphyllae* (Acari: Acaridae) feeding on entomopathogenic nematodes and tissues of insect larvae. *Pedobiologia* 53, 235-240. DOI:10.1016/j.pedobi.2009.11.002

Chavarría-Hernández, N.G., Maciel-Vergara, G., Chavarría-Hernández, J.C., Castro-Rosas, J., Rodríguez-Pastrana, B.R., De la Torre-Martínez, M. & Rodríguez-Hernández, A.I. (2011). Mass production of the entomopathogenic nematode, *Steinernema carpocapsae* CABA01, through the submerged monoxenic culture in two internal loop airlift bioreactors with some geometric differences. *Biochemical Engineering Journal* 55, 145-153. DOI:10.1016/j.bej.2011.03.011

Chavarría-Hernández, N., Pérez-Pérez, N.C., Chavarría-Hernández, J.C., Barahona-Pérez, L.F., & Rodríguez-Hernández, A.I. (2014). Specific oxygen uptake of the entomopathogenic nematode, *Steinernema carpocapsae* CABA01, in submerged culture. *Biocontrol Science and Technology* 24, 723-733. DOI:10.1080/09583157.2014.890697

Chitwood, D.J. & Perry, R.N., 2009. Reproduction, Physiology and Biochemistry. In: Perry R.N, Moens, M., & Starr, J.L., (Eds). *Root-knot Nematodes*. Wallingford, UK: CAB International, pp. 182–200. DOI:10.1079/9781845934927.0182

Ciche, T.A., Darby, C., Ehlers, R.-U., Forst, S. & Goodrich-Blair, H. (2006). Dangerous liaisons: The symbiosis of entomopathogenic nematodes and bacteria. *Biological Control* 38, 22-46. DOI: 10.1016/j.biocontrol.2005.11.016.

Clark, L.C.J. (1956). Monitor and control of blood and tissue oxygen tensions. *Transactions of the American Society of Artificial Internal Organs* 2, 41-46.

Dancy, B., Kayser, E., Sedensky, M. & Morgan, P. (2013). Live worm respiration in 24-well format. *Worm Breeder's Gazette* 19, 11-12.

De la Torre, M. (2003). Challenges for mass production of nematodes in submerged culture. *Biotechnology Advances* 21, 407-416. DOI:10.1016/S0734-9750(03)00057-0

Dusenbery, D.B., Anderson, G.L. & Anderson, E.A. (1978). Thermal acclimation more extensive for behavioral parameters than for oxygen consumption in the nematode *Caenorhabditis elegans*. *Journal of Experimental Zoology* 206, 191-197. DOI:10.1002/jez.1402060208

Ehlers, R.-U. (2007). Entomopathogenic nematodes: From science to commercial use. In: Vincent, C., Goettel, M.S. & Lazarovits, G. (Eds). *Biological Control: A Global Perspective: Case Studies from*

Around the World. Wallingford, CABI Publishing, pp. 136-151. DOI: 10.1079/9781845932657.0136.

Ekmen, Z., Cakmak I., Karagoz, M., Hazir, S., Ozer, N. & Kaya, H.K. (2010a). Food preference of *Sancassania polyphyllae* (Acari: Acaridae): Living entomopathogenic nematodes or insect tissues? *Biocontrol Science and Technology* 20**,** 553-566. DOI:10.1080/09583151003624696

Ekmen, Z.I., Hazir, S., Cakmak, I., Ozer, N., Karagoz, M. & Kaya, H.K. (2010b). Potential negative effects on biological control by *Sancassania polyphyllae* (Acari: Acaridae) on an entomopathogenic nematode species. *Biological Control* 54, 166-171. DOI:10.1016/J.BIOCONTROL.2010.05.004

Ellingsen, I.-J. (1978). Oxygen consumption in active and quiescent protonymphs of the American house-dust mite. *Journal of Insect Physiology* 24, 13-16. DOI:10.1016/0022-1910(78)90005-7

Epsky, N.D., Walter, D.E. & Capinera, J.L. (1988). Potential role of nematophagous microarthropods as biotic mortality factors of entomogenous nematodes (Rhabditida: Steinernematidae, Heterorhabditidae). *Journal of Economic Entomology* 81, 821-825. DOI:10.1093/jee/81.3.821

Evans, A.A.F. & Perry, R.N. (1976). Survival strategies in nematodes. In: Croll, N.A. (Ed). *The Organization of Nematodes.* London, Academic Press, pp. 383-401.

Ferris, H., Lau, S. & Venette, R. (1995). Population energetics of bacterial-feeding nematodes: Respiration and metabolic rates based on CO_2 production. *Soil Biology and Biochemistry* 27, 319-330. DOI:10.1016/0038-0717(94)00186-5

Fourie, H., Van Aardt, W., Venter, C. & Tiedt, L. (2014). The effects of CropGuard® on the motility, ultrastructure, and respiration of two *Meloidogyne* species. *Nematropica* 44**,** 85-92.

Friedman, M.J., Langston, S.E. & Pollit, S. (1989). *Mass Production in Liquid Culture of Insect Killing Nematodes*. International Patent No. WO 89/04602.

Gaugler, R., Grewal, P., Kaya, H.K. & Smith-Fiola, D. (2000). Quality assessment of commercially produced entomopathogenic nematodes. *Biological Control* 17**,** 100-109. DOI:10.1006/bcon.1999.0768

Gilmore, S. & Potter, D. (1993). Potential role of Collembola as biotic mortality agents for entomopathogenic nematodes. *Pedobiologia* 37, 30-38.

Glazer, I. (2002). Survival biology. In: Gaugler, R. (Ed.). *Entomopathogenic nematology*. Wallingford, UK, CABI Publishing, pp. 169-187. DOI: 10.1079/9780851995670.0169.

Grewal, P.S. (2002). Formulation and application technology. In: Gaugler, R. (Ed.). *Entomopathogenic Nematology.* Wallingford, CABI Publishing, pp. 265-287. DOI: 10.1079/9780851995670.0265.

Grewal, P.S., Wang, X. & Taylor, R.A.J. (2002). Dauer juvenile longevity and stress tolerance in natural populations of entomopathogenic nematodes: Is there a relationship? *International Journal for Parasitology* 32, 717-725. DOI: 10.1016/S0020-7519(02)00029-2.

Grewal, P.S., Bai, X. & Jagdale, G.B. (2011). Longevity and stress tolerance of entomopathogenic nematodes. In: Perry, R.N. & Wharton, D.A. (Eds). *Molecular and Physiological Basis of Nematode Survival.* Wallingford, CABI Publishing, pp. 157-181. DOI: 10.1079/9781845936877.0157.

Griffin, C. (2015). Behaviour and population dynamics of entomopathogenic nematodes following application. In: Campos-Herrera, R. (Ed.). *Nematode Pathogenesis of Insects and Other Pests*. Dordrecht, Springer International Publishing, pp. 57-97. DOI:10.1007%2F978-3-319-18266-7_3

Hodgson, M., Day, B., White, D. & Tisa, L. (2003). Effect of growth conditions on the motility of *Photorhabdus temperata*. *Archives of Microbiology* 180, 17-24. DOI:10.1007/s00203-003-0558-z

Jenkins, N. & Goettel, M. (1997). Methods for mass-production of microbial control agents of grasshoppers and locusts. *Memoirs of the Entomological Society* 171, 37-48. DOI:10.4039/entm129171037-1

Kagimu, N., Ferreira, T. & Malan, A.P. (2017). The attributes of survival in the formulation of entomopathogenic nematodes utilised as insect biocontrol agents. *African Entomology* 25, 275-291. DOI: 10.4001/003.025.0275.

Kanungo, K. (1965). Oxygen uptake in relation to water balance of a mite (*Echinolaelaps echidninus*) in unsaturated air. *Journal of Insect Physiology* 11, 557-568. DOI:10.1016/0022-1910(65)90138-1

Karagoz, M., Gulcu, B., Cakmak, I., Kaya, H.K. & Hazir, S. (2007). Predation of entomopathogenic nematodes by *Sancassania* sp. (Acari: Acaridae). *Experimental and Applied Acarology* 43**,** 85-95. DOI:10.1007/s10493-007-9105-y

Karimi, J. & Salari, E. (2015). Entomopathogenic nematodes in Iran: Research and applied aspects. In: Campos-Herrera, R. (Ed.). *Nematode Pathogenesis of Insects and Other Pests*. Dordrecht, Springer International Publishing, pp. 451-476. DOI:10.1007/978-3-319-18266-7_19

Kaya, H.K. & Koppenhöfer, A.M. (1996). Effects of microbial and other antagonistic organism and competition on entomopathogenic nematodes. *Biocontrol Science and Technology* 6, 357-372. DOI:10.1080/09583159631334

Kaya, H.K. & Stock, S.P. 1997. Techniques in insect nematology. In: Lacey, L.A. (Ed.). *Manual of Techniques in Insect Pathology*. London: Academic Press, pp. 281-324. DOI: 10.1016/B978-012432555-5/50016-6.

Klekowski, R.Z., Paplinska, E. & Wasilewska, L. (1972). Oxygen consumption by soil-inhabiting nematodes. *Nematologica* 18**,** 391-403. DOI:10.1163/187529272x00665

Kung, S., Gaugler, R. & Kaya, H. (1990). Influence of soil pH and oxygen on persistence of *Steinernema* spp. *Journal of Nematology* 22, 440-445. PMC2619085

Lindegren, J.E., Rij, R.E., Ross, S.R. & Fouse, D.C. (1986). Respiration rate of *Steinernema feltiae* infective juveniles at several constant temperatures. *Journal of Nematology* 18, 221-224.

PMC2618519

Malan, A.P., Knoetze, R. & Moore, S.D. (2011). Isolation and identification of entomopathogenic nematodes from citrus orchards in South Africa and their biocontrol potential against false codling moth. *Journal of Invertebrate Pathology* 108**,** 115-125. DOI: 10.1016/j.jip.2011.07.006.

Malan, A.P., Knoetze, R. & Tiedt, L.R. (2016). *Steinernema jeffreyense* n. sp. (Rhabditida: Steinernematidae), a new entomopathogenic nematode from South Africa. *Journal of Helminthology* 90**,** 262-278. DOI:10.1017/S0022149X15000097.

Moodley, L., Steyaert, M., Epping, E. & Middelburg, J. (2008). Biomass-specific respiration rates of benthic meiofauna: Demonstrating a novel oxygen micro-respiration system. *Journal of Experimental Marine Biology and Ecology* 357, 41-47. DOI:10.1016/j.jembe.2007.12.025

Nordmeyer, D. & Dickson, D.W. (1989). Effect of carbamate, organophosphate, and avermectin nematicides on oxygen consumption by three *Meloidogyne* spp. *Journal of Nematology* 21, 472-476. PMC2618954

Perry, R.N., Wright, D.J. & Chitwood, D.J. (2013). Reproduction, physiology and biochemistry. In: Perry, R. N. & Moens, M. M. (Eds). *Plant Nematology*. Wallingford, UK: CAB International, pp. 219–245. DOI:10.1079/9781780641515.0219

Piedra-Buena, A., López-Cepero, J. & Campos-Herrera, R. (2015). Entomopathogenic nematode production and application: regulation, ecological impact and non-target effects. In: Campos-Herrera, R. (Ed.). *Nematode Pathogenesis of Insects and Other Pests*. Dordrecht, Springer International Publishing, pp. 255-282. DOI:10.1007/978-3-319-18266-7_10

Poinar, G.O. (1976). Description and biology of a new insect parasite rhabditoid, *Heterorhabditis bacteriophora* n. gen. n. sp. (Rhabditida; Heterorhabditidae. fam.). *Nematologica* 21, 463-470. DOI:10.1163/187529275x00239

Qiu, L. & Bedding, R.A. (2000). Energy metabolism and its relation to survival of the infective juveniles of *Steinernema carpocapsae* under oxygen-deficient conditions. *Journal of Nematology* 32, 271-280. DOI:10.1163/156854100509330

Rankin, C.H. (2005). Nematode memory: Now, where was I? *Current Biology* 15, R374-R375. DOI:10.1016/j.cub.2005.05.005

Rogers, G.W., Brand, M.D., Petrosyan, S., Ashok, D., Elorza, A.A., Ferrick, D.A. & Murphy, A.N. (2011). High throughput microplate respiratory measurements using minimal quantities of isolated mitochondria. *PLoS ONE* 6, e21746. DOI:10.1371/journal.pone.0021746

Schiemer, F. & Duncan, A. (1974). The oxygen consumption of a freshwater benthic nematode, *Tobrilus gracilis* (Bastian). *Oecologia* 15, 121-126. DOI:10.1007/BF00345740

Shapiro-Ilan, D.I., Han, R. & Dolinksi, C. (2012). Entomopathogenic nematode production and application technology. *Journal of Nematology* 44**,** 206-217. PMCID: PMC3578468.

Sharma, M.P., Sharma, A.N. & Hussaini, S.S. (2011). Entomopathogenic nematodes, a potential

microbial biopesticide: Mass production and commercialisation status – a mini review. *Archives of Phytopathology and Plant Protection* 44, 855-870. DOI:10.1080/03235400903345315

Silver, S.C. (1999). *Granular Formulations of Biological Entities with Improved Storage Stability*. World Patent No 95/0577.

Stock, S.P. (2015). Diversity, biology and evolutionary relationships. In: Campos-Herrera, R. (Ed.). *Nematode Pathogenesis of Insects and Other Pests*. Dordrecht, Springer International Publishing, pp. 3-27. DOI:10.1007/978-3-319-18266-7_1

Strauch, O. & Ehlers, R.-U. (2000). Influence of the aeration rate on the yields of the biocontrol nematode *Heterorhabditis megidis* in monoxenic liquid cultures. *Applied Microbiology and Biotechnology* 54, 9-13. DOI:10.1007/s002530000352

Stuart, R.J., Barbercheck, M.E. & Grewal, P.S. (2015). Entomopathogenic nematodes in the soil environment: Distributions, interactions and the influence of biotic and abiotic factors. In: Campos-Herrera, R. (Ed.). *Nematode Pathogenesis of Insects and Other Pests*. Dordrecht, Springer International Publishing, pp. 97-137. DOI:10.1007/978-3-319-18266-7_4

Suda, H., Shouyama, T., Yasuda, K. & Ishii, N. (2005). Direct measurement of oxygen consumption rate on the nematode *Caenorhabditis elegans* by using an optical technique. *Biochemical and Biophysical Research Communications* 330**,** 839-843. DOI:10.1016/j.bbrc.2005.03.050

Surrey, M.R. & Wharton, D.A. (1995). Desiccation survival of the infective larvae of the insect parasitic nematode, *Heterorhabditis zealandica* Poinar. *International Journal for Parasitology* 25, 749-752. DOI:10.1016/0020-7519(94)00190-Y

Thomas, G.M. & Poinar, G.O. (1983). Amended description of the genus *Xenorhabdus* Thomas and Poinar. *International Journal of Systematic Bacteriology* 33, 878-879. DOI:10.1099/00207713-33-4-878

Travassos, L. (1927). Sobre o gênero Oxysomatium. *Boletim Biologico, Sao Paulo* 5, 20-21.

Ulug, D., Hazir, S., Kaya, H.K. & Lewis, E. (2014). Natural enemies of natural enemies: The potential top-down impact of predators on entomopathogenic nematode populations. *Ecological Entomology* 39, 462-469. DOI:10.1111/een.12121

Upadhyay, D., Kooliyottil, R., Mandjiny S. III, F.L.I. & Holmes, L.D. (2013). Mass production of the beneficial nematode *Steinernema carpocapsae* utilizing a fed-batch culturing process. *International Journal of Phytopathology* 2, 52-58.

Van Aardt, W.J., Fourie, H., Pretorius, M., Louw, R. & Van Dyk, H. (2016). The effects of stirring, population levels and a potential anti-nematodal product on the respiration of second-stage juveniles of *Meloidogyne incognita* measured with different technology. *Nematology* 18, 1053-1061. DOI:10.1163/15685411-00003014

Van Zyl, C. & Malan, A.P. (2015). Cost-effective culturing of *Galleria mellonella* and *Tenebrio Molitor* and entomopathogenic nematode production in various hosts. *African Entomology* 23**,** 361-375.

DOI: 10.4001/003.023.0232.

Wang, Y. & Zhang, X. (2007). Influence of agitation and aeration on growth and antibiotic production by *Xenorhabdus nematophila*. *World Journal of Microbiology and Biotechnology* 23, 221-227. DOI:10.1007/s11274-006-9217-2

Wharton, D.A. (1986). *A Functional Biology of Nematodes*. London, Croom Helm, pp. 1-192.

Wilson, M. & Gaugler, R. (2004). Factors limiting short-term persistence of entomopathogenic nematodes. *Journal of Applied Entomology* 128**,** 250-253. DOI:10.1111/j.1439-0418.2004.00814.x

Womersley, C.Z. (1990). Dehydration survival and anhydrobiotic potential. In: Gaugler, R. & Kaya, H.K. (Eds). *Entomopathogenic Nematodes in Biological Control.* Boca Raton, FL, CRC Press, pp. 117-137.

Wright, D. (1998). Respiratory physiology, nitrogen excretion and osmotic and ionic regulation. In: Perry, R.N. & Wright, D.J. (Eds). *The Physiology and Biochemistry of Free-Living and Plant-Parasitic Nematodes*. Wallingford, CAB International, pp. 103-131.

Zhdanov, A.V., Hynes, J., Dmitriev, R.I. & Papkovsky, D.B. (2012). O_2 analysis on a fluorescence spectrometer or plate reader. In: Papkovsky, D.B., Zhdanov, A.V., Fercher, A., Dmitriev, R.I. & Hynes, J. (Eds). *Phosphorescent Oxygen-Sensitive Probes.* Springer briefs in biochemistry and molecular biology. New York, Springer, pp. 29-70. DOI:10.1007/978-3-0348-0525-4_2

Chapter 5

Effect of water activity and desiccation on the stability of *Steinernema yirgalemense* and *S. jeffreyense* formulated in diatomaceous earth at room temperature

Abstract

Microbial contamination, as well as desiccation, are, among other factors, often responsible for the quality and short shelf life of entomopathogenic nematodes (EPNs) in formulation. The water activity (a_w) of a food system governs microbial growth and toxin production. In the current study, the concept of determining moisture content at the corresponding a_w-values, using the Guggenheim-Anderson-Boer (GAB) isotherm model, has been studied, with regards to diatomaceous earth (DE), as well as to the survival of *Steinernema yirgalemense*. Scanning electron microscopy has been employed to determine the effect of DE on *S. jeffreyense* during storage in formulation. A decline in the survival rate of *S. yirgalemense* at high a_w-values, due to bacterial sporulation and toxin production, is reported. Scanning micrographs show the desiccation of *S. jeffreyense*, beyond rejuvenation on the addition of water. The effect of desiccation was, however, not widely distributed throughout the sample, and it was equally random between the different test dates. Future research should investigate this problem using high densities of IJs/g of formulation, which are reported to be relatively stable. Equally, the moisture content at different a_w-values for each of the ingredients used in the formulation should be investigated separately, so as to enable stabilising of the formulation and improvement of the shelf life.

Key words: Guggenheim-Anderson-Boer isotherm, scanning electron microscopy, diatomaceous earth, formulation, microbials, toxin, adsorption, desorption

Introduction

Entomopathogenic nematodes (EPNs) (Rhabditida: Steinernematidae) from the *Steinernema* Travassos, 1927 and *Heterorhabditis* Poinar, 1976 and their associated symbiotic bacteria of *Xenorhabdus* Thomas & Poinar, 1983 and *Photorhabdus* Boemare, Akhurst & Mourant, 1993 (Boemare *et al.*, 1993; Akhurst *et al.*, 1996; Ehlers, 2007), are regarded as excellent biological control agents of pest insects. Their many advantages are compromised by their short shelf life, which is a major shortfall to their large-scale commercial utilisation (Grewal, 2000a,b). Their poor survival rate at room temperature, coupled with them being prone to desiccation, hinders their potential utilisation as bio-insecticides (Grewal, 2002; Surrey & Wharton, 1995; Womersley, 1990). Research into the mass culturing of EPNs for commercialisation in medium to large-scale farming systems is under way in South Africa, where strides have been made in several sectors of production not limited to formulation (Hatting & Malan, 2017, Hatting *et al.*, 2018, Malan & Ferreira, 2017). In a recent review (Kagimu *et al*., 2017), microbial contamination, as well as desiccation, were identified as being among other factors affecting the survival of formulated EPNs.

Maintenance of the high quality of EPNs in formulation is imperative for their successful commercialisation. Such a necessity can be identified in terms of their virulence and pathogenicity (Grewal & Peters, 2005; Kagimu *et al*., 2017). The impact of contamination and toxicity of antimicrobial agents on IJs was of utmost importance to the present study (Grewal & Peters, 2005; Kagimu *et al*., 2017). Microbial contamination of nematode formulations with high moisture content, especially at room temperature shelf life, is of significance (Grewal, 2002). The above is due to the propensity of microbials to compete for the existing oxygen supply, which renders the formulations concerned decreasingly usable, as well as leading to the clogging of spray nozzles, which decreases the suitability of the formulated product. Noteworthy, some antimicrobial agents may also decrease the extent of nematode survival in the formulations (Grewal, 2002).

Water activity is defined as the ratio of partial pressure of water vapour in the product to that in the presence of pure water (Mathlouthi, 2001). Microbial growth is mainly determined by a_w, which is a better index for microbial growth than is water content (Heidemann & Jarosz, 1991). Water activity has extensively been employed in optimising product formulation, thereby improving the antimicrobial effectiveness of such formulations. Scott (1953) demonstrated that it was not the water content, but rather the a_w, of a food system that governs microbial growth and toxin production. In addition, the researcher also showed that microorganisms have a limiting a_w-level, below which they will neither grow, nor produce toxins.

Although water activity changes osmotic stress-prompting sporulation response in spore-forming microorganisms, both bacterial endospores and certain fungal spores have special requirements, such as a_w-values for the initiation of germination and outgrowth (consisting of the minimal a_w for germination, which is often higher than the minimum a_w for sporulation) (Tapia *et al*., 2007). Sporulation, germination and toxin production by microbes often occurs at different minimal a_w-values. Such processes are affected by a_w, along with other environmental factors like type of solute, temperature, pH, and nutrient availability, among others, which strongly affect the responses involved, as reviewed by Beuchat (1987, 2002) and Tapia *et al*. (2007).

According to Tapia *et al*. (2007), the minimal a_w-value defines, in theory, the level below which certain biologicals can no longer reproduce, even though other microbes that are more resistant, and that are more adapted, to a_w reduction can grow, thereby leading to the spoiling or compromising of shelf life. Tapia *et al*. (2007) and Troller (1987) further stress the need for laboratory model systems for obtaining a_w, in terms of which the abiotic factors influencing microbial response are regarded as being at their optimum, given the fact that it is extremely difficult to isolate responses to a_w alone within a formulation (Troller, 1987). Most putrefying bacteria are generally accepted to thrive below 0.95 a_w, as well as are bacteria to be the dominant flora in mostly high-moisture formulations. Other bacteria may reach values ranging from 0.90 to 0.85 a_w. However, exceptions exist in the case of moderate-high halophilic bacteria that are comparable to those that spoil brines and salt-rich foods.

Other than the above-mentioned, bacteria do not tend to thrive in a high osmotic, or a low a_w, environment. Fungi are the predominant microflora in ranges of a_w-values of 0.85 to 0.61, in which yeast and moulds tend to bloom equally well (Fontana, 2002; Tapia *et al*., 2007). No microbes can grow at ≤ 0.6 a_w, which is a critical point at which there is potential for the growth of microbes, if the moisture content increases still further. For a broader range of examples of microorganisms inhibited by the lowest a_w for their growth and/or the a_w of some foods, selected consumer and pharmaceutical products refer to the works of Beuchat (2002), Fontana (2007c), Fontana & Schmidt (2007), Schmidt & Fontana (2007) and Tapia *et al.* (2007).

Welti-Chanes *et al*. (2007) reviewed innumerable management applications of a_w in the food industry, and various government regulations in addition to the Food and Drug Administration (FDA) and the US Department of Agriculture (USDA) regulations, as well as the Hazzard Analysis and Critical Control Points (HACCP). The above, among other relevant bodies (Fontana, 2000; Labuza & Altunakar, 2007), have included consideration of a_w in their practices. Fontana & Schmidt (2007) in contrast, illustrated several applications of a_w in non-food systems including: water potential and soil–plant water applications; the association

between a_w and seeds (in terms of the latter's viability, coating and priming); and medical and pharmaceutical applications. Naturally, the concept of a_w in the formulation of EPNs in diatomaceous earth (DE), comprising a food and soil system, is equally important to the above.

Several methods have been employed to determine a_w in formulations, with them not being limited to isopiestic equilibration, freezing point, hair or polymer, electrolytic, capacitance, or dew point hygrometers (Rahman, 1995). The use of water vapour sorption to estimate soil-specific surface area (SA) from single-point measurements (Puri & Murari, 1964; Newman, 1983) and/or modelling approaches has recently proved to be of much interest (Arthur *et al*., 2013; Akin & Likos, 2014, 2017; Khorshidi *et al*., 2017; Arthur *et al*., 2018). The Guggenheim-Anderson-Boer (GAB) equation developed by Anderson (1946), De Boer (1953), and Guggenheim (1966) is, at present, comprehensively applied as being the most efficient equation for sorption isotherm prediction (Kiranoudis *et al*., 1993). The GAB model has several major advantages, which are not limited to having a viable theoretical background, but also to its applicability to virtually all foods, with a range of 0 to 0.95 a_w, coupled with its ease in engineering calculations. The above is due to its three parameters having a mathematical form, together with its parameters having physical meaning in terms of sorption processes. In addition, the model has the ability to designate temperature effects on isotherms by means of employing Arrhenius-type equations (Van den Berg & Bruin, 1981; Al-Muhtaseb *et al.*, 2002; Labuza & Altunakar, 2007; Andrade *et al*., 2011). For the above-mentioned reasons, the GAB equation has been recommended by the European Project Group COST 90 on Physical Properties of Food (Wolf *et al*., 1984). The equation was equally expeditiously accepted at the International Symposium on the Properties of Water (ISOPOW) in 1983 as being the best equation for modelling the moisture isotherm.

In conformity with the arguments of Labuza & Altunakar (2007), a_w estimates the limiting reaction(s) that a formulation can support at a point. The a_w <0.2 would tend to be degraded by lipid oxidation, whereas a_w >0.85 would be likely to be spoiled by bacterial growth, due to the amount of available free water. Therefore, focusing the current study on the water relationships involved in relation to the a_w levels concerned, considering the range of water content to which a DE formulation might be subjected on a large scale, was of interest. The water vapour sorption isotherm (WSIs) of soil defines the relationship between a_w and water content over a range of a_w values, at a given temperature, along an adsorption/desorption, path (Arthur *et al*., 2016). Conforming to the methodology of Arthur *et al*. (2016), the soil clay mineralogy fraction was, therefore, correlated to the water vapour sorption at a given temperature along such an adsorption or desorption path. Recent studies have shown the possibilities implicit in predicting clay content from the equilibrium water content at a given a_w-value (Wuddivira *et al*., 2012; Arthur *et al*., 2015), as well as in evaluating the water content of

clay, in addition to other soil textural fractions (Schneider and Goss, 2012; Jensen *et al*., 2015). The water vapour sorption isotherm can be adequately characterised using models that establish the relationships between the model parameters and the clay content, which are more common and more readily measured (Arthur *et al*., 2016).

When the vapour pressure is equal in the water isotherm, the amount of adsorption/desorption experienced for the same food might differ. Such difference is referred to as the sorption hysteresis. Hysteresis has some practical aspects. For example, if a moist low a_w product is desired, as in the case of a DE formulation, a large desorption hysteresis would be beneficial, as then an increased amount of water would be available at the same a_w level. However, at the same a_w, the relatively high moisture content (desorption) also tends to result in a relatively high rate of loss for some chemical reactions, thus reducing the shelf life concerned, which is undesirable (Kapsalis, 1981). In terms of hysteresis, the calculations of water content from a model fit of GAB to the adsorption-desorption isotherms of any system, such as DE formulation of EPNs, is feasible. The clay mineralogy in DE, even though of minimal percentage, is likely to be comparable to that which was recently found by Arthur *et al*. (2018), in the case of silt-clay soils. According to Arthur *et al*. (2018), the GAB equation accurately describes the water sorption isotherms (with a_w ranging from 0.03 to 0.93 or 0.95) for a wide range of natural (Arthur *et al*., 2016) and swelling soils (Akin & Likos, 2017), especially in relation to the desorption data concerned. For the above, Arthur *et al*. (2018) recently evaluated the water activity corresponding to the GAB monolayer water content for different soil groups (kaolinite-rich, illite-rich and mixed clay samples, smectite-rich and organic soil samples). The authors measured the soil water vapour sorption isotherms for both adsorption and desorption for a_w ranging from 0.03 to 0.93, at a temperature of 25°C, using a vapour sorption analyser (METER Group Inc., Pullman, WA, USA), for 321 soil samples. The authors affirm that the GAB sorption model accurately characterises soil water vapour sorption isotherms and suggest the adoption of such a model as an alternate approach to determining the SA from water vapour sorption.

A recent review by Kagimu *et al*. (2017) mentions that osmotic stress, microbial growth and desiccation, among other factors, tend to affect the survival rate of EPNs in formulations. In nematology, osmotic solutions are often attained using polyethylene glycol (PEG), which tends to increase the osmotic potential, and to lower the a_w, concerned (Feng *et al.*, 2006; Kagimu *et al*., 2017). For the above reason, PEG has been extensively utilised in formulating EPNs. The current study sought to adopt a different approach to testing the survival rate of EPNs at different a_w levels, using the equilibrated saturated salts of known a_w at a determined temperature. Due to the hidden costs involved in using PEG, the current researchers resolved

to exploit the use of isotherms in determining a_w and its relationship with water content, despite them apparently never before having been tested in relation to EPNs.

EPNs have a short shelf life, due to their disposition to low desiccation tolerance, which greatly affects their IJ longevity, host-finding, penetration, pathogenicity, and/or virulence traits (Strauch *et al.*, 2004; Grewal *et al.*, 2011). As EPNs experience partial anhydrobiosis in a quiescent stage (Womersley, 1990), they tend to perform poorly in terms of desiccation. However, the extent of damage that is exerted on the EPNs by DE, which is the preferred carrier in EPN formulation, is unknown. Therefore, the aim of the current study was to improve the viability of the DE formulation by assessing the a_w-stability of locally isolated *Steinernema* species at room temperature, and by determining the desiccative effect of DE on EPNs. The above was done by using the equilibration method transfer of the a_w of saturated salts at 25°C into DE, and by determining the EPN survival rate for four weeks. The GAB isotherm model was used to calculate the amount of water required during the formulation of IJs in DE at that a_w level. Lastly, scanning electron microscopy (SEM) was used in reporting the desiccative effect of DE on IJs during storage.

Materials and methods

SOURCE OF NEMATODES AND HOST INSECTS

The nematodes, *Steinernema yirgalemense* Nguyen, Tesfamariam, Gozel, Gaugler & Adams, 2004 and *Steinernema jeffreyense* Malan, Tiedt & Knoetze, 2016, which are both endemic to South Africa, were used in the current study (Malan *et al*., 2011, 2016). *Galleria mellonella* L. (Lepidoptera: Phyralidae) larvae were cultured, according to the methodology employed by Van Zyl & Malan (2015), on an artificial diet at 25°C in a growth chamber. The *Galleria* larvae were inoculated with *S. yirgalemense* IJs in 9-mm-diameter Petri dishes, lined with moist filter paper. Freshly harvested *S. yirgalemense* IJs were cultured *in vivo*, using the last-instar larvae of *Galleria*, kept at 25°C in a growth chamber. Modified White traps (Kaya & Stock, 1997) were used to harvest the emerged EPNs. The harvested IJs were stored in distilled water at 14°C, being collected in 5-L Erlenmeyer flasks that were constantly stirred, using a 70 × 10 mm cylindrical magnetic stirring bar, on an AGE magnetic stirrer (VELP® Scientifica). The above-mentioned procedure was followed for approximately three weeks, until the desired concentration of IJs for each batch was achieved. However, the *S. jeffreyense* IJs were cultured *in vitro*, in 50 ml liquid medium, in 250-ml Erlenmeyer flasks, according to the methodology employed by Ferreira *et al*. (2014, 2016). The harvested IJs were concentrated, and the associated bacteria removed, by letting them settle in Erlenmeyer flasks. The supernatant was siphoned off, and distilled water was added, in several cleaning cycles, until the suspension was clear. This study was challenged with *in vitro* mass culture of

S. yirgalemense, unlike *S. jeffreyense*. This explains the difference in the culture methods explained above.

EQUILIBRATING DE WITH THE A_W-VALUE OF A SATURATED SOLUTION

The equilibration process involved keeping 100 g of DE (Celite 209) in a Petri dish in an oven at 100°C for 48 h. After drying, the weight of the sample was determined. The difference in initial and final weight, corresponding to the amount of water that was associated with the DE, was determined. Samples of 50 g of bone-dried DE placed in separate Petri dishes, along with 100 ml of saturated solution of potassium bromide (KBr), potassium chloride (KCl), strontium nitrate ($Sr(NO_3)_2$), potassium nitrate (KNO_3), or potassium sulphate (K_2SO_4) in a 200-ml beaker, were kept in desiccators. They were covered with a lid and with Parafilm, after being greased with silicone to make the containers airtight. The entire set-up was kept inside an incubator at 25°C for 48 h, to enable the DE to be equilibrated with the corresponding a_w from the saturated salt solution (KBr, a_w = 0.809; KCl, a_w = 0.843; $Sr(NO_3)_2$, a_w = 0.851; KNO_3, a_w = 0.936; K_2SO_4, a_w = 0.973) (Greenspan, 1977; Fontana, 2007a). After incubation, the DE that had acquired the desired a_w (DEa_w) by means of equilibrium, after being mixed with 5 000 000 IJs (paste) of *S. yirgalemense*, was kept in an airtight container for a period of 4 weeks at 25°C. The survival rate of the IJs was checked after 24 h and 48 h, as well as after 1 to 4 weeks, respectively. The experiment was conducted twice on different test dates, using different batch of nematodes.

FORMULATING NEMATODES IN DE FOR SEM ANALYSIS DE_{SEM}

Steinernema jeffreyense was concentrated into a paste using a 32-µm sieve (Clear Edge Filtration SA (Pty) Ltd, South Africa). The technique described in Chapters 2 and 3 was used to formulate 64 million IJs of *S. jeffreyense* in DE (Celite 209 − Imerys Refractory Minerals SA (Pty) Ltd). The proportions of the ingredients used in the formulation were employed according to the methodology favoured by Grewal & Jagdale (2002), which was an improvement on the procedure reported on in chapters 2 and 3, attaining a final density of 3 700 000 IJs/g of formulation. The a_w-value of 0.970 that was used in the formulation induced the IJs into a state of partial anhydrobiosis and subjected them to slow desiccation (Grewal, 2000a,b). After hand-mixing the nematode paste together with all the ingredients, 10 g of the formulated nematodes were placed in lidded containers (Mambo's Plastics) (n = 5), which were stored at 14°C. The formulated nematodes were scanned after 1 to 8 weeks to determine the desiccative effect of DE_{SEM}. The control in this experiment consisted of freshly harvested IJs. The experiment was repeated thrice on different test dates, using different batches of nematodes, which were stored under the same conditions as those described above.

SURVIVAL OF NEMATODES IN DE AT DIFFERENT A_W

The formulated nematodes were counted according to a modification method of Peters (2004). One g from a container with 50 g, containing 5 000 000 IJs, was dissolved in 10 ml of distilled water in a 50-ml beaker. Air was bubbled in from an aquarium air pump (Second Nature Whisper™ 1000), whose tube led to the bottom of the beaker. One 100-µl sample was pipetted into 5 ml distilled water in a clean beaker, which was then brought into suspension, with 1 ml of the suspension being diluted in 2 ml of distilled water. The IJs were counted after the first 24 h and 48 h, and later weekly, for 4 weeks, using a binocular microscope to determine the IJ survival percentage in each of the *DE*a_w samples at a temperature of 25°C.

Moisture content was estimated from the water sorption data obtained, based on the GAB model (Van den Berg & Bruin, 1981). The GAB isotherm equation:

$$m = \frac{m_0 k_b c a_w}{[(1 - k_b a_w)(1 - k_b a_w + c k_b a_w)]}$$

where k_b is a constant in the range 0.70 to 1, and *c* is a constant in the range 1 to 20, was used. In the above equation, m_o is the monolayer water content [water per solid (kg kg−1)] and a_w is the water activity at moisture (*m*) level (Van den Berg & Bruin, 1981). The three-parameter equation is often calculated using a nonlinear solution. The above was done by calculating the moisture content (*m*) at a minimum of five water activity (a_w) values in the range 0.1 to 1. Nonlinear regression could then be used to determine the values of k_b, c, and m_o. Once the constant values are known for the isotherm concerned, any water activity value can be inserted into the GAB equation to determine the corresponding moisture content (Fontana, 2007b; Labuza & Altunakar, 2007).

The moisture content of DE in the current study was extrapolated from the GAB graph for both the adsorption and the desorption isotherms concerned, according to Arthur *et al*. (2018), since the graph is linear, and was prepared for clay-rich soils.

CALCULATION OF A_W FROM THE MODEL FIT OF GAB

The a_w-values were extrapolated from the research of Arthur *et al*. (2018) (Figs 5.1 and 5.2), as the relationship between a_w and water content is linear.

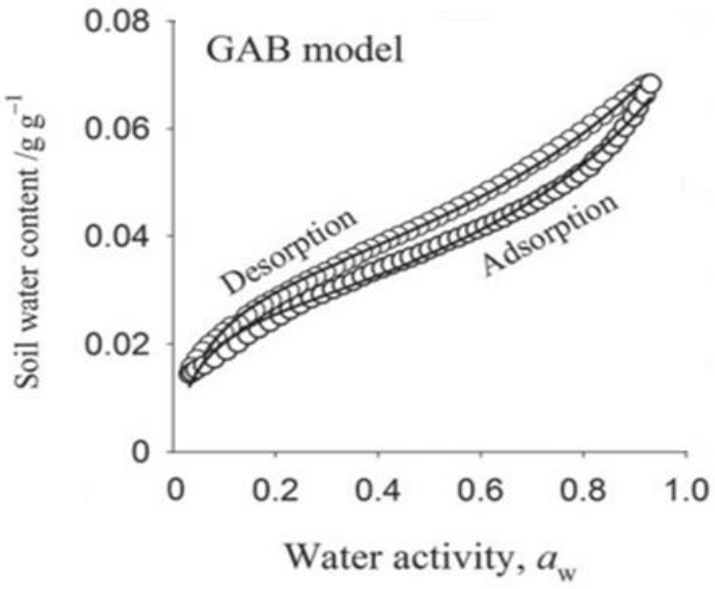


Fig 5. 1 Model fit of the Guggenheim–Anderson–Boer (GAB) model to the adsorption-desorption isotherms of a silty clay loam soil. Applicability of the GAB water vapour sorption model for the estimation of soil-specific surface area (*European Journal of Soil Science*. Source: Adapted from Arthur *et al*., 2018).

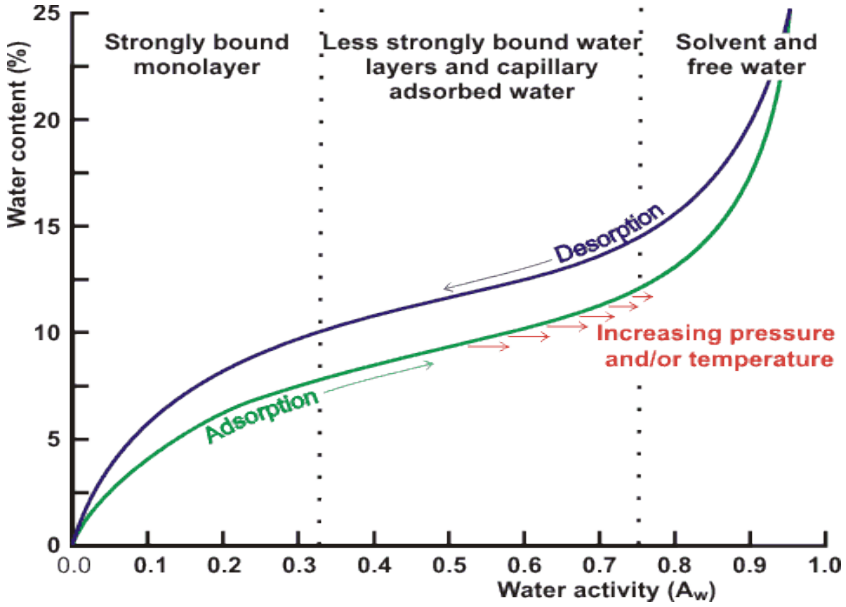


Fig 5. 2 Water activity versus sorption isotherm, displaying the hysteresis often encountered, depending on whether the water is being added to the dry material or removed (in drying) from the wet material, as well as on the effect of the associated temperature and pressure shifts in a hysteresis. Of much interest to the current study is the section on solvent and free water (source: http://www1.lsbu.ac.uk/water/water/activity.html).

SEM OF THE FORMULATED IJS (DE_{SEM})

After fixed the samples in 4% paraformaldehyde (PFA) with 2% glutaraldehyde in 0.1M phosphate buffer (pH7.4) at 4°C overnight, they were post-fixed in 1% aqueous osmium

tetroxide (OsO_4), and then dehydrated in an increasing ethanol series (50%, 70%, 90%, 100%, with intervals of 5 min between each) before undergoing final treatment with hexamethyldisilazane (HMDS), and overnight drying in a desiccator. The samples were then mounted on standard 15-mm aluminium SEM stubs, and coated with a thin layer of gold, using an Edwards S150A sputter coater to enhance conductivity. Imaging was done using a Zeiss MERLIN field emission scanning electron microscope (FESEM), operated at 2-4 kV, and using InLens Secondary (SE) and SE2 detection. The above was done using the facilities of the Centre for Analytical Facilities, Stellenbosch University.

Data analysis

Statistical analyses were conducted using STATISTICA 13.2 software (StatSoft. Inc). The results of the survival of *S. yirgalemense* in *DE*a_w were analysed by means of a two-way repeated measures ANOVA, and the means were separated by means of Fisher's least significant difference post hoc test. The moisture content data extrapolated from the GAB adsorption-desorption isotherm was not analysed, due to the few entries obtained.

Results

Survival of nematodes in DE at different A_w levels

No significant differences ($p > 0.05$) were obtained between the two batches with the main effects of treatment and date, thus enabling the data from the two batches to be pooled and analysed. Two groups of data were separated based on the dates concerned, meaning that the data from 24 and 48 h, and those from 1 to 4 weeks, were analysed separately. The analysis of the 24 h and 48 h data showed no significant differences ($F_{(4, 15)} = 0.87600$, $p = 0.50125$) between the treatments, with regards to survival. In general, the mean percentage survival was higher after 24 h than it was after 48 h of incubation. After 24 h, the mean percentage survival of *S. yirgalemense* in *DE*a_w, treated at a_w 0.809, did not differ significantly from the a_w 0.843 ($p = 0.82$), the a_w 0.851 ($p = 0.36$), the a_w 0.936 ($p = 0.97$), or the a_w 0.973 ($p = 0.72$), of which none differed significantly ($p > 0.05$) from the others. The highest a_w mean percentage survival was obtained with a_w 0.973 (89.38% ± 2.55%), followed by that which was obtained with a_w 0.843 (88.77% ± 2.55%), with a_w 0.936 (87.97% ± 2.55%), with a_w 0.809 (87.80% ± 2.55%), and with a_w 0.851 (83.82% ± 2.55%), respectively. Likewise, after 48 h, the survival of *S. yirgalemense* in *DE*a_w, treated at a_w 0.809, did not differ significantly from the a_w 0.843 ($p = 0.70$), the a_w 0.851 ($p = 0.66$), the a_w 0.936 ($p = 0.33$), or the a_w 0.973 ($p = 0.82$). The highest mean percentage survival was obtained with a_w 0.973 (85.12% ± 3.40%), followed by a_w 0.809 (84.16% ± 3.40%), a_w 0.843 (82.53% ± 3.40%), a_w 0.851 (82.28% ± 3.40%), and a_w 0.936 (79.96% ± 3.40%), respectively. Only the mean percentage survival at a_w 0.843, and

a_w 0.936, after 24 h differed significantly from that which was attained after 48 h (p = 0.033), and (p = 0.009), respectively. The remaining treatments did not differ significantly during the short time interval concerned (Fig. 5.3).

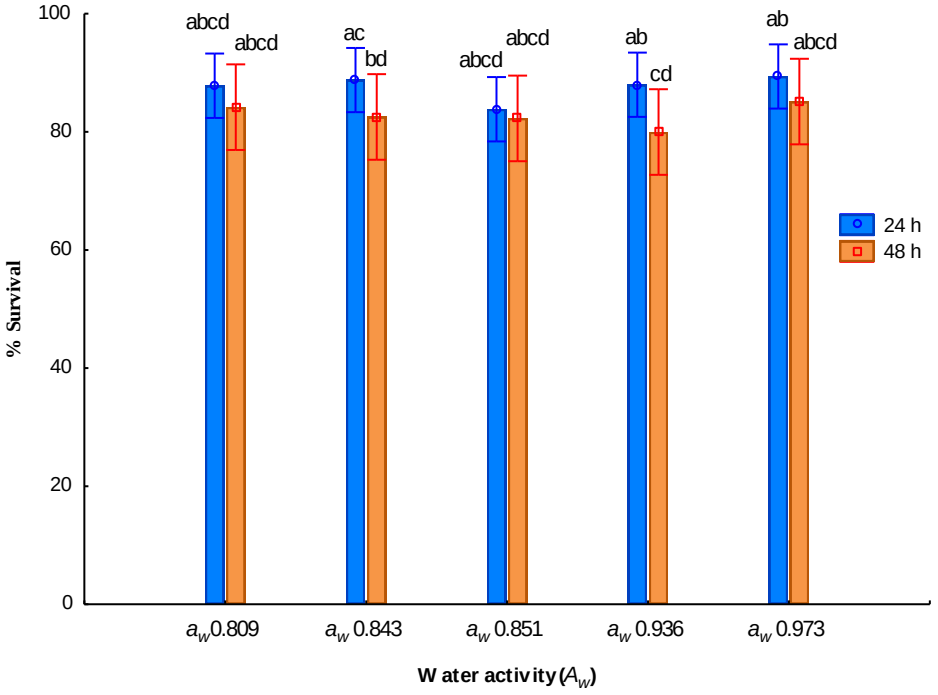


Fig. 5. 3. Mean percentage survival of *Steinernema yirgalemense* in diatomaceous earth at different equilibrated a_w per salt after a period of 24 and 48 h (95% confidence level) and repeated measures one-way ANOVA ($F_{(4, 15)}$ = 0.87600, p = 0.50125). Mean separated by Fisher's least significant difference (LSD) post hoc test; Error: Between; Within; Pooled MS = 36.138, df = 21.840. The same letter above the bar indicates no significant difference (p <0.05).

Equally important, at weeks 1 to 4, no significant differences (p >0.05) were obtained between the two batches, with the main effects of treatment and date in terms of the two-way ANOVA, thus enabling the data from the two batches to be pooled. The analysis of the data from weeks 1 to 4 showed significant differences (repeated measures two-way ANOVA: $F_{(12, 45)}$ = 3.0483, p = 0.003) between the treatments, with regards to survival. Overall, in weeks 1 and 2, no such significant differences (p >0.05) in the mean percentage survival of *S. yirgalemense* was observed as was noted in weeks 3 and 4. A gradual decrease in the survival of IJs occurred with the increasing number of weeks. In week 1, the survival of *S. yirgalemense* in *DE*a_w treated, at a_w 0.809, did not differ significantly from that which was treated at a_w 0.843 (p = 0.25), a_w 0.851 (p = 0.23), a_w 0.936 (p = 0.58), or a_w 0.973 (p = 0.66). In short, the above did not differ significantly (p >0.05) from each other. The highest mean percentage survival was obtained with a_w 0.809 (80.15% ± 2.83%), followed by a_w 0.973 (76.98% ± 2.83%), with

a_w 0.936 (76.26% ± 2.83%), a_w 0.843 (71.91% ± 2.83%), and a_w 0.851 (71.50% ± 2.83%), respectively (Fig. 5.4).

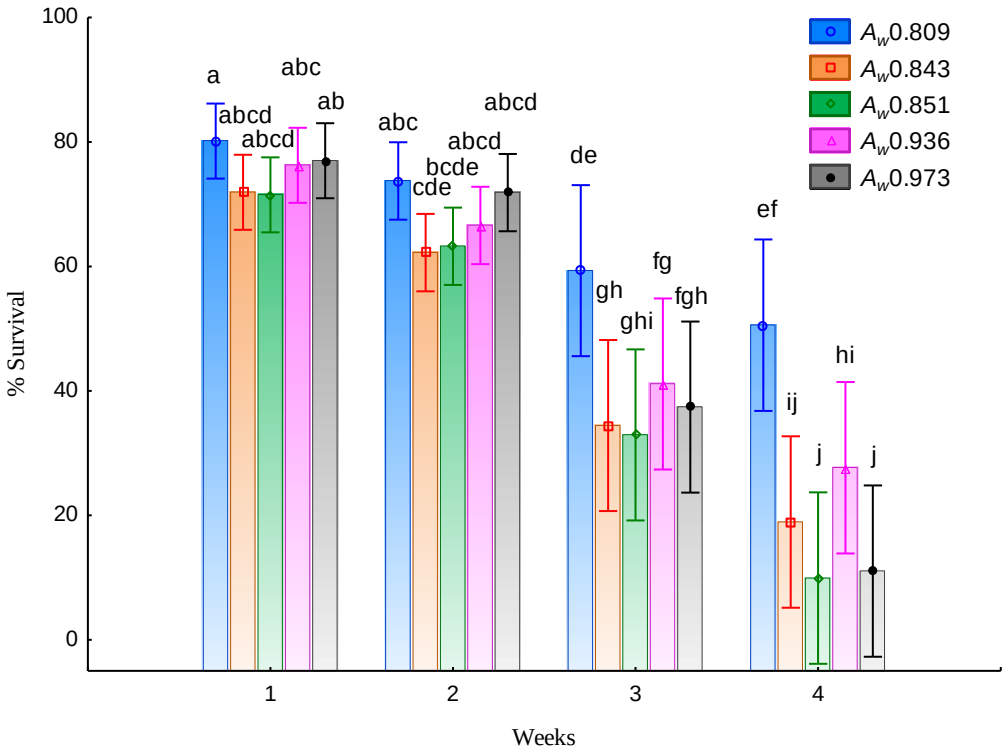


Fig. 5. 4. Mean percentage survival of *Steinernema yirgalemense* IJs in diatomaceous earth at different equilibrated a_w per salt during a period of 1 to 4 weeks (95 % confidence level) and repeated measures two-way ANOVA ($F_{(12, 45)} = 3.0483$, ρ = 0.00329). Mean separated by Fisher's least significant difference (LSD) post hoc test; Error: Between; Within; Pooled MS = 99.815, df = 42.816. Different letters above the bars indicate significant differences (p <0.05).

Besides the above, in week 2, the survival of *S. yirgalemense* in *DEa*$_w$, treated at a_w 0.809, did not differ significantly from that treated at a_w 0.843 (p = 0.11), at a_w 0.851 (p = 0.14), at a_w 0.936 (p = 0.32), or at a_w 0.973 (p = 0.79), which did not differ significantly (p >0.05) from each other. The highest mean percentage survival rate was obtained with a_w 0.809 (73.74% ± 2.92%), followed by a_w 0.973 (71.86% ± 2.92%), a_w 0.936 (66.59% ± 2.92%), a_w 0.851 (63.25% ± 2.92%), and a_w 0.843 (62.22% ± 2.92%), respectively. Notwithstanding the observations made in weeks 1 and 2, in weeks 3 and 4 there was a rapid decline in the survival rate of *S. yirgalemense* in *DEa*$_w$ for all water activities, apart from a_w 0.809, whose survival rate remained high. The effect was dire for a_w 0.851 and for a_w 0.973 in week 4.

CALCULATION OF A_W FROM THE MODEL FIT OF GAB

Extrapolated a_w-values from inference from the GAB model are depicted in Table 5.1.

Table 5. 1. Water content and a_w from the model fit of GAB to the desorption isotherms of a silty clay loam soil.

Water activity a_w	Water content /gg^{-1}	
	Desorption	Adsorption
0.81	0.061	0.055
0.84	0.064	0.059
0.85	0.065	0.060
0.94	0.074	0.069
0.97	0.077	0.072

SEM OF FORMULATED IJS (DE_{SEM})

Few observable differences could be seen between the different test dates and the nematodes in terms of desiccation. Therefore, the results are presented with the unformulated IJs as control, and with the formulated IJs showing desiccative effect (Fig. 5.5, 5.6 and 5.7).

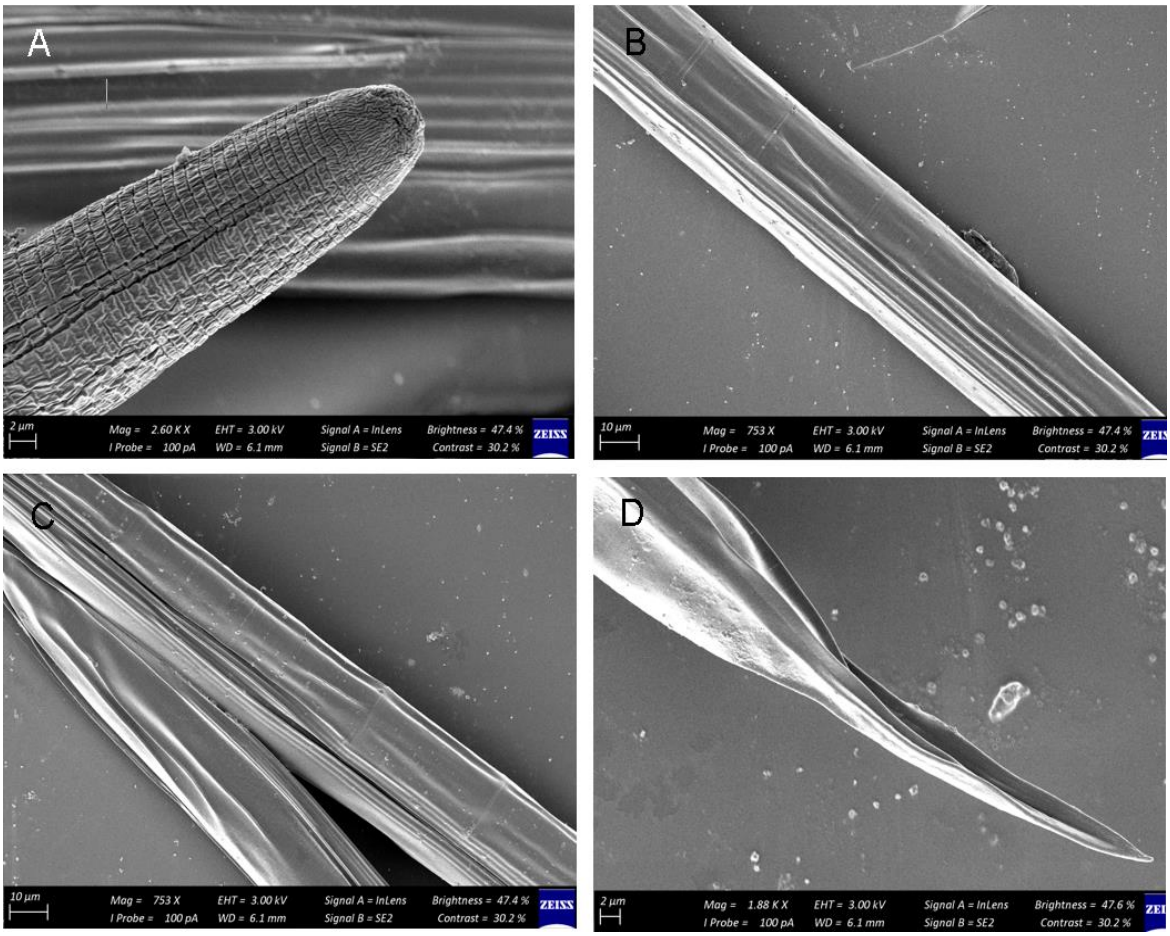


Fig 5. 5.*Steinernema jeffreyense* infective juvenile (IJ) used as the control: (A) anterior of exsheathed IJ; (B) and; (C) shift in orientation of ridges in the mid-body; (D) tail region. The IJ was not freshly harvested prior to the scanning preparation.

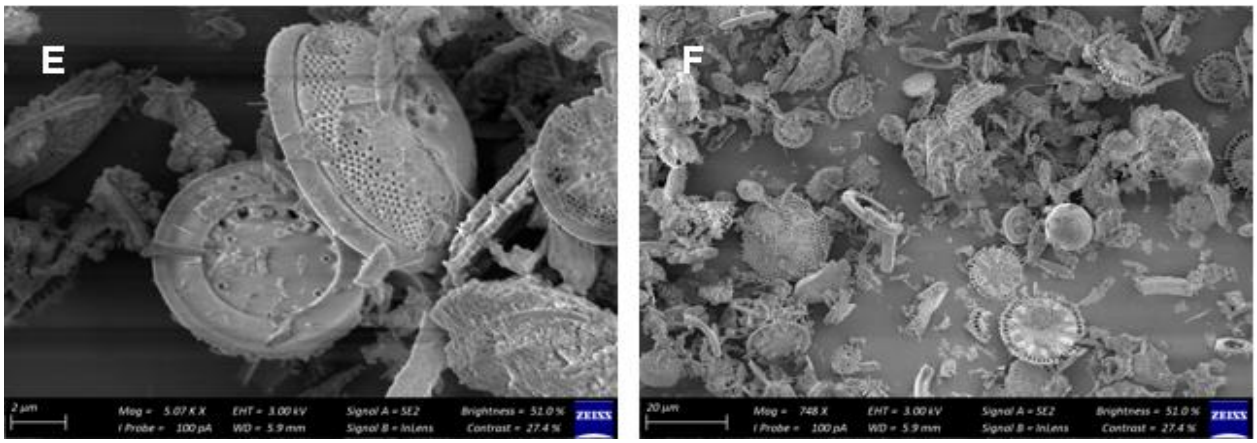


Fig 5. 6. Different magnification of diatomaceous earth: (E) and (F).

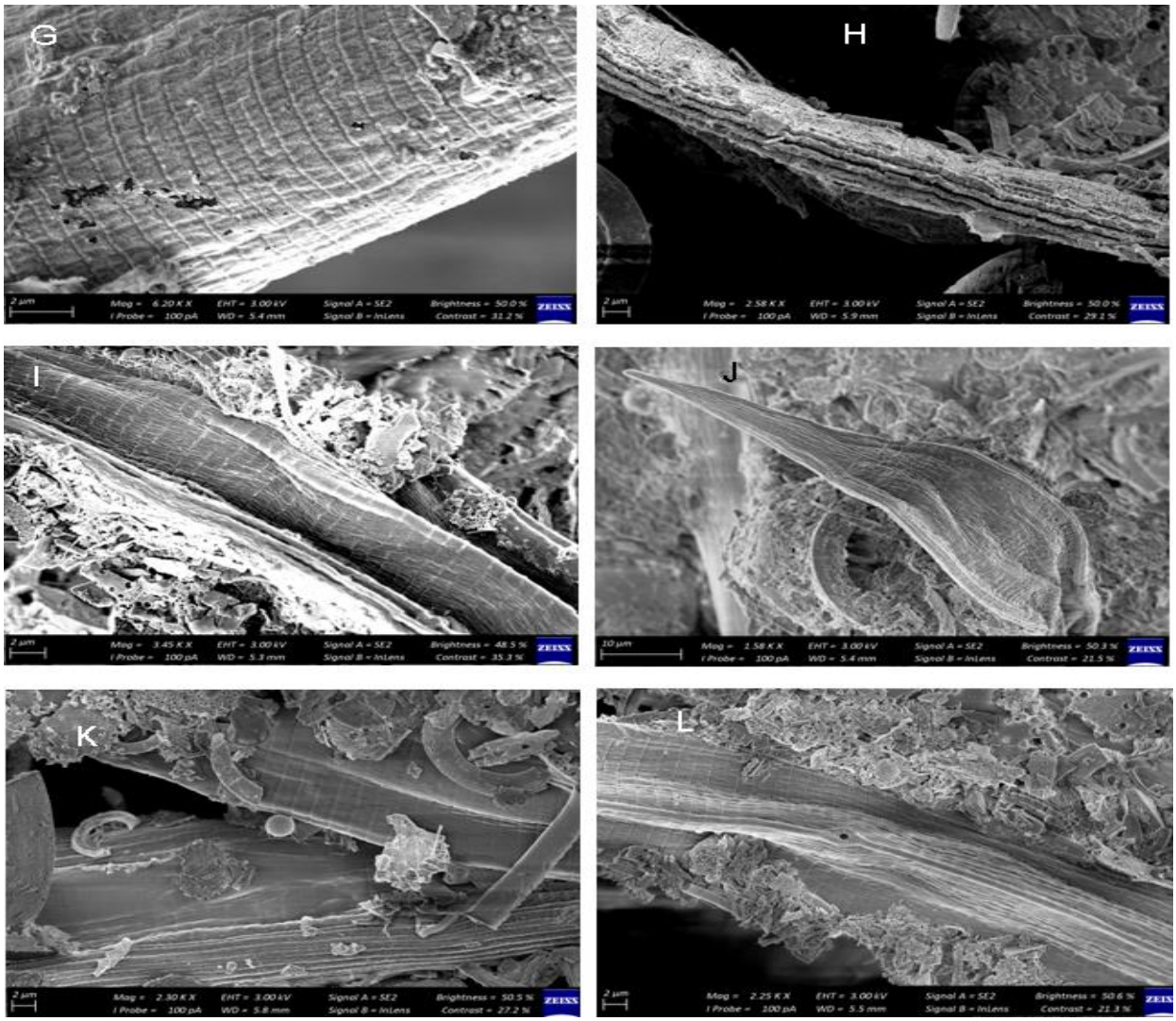


Fig 5. 7. *Steinernema jeffreyense* infective juvenile (IJ): (G) anterior of region of unsheathed IJ with damaged cuticle; (H) mid-body region desiccated, with only lateral lines extant; (I) strongly desiccated mid-body area; (J) only lateral lines remaining in desiccated IJ; (K) anterior region of unsheathed desiccated IJ, showing the excretory pore. The IJs were formulated in diatomaceous earth for a varying number of weeks prior to scanning preparation.

Discussion

The EPNs are poor desiccants and it is expected that a low density of IJs in DE formulation (IJs/g) will lead to low survival rate (Kagimu, personal communication). The study reports on the gradual loss of survival of *S. yirgalemense* in DE during the first 2 weeks of storage. However, the loss was steep between the third and fourth weeks. Still, even though DE is very desiccative, and it is widely used as an insecticide, due to the hygroscopic nature of some of its grades, the loss in survival of the IJs experienced in the present research might not, in its entirety, have been due to the DE. Coincidentally, the survival rate was less than was observed in the fourth week in the study, than what was reported in Chapter 2, where the survival remained high, at 60%, by the fourth week. However, the survival rate was ascribed to the density of 50 000 IJs/g formulated, compared to in the present study, which employed a density of 100 000 IJs/g. Survival ranged between 1% and 10% by the fourth week, at the same temperature as was maintained for the storage. An even greater difference occurred at a density of 200 000 IJs/g, which was reported in Chapter 3, in terms of which an 80% survival rate was attained during the fourth week, at a temperature of 25°C.

The only plausible source of the observed variation in IJ survival is the corresponding a_w-values tested in the current study, which caused a significant and sharp decrease in the survival rate of *S. yirgalemense* in DE. The repeated measures experimental design used in the current study probably had an inherent effect on the physical and chemical reactions in the different treatments during the study, due to the opening and closing of containers during subsequent samplings. Since the stability of dehydrated foods is predisposed to a_w, both chemical reaction rates, as well as microbial activity, are directly controlled thereby (Labuza *et al*., 1985). Formulations of EPNs that are stabilised at a certain a_w, should be sealed in non-moisture-permeable packaging material, so as to avoid absorbing moisture from the atmosphere, which would otherwise be likely to lead to an increase in a_w, thereby decreasing the shelf life of the EPNs so affected. In addition, the formulated EPNs in DE, if they are sealed in impermeable packaging material, should not be subjected to temperature modifications either in storage, or in transportation, as such temperature variance might equally alter the a_w at which they were stabilised, hence leading to a decline in their shelf life. The above is because a_w increases with increasing temperature and pressure. Notwithstanding the above, Labuza *et al*. (1985) had to contend with the decreasing a_w of salts, with increasing temperature being a factor that is entirely different in a food system.

The results from the survival of *S. yirgalemense* in DE at different a_w per salt during the 1 to 4 weeks showed a_w 0.973 and a_w 0.809, respectively, causing a lower and higher than expected mean percentage survival of IJs. The observed trend is that which was hypothesised for the study. Since EPNs tend to respond poorly to desiccation, which is a tendency that is

easily achieved by means of lowering the a_w during stabilisation of the formulation, a higher survival rate at a higher a_w than what was observed was anticipated. The effect was probably due to the sporulation and toxin production by bacteria at the a_w ranges at which such bacteria tend to thrive (Beuchat, 1987, 2002).

The above phenomenon is explained by Labuza & Altunakar (2007), who found that, at somewhat high a_w-levels in the 0.6 to 0.8 range, a small increase in a_w tends to translate into a large gain in moisture content, resulting in the growth of bacteria that cause the spoiling of some foods. Such behaviour was observed at high a_w levels, throughout the current study, as, with high a_w-values, solvents and free water are likely to favour bacterial growth. According to the literature, many empirical equations inaccurately attempt to describe hysteresis behaviour. However, the water activity isotherm should be experimentally determined for each of the ingredients (EPNs in this case) required in DE formulation to estimate the shelf life of foods free of fungal and bacterial growth (Seiler, 1976; Bell & Labuza, 2000; Labuza & Altunakar, 2007; Suntaro *et al*., 2014; Zhang *et al*., 2015). Since 1 g was removed with each subsequent sampling, which could have led to the development of error in estimating water loss in this repeated measure experimental set-up, the researchers chose to infer the water content of the corresponding a_w values to the GAB model fit isotherm of Arthur *et al*. (2018). However, the study insight was gained into the survival trends of *S. yirgalemense* in DE at different a_w-values. As the results from the present study do not contribute to understanding the improved long-term shelf life of the formulation, making short survival gains at the corresponding a_w values would be of little value.

The extrapolated results in Table 5.2 show that the water content at the desorption isotherm are slightly higher than those at the adsorption isotherm at any given a_w. Despite analysis of the above, in practice, care should be taken to find the equilibrium point between the two isotherms, which is often called the working isotherm (Labuza & Altunakar, 2007).

The SEM results revealed the severe damage caused by the desiccative effect of DE on *S. jeffreyense*, and that the effect is random with the sample. The effect is not spread throughout the treatment, but, where it happens, it can be devastating. The damaged IJs have no possibility of survival, if they are damaged beyond repair, the likelihood of which increases with the length of storage.

Incidentally, the study showed that variations in terms of batches, when it comes to desiccation, do not necessarily occur chronologically. Rather, some newer batches have been found to be desiccated, while some of the older ones were not. Such results are comparable with those found in the relevant literature, which reveals that the commercial utilisation of

EPNs in biological control is restricted, due to their comparatively short shelf life, which relates to their low tolerance of desiccation (Strauch *et al.*, 2004).

Desiccation can have a robust effect on EPN IJ longevity (Grewal *et al.*, 2011). As EPNs experience partial anhydrobiosis in a quiescent stage (Womersley, 1990), they tolerate desiccation poorly. Although the physiological mechanisms that are involved in the initiation of anhydrobiosis are not yet fully understood (Grewal *et al.*, 2011), a relationship between the accumulation of polyols and sugars, and their function in the protection of intracellular proteins, in addition to that of biological membranes during dehydration, has been documented in various anhydrobiotic nematodes (Womersley, 1990).

As reviewed by Grewal *et al.* (2011) and Kagimu *et al*. (2017), the ability of anhydrobiotic organisms to tolerate desiccation is largely associated with the accumulation of carbohydrates, including trehalose and water stress-related proteins. Trehalose protects membranes and proteins from desiccation and cold or freezing injury, by means of substituting the structural water that is associated with the phospholipid bilayer, thus upholding membrane fluidity. In addition, it retains the duo layer in the liquid crystalline state, and by means of forming glass (through the process of vitrification) to stabilise the cell content. During desiccation, trehalose equally safeguards proteins by replacing ‘bound water', in addition to decreasing the 'browning' or Maillard reaction (Womersley, 1990; Perry *et al.*, 2012). The results obtained in the current study have clearly shown that IJs tend to desiccate to the point where the lateral lines are prominent at the time of storage increases. Certainly, such IJs are no longer viable, as they will have lost all their energy reserves, like lipids, which are undoubtedly essential to the survival of the non-feeding IJs (Perry *et al.*, 2012; Kagimu *et al*., 2017), and are then subjected to the loss of traits for host-finding or for persistence in the soil once hosts are unavailable. Furthermore, several reports have shown (Patel & Wright, 1997a, b; Patel *et al.*, 1997) that the nematode infectivity levels tend to drop as energy reserves become depleted in storage. The accessibility of energy reserves is indispensable to supporting the physiological and behavioural processes that are associated with adaptation to environmental stress (Glazer, 2002; Kagimu *et al*., 2017).

In conclusion, the present study reports on the gradual loss of survival of *S. yirgalemense* in DE, probably due to changes in their chemical and physical responses at the corresponding a_w-values. The study equally reports on the significant differences observed in the survival of *S. yirgalemense* at the corresponding a_w-values. A repeated measures experimental design does not favour the simultaneous study of both the survival of EPNs and of the corresponding water content of the system at the corresponding a_w-values. For such simultaneous study, we recommend studying each aspect separately, while using the water vapour isotherm models, and then employing the joint results to stabilise the system. The current study has affirmed

the notion that EPNs are poor desiccators, and it has indicated that nematodes do not desiccate at the same rate, meaning that some are likely to survive adverse circumstances prior to application.

With these findings, and with what is already known from the literature, increasing the density of IJs/g in DE formulation is recommended. An increase in the number of IJs has already been shown in chapters 2 and 3, and in other trials not reported here, to stabilise such formulations. The current study further suggests the creating of awareness among farmers, and the adequate labelling of the formulated EPN product. Having an unlimited number of unformulated *in vitro* mass-cultured nematodes available is advisable for this kind of study. Future research should be aimed at investigating DE formulation still further, as positive results have already been achieved in stabilising such.

References

Akhurst, R.J., Mourant, R.G., Baud, L. & Boemare, N.E. (1996). Phenotypic and DNA relatedness between nematode symbionts and clinical strains of the genus *Photorhabdus* (Enterobacteriaceae). *International Journal of Systematic Bacteriology* 46, 1034-1041. DOI: 10.1099/00207713-46-4-1034.

Akin, I.D. & Likos, W.J. (2014). Specific surface area of clay using water vapor and EGME sorption methods. *Geotechnical Testing Journal* 37(6), 1016-1027. DOI: 10.1520/GTJ20140064.

Akin, I.D. & Likos, W.J. (2017). Evaluation of isotherm models for water vapor sorption behavior of expansive clays. *Journal of Performance of Constructed Facilities* 31, D4016001. DOI: 10.1061/(ASCE)CF.1943-5509.0000899.

Al-Muhtaseb, A.H., McMinn, W.A.M. & Magee, T.R.A. (2002). Moisture sorption isotherm characteristics of food products: a review. *Food and Bioproducts Processing* 80,118-128. DOI: 10.1205/09603080252938753.

Anderson, R.B. (1946). Modifications of the Brunauer, Emmett and Teller equation. *Journal of the American Chemical Society* 68, 686-691. DOI: 10.1021/ja01208a049.

Andrade, R.D.P., Roberto, L.M. & Pérez, C.E.C. (2011). Models of sorption isotherms for food: uses and limitations | Modelos de isotermas de sorcion para alimentos: usos y limitaciones. *Vitae* 18, 325-334.

Arthur, E., Tuller, M., Moldrup, P., Resurreccion, A.C., Meding, M.S., Kawamoto, K., Komatsu, T. & De Jonge, L.W. (2013). Soil specific surface area and non-singularity of soil-water retention at low saturations. *Soil Science Society of America Journal* 77, 43-53. DOI: 10.2136/sssaj2012.0262.

Arthur, E., Tuller, M., Moldrup, P., Jensen, D.K. & De Jonge, L.W. (2015). Prediction of clay content from water vapour sorption isotherms considering hysteresis and soil organic matter content. *European Journal of Soil Science* 66, 206-217. DOI: 10.1111/ejss.12191.

Arthur, E., Tuller, M., Moldrup, P. & De Jonge, L.W. (2016). Evaluation of theoretical and empirical water vapor sorption isotherm models for soils. *Water Resources Research* 52, 190-205. DOI: 10.1002/2015WR017681.

Arthur, E., Tuller, M., Moldrup, P., Greve, M.H., Knadel, M. & De Jonge, L.W. (2018). Applicability of the Guggenheim-Anderson-Boer water vapour sorption model for estimation of soil specific surface area. *European Journal of Soil Science* 69, 245-255. DOI: 10.1111/ejss.12524.

Bell, L.N. & Labuza, T.P. (2000). *Practical aspects of moisture sorption isotherm measurement and use*. 2nd ed. Eagan, MN, AACC Eagan Press.

Beuchat, L.R. (1987). Influence of water activity on sporulation, germination, outgrowth, and toxin production. In: Rockland, L.B. & Beuchat, L.R. (Eds). *Water activity theory and applications to food.* New York, Routledge CRC Press, pp.137-151. DOI: 10.1201/9780203734148-7.

Beuchat, L.R. (2002). Water activity and microbial stability. In: *Fundamentals of water activity, IFT Continuing Education Committee, June*, pp. 14-15.

Boemare, N.E., Akhurst, R.J. & Mourant, R.G. (1993). DNA relatedness between *Xenorhabdus* spp. (Enterobacteriaceae), symbiotic bacteria of entomopathogenic nematodes, and a proposal to transfer *Xenorhabdus luminescens* to a new genus, *Photorhabdus* gen. nov. *International Journal of Systematic Bacteriology* 43, 249-255. DOI: 10.1099/00207713-43-2-249.

De Boer, J.H. 1953. *The dynamic character of adsorption*. Oxford, Oxford University Press.

Ehlers, R.-U. (2007). Entomopathogenic nematodes: from science to commercial use. In: Vincent, C., Goettel, M.S. & Lazarovits, G. (Eds). *Biological control: a global perspective: case studies from around the world*. Wallingford, UK, CABI Publishing, pp. 136-151. DOI: 10.1079/9781845932657.0136.

Feng, S.-P., Han, R.-C., Qiu, X.-H., Cao, L. I., Chen, J.-H. & Wang, G.-H. (2006). Storage of osmotically treated entomopathogenic nematode *Steinernema carpocapsae*. *Insect Science* 13, 263-269. DOI: 10.1111/j.1744-7917.2006.00093.x

Ferreira, T., Addison, M.F. & Malan, A.P. (2014). In vitro liquid culture of a South African isolate of *Heterorhabditis zealandica* for the control of insect pests. *African Entomology* 22, 80-92. DOI: 10.4001/003.022.0114.

Ferreira, T., Addison, M.F. & Malan, A.P. (2016). Development and population dynamics of *Steinernema yirgalemense* (Rhabditida: Steinernematidae) and growth characteristics of its associated *Xenorhabdus indica* symbiont in liquid culture. *Journal of Helminthology* 90, 364-371. DOI: 10.1017/S0022149X15000450.

Fontana, A., J. (2000). Water activity's role in food safety and quality. In: *Second NSF International Conference on Food Safety, October 11-13, 2000*. Savannah, GA.

Fontana, A., J. (2002). Measurement of water activity. In: *Fundamentals of water activity, IFT Continuing Education Committee, June*, pp. 14-15.

Fontana, A., J. (2007a). Appendix A: Water activity of saturated salt solutions. In: Barbosa-Cánovas, G.V, Fontana A.J., Schmidt, S.J. & Labuza T.P. (Eds). *Water activity in foods: fundamentals and applications.* Oxford, UK, Blackwell Publishing Ltd, pp. 391-393. DOI: 10.1002/9780470376454.app1.

Fontana, A., J. (2007b). Appendix C: Water activity and isotherm equations. In: Barbosa-Cánovas, G.V., Fontana, A., J., Schmidt S.J. & Labuza T.P. (Eds). *Water activity in foods: fundamentals and applications.* Oxford, UK, Blackwell Publishing Ltd, pp. 399-403. DOI: 10.1002/9780470376454.app3.

Fontana, A., J. (2007c). Appendix D: Minimum water activity limits for growth of microorganisms. In: Barbosa-Cánovas, G.V., Fontana, A.J., Schmidt S.J. & Labuza, T.P. (Eds). *Water activity in foods: fundamentals and applications.* Oxford, UK, Blackwell Publishing Ltd, pp. 405-405. DOI: 10.1002/9780470376454.app4.

Fontana, A., J. & Schmidt, S.J. (2007). Appendix F: Water activity values of select consumer and pharmaceutical products. In: Barbosa-Cánovas, G.V., Fontana, A.J., Schmidt S.J. & Labuza, T.P. (Eds). *Water activity in foods: fundamentals and applications.* Oxford, UK, Blackwell Publishing Ltd, pp. 421-422. DOI: 10.1002/9780470376454.app6.

Glazer, I. (2002). Survival biology. In: Gaugler, R. (Ed.). *Entomopathogenic nematology*. Wallingford, UK, CABI Publishing, pp. 169-187. DOI: 10.1079/9780851995670.0169.

Greenspan, L. (1977). Humidity fixed points of binary saturated aqueous solutions. *Journal of Research of the National Bureau of Standards – A. Physics and Chemistry* 81, https://pdfs.semanticscholar.org/9a90/c8e8fb71c152ae3bacf9904e6c761cdf9de7.pdf [2018, July 21].

Grewal, P.S. (2000a). Enhanced ambient storage stability of an entomopathogenic nematode through anhydrobiosis. *Pest Management Science* 56, 401-406. DOI: 10.1002/(SICI)1526-4998(200005)56:5<401::AID-PS137>3.0.CO;2-4.

Grewal, P.S. (2000b). Anhydrobiotic potential and long-term storage of entomopathogenic nematodes (Rhabditida : Steinernematidae). *International Journal for Parasitology* 30, 995-1000. DOI: 10.1016/S0020-7519(00)00080-1.

Grewal, P.S. (2002). Formulation and application technology. In: Gaugler, R. (Ed.). *Entomopathogenic nematology*. Wallingford, UK, CABI Publishing, pp. 265–287. DOI: 10.1079/9780851995670.0265.

Grewal, P.S. & Jagdale, G.B. (2002). Enhanced trehalose accumulation and desiccation survival of entomopathogenic nematodes through cold preacclimation. *Biocontrol Science and Technology* 12, 533-545. DOI: 10.1080/0958315021000016207.

Grewal, P.S. & Peters, A. (2005). Formulation and quality. In: Grewal, P.S. & Ehlers, R.-U. (Eds). *Nematodes as biocontrol agents.* Wallingford, UK, CABI Publishing, pp. 79-89. DOI: 10.1079/9780851990170.0079.

Grewal, P.S., Bai, X. & Jagdale, G.B. (2011). Longevity and stress tolerance of entomopathogenic nematodes. In: Perry, R.N. & Wharton, D.A. (Eds). *Molecular and physiological basis of nematode survival.* Wallingford, UK, CABI Publishing, pp. 157-181. DOI: 10.1079/9781845936877.0157.

Guggenheim, E. (1966). *Applications of statistical mechanics*. London, Oxford University Press. http://cds.cern.ch/record/231343 [2018, July 10].

Hatting, J.L. and Malan, A.P. (2017). Status of entomopathogenic nematodes in integrated pest management strategies in South Africa. In: Abd-Elgawad, M.M.M., Askary, T.H. & Coupland, J. (Eds). B*iocontrol agents: entomopathogenic and slug parasitic nematodes*, Wallington, UK, CABI Publishing, pp. 409-428. DOI: 10.1079/9781786390004.0409

Hatting, J.L., Moore, S.D. & Malan, A.P. (2018). Microbial control of phytophagous invertebrate pests in South Africa: current status and future prospects. *Journal of Invertebrate Pathology*, In press, Corrected proof. DOI: 10.1016/j.jip.2018.02.004.

Heidemann, D.R. & Jarosz, P.J. (1991). Preformulation studies involving moisture uptake in solid dosage forms. *Pharmaceutical Research* 8, 292-297. DOI: 10.1023/A:1015877011807.

Jensen, D.K., Tuller, M., De Jonge, L.W., Arthur, E. & Moldrup, P. (2015). A new two-stage approach to predicting the soil water characteristic from saturation to oven-dryness. *Journal of Hydrology* 521, 498-507. DOI: 10.1016/J.JHYDROL.2014.12.018.

Kagimu, N., Ferreira, T. & Malan, A.P. (2017). The attributes of survival in the formulation of entomopathogenic nematodes utilised as insect biocontrol agents. *African Entomology* 25, 275-291. DOI: 10.4001/003.025.0275.

Kapsalis, J.G. (1981). Moisture sorption hysteresis. In: Rockland, L.B. & Stewart, G.F. (Eds). *Water activity: influences on food quality*. Cape Town, Academic Press, pp. 143-177. DOI: 10.1016/B978-0-12-591350-8.50011-5.

Kaya, H.K. & Stock, S.P. (1997). Techniques in insect nematology. In: Lacey, L. (Ed.). *Manual of techniques in insect pathology*. San Diego, CA, Academic Press, pp. 281-324. DOI: 10.1016/B978-012432555-5/50016-6.

Khorshidi, M., Lu, N., Akin, I.D. & Likos, W.J. (2017). Intrinsic relationship between specific surface area and soil water retention. *Journal of Geotechnical and Geoenvironmental Engineering* 143, 04016078. DOI: 10.1061/(ASCE)GT.1943-5606.0001572.

Kiranoudis, C.T., Maroulis, Z.B., Tsami, E. & Marinos-Kouris, D. (1993). Equilibrium moisture content and heat of desorption of some vegetables. *Journal of Food Engineering* 20, 55-74. DOI: 10.1016/0260-8774(93)90019-G.

Labuza, T.P. & Altunakar, L. (2007). Water activity prediction and moisture sorption isotherms. In: Barbosa-Cánovas, G.V., Fontana, A.J., Schmidt, S.J. & Labuza, T.P. (Eds). *Water activity in foods: fundamentals and applications.* Oxford, UK, Blackwell Publishing Ltd, pp. 109-154. DOI: 10.1002/9780470376454.ch5.

Labuza, T.P., Kaanane, A. & Chen, J.Y. (1985). Effect of temperature on the moisture sorption isotherms and water activity shift of two dehydrated foods. *Journal of Food Science* 50, 385-392. DOI: 10.1111/j.1365-2621.1985.tb13409.x.

Malan, A., P., Ferreira, T. (2017). Entomopathogenic nematodes. In: Fourie, H. Spaull, V.W. Jones, R.K. Daneel, M.S. & De. Wale, D. (Eds.) Nematology in South Africa: A view from the 21st century. Cham, Switzerland, Springer International, pp. 459-480. DOI: 10.1007/978-3-319-44210-5_22

Malan, A., P., Knoetze R. & Moore, S.D. (2011). Isolation and identification of entomopathogenic nematodes from citrus orchards in South Africa and their biocontrol potential against false codling moth. *Journal of Invertebrate Pathology* 108, 115-125. DOI: 10.1016/j.jip.2011.07.006.

Malan, A.P., Knoetze, R. & Tiedt, T.R. (2016). *Steinernema jeffreyense* n. sp. (Rhabditida: Steinernematidae), a new entomopathogenic nematode from South Africa. *Journal of Helminthology* 90, 262-278. DOI:10.1017/S0022149X15000097.

Mathlouthi, M. (2001). Water content, water activity, water structure and the stability of foodstuffs. *Food Control* 12, 409-417. DOI: 10.1016/S0956-7135(01)00032-9.

Newman, A.C.D. (1983). The specific surface of soils determined by water sorption. *Journal of Soil Science* 34, 23-32. DOI: 10.1111/j.1365-2389.1983.tb00809.x.

Patel, M.N. & Wright, D.J. (1997a). Fatty acid composition of neutral lipid energy reserves in infective juveniles of entomopathogenic nematodes. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology* 118, 341-348. DOI: 10.1016/S0305-0491(97)00057-6.

Patel, M.N. & Wright, D.J. (1997b). Phospholipids and their fatty acids in infective juveniles of entomopathogenic steinernematid nematodes. *Comparative Biochemistry and Physiology B-Biochemistry & Molecular Biology* 118, 649-657. DOI: 10.1016/s0305-0491(97)00270-8.

Patel, M.N., Perry, R.N. & Wright, D.J. (1997). Desiccation survival and water contents of entomopathogenic nematodes, *Steinernema* spp. (Rhabditida: Steinernematidae). *International Journal for Parasitology* 27, 61-70. DOI: 10.1016/S0020-7519(96)00154-3.

Perry, R.N., Ehlers, R.-U. & Glazer, I. (2012). A realistic appraisal of methods to enhance desiccation tolerance of entomopathogenic nematodes. *Journal of Nematology* 44,185-190.

Peters, A. (2004). Protocol quality assessment of entomopathogenic nematodes. In: *COST Action 850 meeting programme and mushroom protocols*. Wädenswil, Switzerland, Agroscope Faw, pp. 4-6.

Puri, B.R.A.I. & Murari, K. (1964). Studies in surface area measurements of soils: 2. Surface area from a single point on the water isotherm. *Soil Science* 97, 341-343. https://journals.lww.com/soilsci/Fulltext/1964/05000/STUDIES_IN_SURFACE_AREA_MEASUREMENTS_OF_SOILS__2_.8.aspx.

Rahman, S. (1995). Water activity and sorption properties of foods. *Food properties handbook* 1-86.

Schmidt, S.J. & Fontana, A.J. (2007). Appendix E: Water activity values of select food ingredients and products. In: Barbosa-Cánovas, G.V., Fontana, A.J., Schmidt, S.J. & Labuza, T.P. (Eds). *Water*

activity in foods: fundamentals and applications. Oxford, UK, Blackwell Publishing Ltd, pp. 407-420. DOI: 10.1002/9780470376454.app5.

Schneider, M. & Goss, K.-U. (2012). Prediction of the water sorption isotherm in air dry soils. *Geoderma* 170, 64-69. DOI: 10.1016/J.GEODERMA.2011.10.008.

Scott, W. (1953). Water relations of *Staphylococcus aureus* at 30°C. *Australian Journal of Biological Sciences* 6, 549-564. DOI: 10.1071/BI9530549.

Seiler, D. (1976). The stability of intermediate moisture foods with respect to mold growth. *Intermediate moisture foods*. London, UK, Applied Science Publishers Ltd. http://agris.fao.org/agris-search/search.do?recordID=US201302481890 [2018, July 20].

Strauch, O., Oestergaard, J., Hollmer, S. & Ehlers, R.U. (2004). Genetic improvement of the desiccation tolerance of the entomopathogenic nematode *Heterorhabditis bacteriophora* through selective breeding. *Biological Control* 31, 218-226. DOI: 10.1016/j.biocontrol.2004.03.009.

Suntaro, K., Tirawanichakul, S. & Tirawanichakul, Y. (2014). Determination of isosteric heat and entropy of sorption of air dried sheet rubber using artificial neural network approach. *Applied Mechanics and Materials* 374-379, 541-542. DOI: 10.4028/www.scientific.net/AMM.541-542.374.

Surrey, M.R. & Wharton, D.A. (1995). Desiccation survival of the infective larvae of the insect parasitic nematode, *Heterorhabditis zealandica* Poinar. *International Journal for Parasitology* 25, 749-752. DOI: 10.1016/0020-7519(94)00190-Y.

Tapia, M.S., Alzamora, S.M. & Chirife, J. (2007). Effects of water activity (aw) on microbial stability: as a hurdle in food preservation. In: Barbosa-Cánovas, G.V., Fontana, A.J., Schmidt S.J. & Labuza, T.P. (Eds). *Water activity in foods: fundamentals and applications.* Oxford, UK, Blackwell Publishing Ltd, pp. 239-271. DOI: 10.1002/9780470376454.ch10.

Troller, J.A. (1987). Adaptation and growth of microorganisms in environments with reduced water activity. In: Rockland, L.B. & Beuchat, L.R. (Eds). *Water activity theory and applications to food*. New York, Routledge CRC Press, pp. 101-117. DOI: 10.1201/9780203734148-5.

Van den Berg, C. & Bruin, S. (1981). Water activity and its estimation in food systems. In: Rockland, L.B. & Stewart, G.F. (Eds). *Water activity: influences on food quality*. New York, Academic Press, pp. 147-177.

Van Zyl, C. & Malan, A.P. (2015). Cost-effective culturing of *Galleria mellonella* and *Tenebrio Molitor* and entomopathogenic nematode production in various hosts. *African Entomology* 23, 361-375. DOI: 10.4001/003.023.0232.

Welti-Chanes, J., Prez, E., Guerrero-Beltrn, J.A., Alzamora, S.M. & Vergara-Balderas, F. (2007). Applications of water activity management in the food industry. In: Barbosa-Cánovas, G.V., Fontana, A.J., Schmidt, S.J. & Labuza, T.P. (Eds). *Water activity in foods: fundamentals and applications.* Oxford, UK, Blackwell Publishing Ltd, pp. 341-357. DOI: 10.1002/9780470376454.ch13.

Wolf, W., Spiess, W.E.L., Jung, G., Weisser, H., Bizot, H. & Duckworth, R.B. (1984). The water-vapour sorption isotherms of microcrystalline cellulose (MCC) and of purified potato starch. Results of a collaborative study. *Journal of Food Engineering* 3, 51-73. DOI: 10.1016/0260-8774(84)90007-4.

Womersley, C.Z. (1990). Dehydration survival and anhydrobiotic potential. In: Gaugler, R. & Kaya, H.K. (Eds). *Entomopathogenic nematodes in biological control*. Boca Raton, FL, CRC Press, pp. 117-137.

Wuddivira, M.N., Robinson, D.A., Lebron, I., Bréchet, L., Atwell, M., De Caires, S., Oatham, M., Scott B. J., Hiruy, A., Verma, A.K. & Tuller, M. (2012). Estimation of soil clay content from hygroscopic water content measurements. *Soil Science Society of America Journal* 76, 1529-1535. DOI: 10.2136/sssaj2012.0034.

Zhang, L., Sun, D.-W. & Zhang, Z. (2015). Methods for measuring water activity (a_w) of foods and its applications to moisture sorption isotherm studies. *Critical Reviews in Food Science and Nutrition* 57, 1052-1058. DOI: 10.1080/10408398.2015.1108282.

Chapter 6

Potential of attenuated total reflectance-Fourier transform infrared spectroscopy and hyperspectral imaging techniques for quality testing of formulated entomopathogenic nematodes

Abstract

Quality control is very important in upholding standards, during the manufacturing of products according to specifications, including the commercialisation of entomopathogenic nematodes (EPNs). For nematode quality, virulence remains the most important standard for measurement, which is often determined through using either one-on-one, or sand-well, bioassays, which are costly in terms of laboratory consumables and time. Such determination calls for the use of quick, non-destructive and effective quality control techniques, which could include the application of attenuated total reflectance (ATR), in conjunction with Fourier transform infrared spectroscopy (FTIR) and hyperspectral imaging (HSI) tools, which have been proven to have a wide application in other fields of research. In this study, the potential for the quality control of formulated *Steinernema jeffreyense* and *S. yirgalemense* in diatomaceous earth (DE), and the characterisation of different species using ATR-FTIR and HSI have been investigated. Results report, for the first time, the use of ATR-FTIR spectral analysis in detecting chemometric changes in the formulated EPN product and changes occurring over time, during storage. The changes are mainly for reasons of nematode survival, in response to environmental stresses. HSI was able to differentiate between variables, in terms of differences in nematode densities, in the formulated sample. For EPN characterisation, the study reports close similarities among the species, as detected by the ATR-FTIR.

Key words: virulence, *Heterorhabditis, Steinernema,* formulation, multivariate analysis, trehalose, short-wave infrared, visible near-infrared.

Introduction

The degree of excellence of a product is its quality, and quality control is a system of upholding standards in manufactured products. The associated system requires the testing of a sample of the product, according to specific specifications. Kagimu *et al*. (2017) opine that small companies with limited resources tend to forego the adoption of proper quality control methods and the routine assessment of quality. Quality assessment calls for training of staff and resources, which requires major commitment by management. Virulence, which remains the most important element of nematode quality, is often determined by means of the use of either one-on-one, or sand-well, bioassays (Grewal, 2002; Grewal & Peters, 2005). The two methods are costly, in terms of both laboratory consumables and time. The need exists to investigate quick, non-destructive and efficient quality control techniques used by other laboratories. The tools employed include the use of attenuated total reflectance (ATR), in conjunction with Fourier transform infrared spectroscopy (FTIR), consisting of ATR-FTIR, Raman spectroscopy and hyperspectral imaging (HSI) tools that have been proven to have wide application. Of much interest to the current study is the use of ATR-FTIR and HSI.

The ATR-FTIR micro spectroscopy is considered as a powerful technique that can be used to determine the chemical structure and composition of various materials, including biological samples (Bouyanfif *et al*., 2018). It has had applications in terms of nematology research, especially in relation to *Caenorhabditis elegans* Maupas, 1900. For broader reference, attention should be drawn to the current review by Bouyanfif *et al*. (2018) on FTIR micro spectroscopy applications, which investigates the biochemical changes occurring in *C. elegans*. The applications include: the detection of diet- and genotype-dependent changes in the chemical composition of wild-type *C. elegans* and mutant strains (Bouyanfif *et al*., 2017); the molecular mechanisms of anhydrobiosis; and the role of trehalose and of phospholipid headgroup composition and trehalose in desiccation tolerance (Abusharkh *et al*., 2014), among others. Furthermore, ATR-FTIR has been used to characterise such entomopathogenic nematodes (EPNs) as *Steinernema glaseri* Wouts, Mráček, Gerdin & Bedding 1982 and *Heterorhabditis indica* Poinar, Karunakar & David 1994, and to assess the differences between the nematodes and the *C. elegans* wild strain (San-Blas *et al*., 2011). They equally utilised the same tool to characterise *Xenorhabdus* and *Photorhabdus* bacteria associated with EPNs. Even more so, Virágh *et al*. (2003) used FTIR to analyse the role of polar (phospholipid) membranes, polyunsaturated fatty acids and sterols in the thermo-adaptation of *Steinernema*. The differences in the intensity of spectral peaks of the two strains of *Steinernema feltiae* (Filipjev, 1934) Wouts, Mráček, Gerdin & Bedding, 1982, being *S. feltiae* VIJE (from Norway) and *S. feltiae* IS6 (from Israel), were relied upon in terms of inference, regardless of the culture temperature of the nematodes. Wharton *et al*. (2008) also used ATR

infrared spectroscopy in confirming surface lipid triglyceride and its proportion of fatty acids in the plant-parasitic nematode, *Ditylenchus dipsaci* (Kühn, 1857) Filipjev, 1936, during complete anhydrobiosis.

Hyperspectral imaging, in contrast, has not previously been applied within the ambit of Nematology. However, HSI is extensively utilised in food science, with regards to the quality assessment of products. Su & Sun (2017), in their recent review of FTIR, Raman, and HSI techniques, expound on the application in determining the quality of powdery foods. HSI is used mainly to detect impurities, by means of scanning, and by way of comparing the output with that of a predetermined model. Zhang *et al*. (2015), in their research into the internal defects of food products, report the wide use of HSI for detecting the insect-induced damage of food products. This include field peas, wheat kernels, soybeans, and jujubes, with the common denominator in the products being reflection-based applications. Equally important, Liu *et al*. (2013) report how near-infrared (NIR) spectroscopy and imaging techniques can provide useful information for the estimation of quality attributes in fish and fish products, due to their rapid speed, non-invasiveness, ease of use, and minimal sample preparation.

In addition, the data obtained from the use of such chemometric methods as FTIR, as well as HSI, can be combined with multivariate data analysis tools like principal component analysis (PCA), as well as hierarchical cluster analysis (HCA), to mention but a few. The above can statistically illustrate biochemical events at the molecular level and identify metabolic fingerprints (Ami *et al*., 2004).

The aim of the current study was to test the applicability of ATR-FTIR and HSI in determining the quality of a formulated EPN product. The emphasis was on the impurities present in diatomaceous earth (DE) formulations, such as microbial growth, as well as on the reduction in either the protein or the lipid content of the infective juveniles (IJ), the only living stage present in such an environment, due to the desiccative effect of the DE. The above was accomplished through the formulation and storage of EPNs in DE, and through the scanning of the product after storage, using either tool. The study also included the characterisation of several local EPN species, using ATI-FTIR.

Materials and methods

Source of nematodes and host insects

The nematodes used in the current study included endemic South African EPNs: *Steinernema yirgalemense* Nguyen, Tesfamariam, Gozel, Gaugler & Adams, 2004; *Steinernema jeffreyense* Malan, Knoetze & Tiedt, 2016; *Heterorhabditis bacteriophora* Poinar, 1976; *Heterorhabditis baujardi* Phan, Subbotin, Nguyen & Moens, 2003; *H. indica*;

Heterorhabditis noenieputensis Malan, Knoetze & Tiedt, 2014; *Heterorhabditis safricana* Nguyen, Malan, De Waal, Tiedt, 2008; and *Heterorhabditis zealandica* Poinar 1990 (Malan *et al.*, 2011, 2016; Malan & Ferreira, 2017). *Galleria mellonella* L. (Lepidoptera: Phyralidae) larvae were cultured, according to the methodology advocated by Van Zyl & Malan (2015), on an artificial diet, at 25°C in a growth chamber. The *Galleria* larvae were inoculated with either each species (except for *S. jeffreyense*), in 9-mm-diameter Petri dishes, lined with moist filter paper. Freshly harvested IJs were cultured *in vivo*, using the last-instar larvae of *Galleria*, kept at 25°C in a growth chamber. Modified White traps (Kaya & Stock, 1997) were used to harvest the emerged EPNs. The harvested IJs were stored in distilled water at 14°C and collected in 5-L Erlenmeyer flasks that were constantly stirred, for approximately three weeks, until the desired concentration of IJs for each batch had been collected. *Steinernema jeffreyense* were cultured *in vitro*, in 50 ml liquid medium, in 250-ml Erlenmeyer flasks, according to Ferreira *et al*. (2014, 2016) and Dunn *et al*. (2018). The control for the FTIR experiment consisted of freshly harvested IJs.

FORMULATING USAGE OF DIATOMACEOUS EARTH

Both *S. jeffreyense* and *S. yirgalemense* were concentrated into a paste, using a 32-µm sieve (Clear Edge Filtration SA (Pty) Ltd, South Africa). The technique described in Chapters 2 and 3 was used to formulate 64 million IJs of *S. jeffreyense*, as well as 10 million IJs of *S. yirgalemense* in DE (Celite 209 − Imerys Refractory Minerals SA (Pty) Ltd). A final density of 3 700 000 IJs/g for *S. jeffreyense*, and 300 000 IJs/g for *S. yirgalemense*, was obtained in the formulation. The formulated nematodes were stored at 14°C, and scanned after 2, 4 and 8 weeks, to determine their spectra in both the FTIR-ATR and HSI analyses. The experiment was conducted twice on different test dates, using different batches of nematodes, stored under the same conditions.

EXPERIMENTAL PROCEDURE

FTIR analyses of the samples were performed on a Thermo Nicolet iS10 Spectrometer (Thermo Scientific™ Nicolet™ iS™, Waltham, MA, USA). The Nicolet is 10 FTIR is equipped with a Smart Diamond ATR accessory, and operates in the mid-infrared range of 4 000 to 500 cm^{-1} on both solid and liquid samples. It is easy to operate, and it is designed for precise and fast-paced operation. It has a deuterated-triglycine sulfate (DTGS) detector, KBr beamsplitter, a helium-neon (HeNe) laser, and a diamond HATR crystal of the Thermo Scientific™ Smart iTR™ ATR accessory. The spectra data were collected as absorbance spectra. A background spectrum was collected from the diamond crystal, prior to the recording of the nematode

images. Spectra were recorded at a resolution of 4 cm^{-1}, with 32 scans being recorded for each spectrum. Thermo Scientific OMNIC software (version 8.1) was used for the spectra data collection and processing. A linear baseline was subtracted from the spectral region corresponding to the entire range of the spectra, prior to the fitting of the spectra.

For the NIR HSI system, all the hyperspectral images were acquired with a push broom HySpex VNIR-1800 (visible near-infrared) and SWIR-384 (short-wave infrared) imaging system (Norsk Elektro Optikk, Norway) using Breeze® (Prediktera), software version 2018.17.1. The cameras were mounted on a laboratory rack, with a translation stage, and fitted with a 30-cm focal-length lens. The field of view (FOV) for the VNIR was 9.773 cm, with it being 9.447 cm for the SWIR. The imaging system consisted of an imaging spectrograph, which was coupled to a CMOS (complementary metal-oxide-semiconductor) detector for the VNIR-1800, and to an MCT (mercury cadmium telluride) sensor for the SWIR-384. The spectral range for the VNIR camera was from 400 nm to 1000 nm, and the spectral resolution was 3.26 nm, resulting in 182 bands. The spatial resolution for the VNIR-1800 using the 30-cm lens was 54 µm, and each image consisted of 1800 pixels. The spectral range for the SWIR camera was from 850-2500 nm, with a spectral resolution of 5.45 nm, resulting in 288 spectral bands. The spatial resolution for the SWIR-384, using a 30-cm lens, was pixel size 0.247 mm, with each image consisting of 384 pixels. Samples were illuminated by means of two 150-W halogen lamps (Ushio lighting Inc., Japan), which had the capacity to emit light in the 400-2500 nm wavelength range. The integration time, which is the duration of time during which the sensor stores light energy, was fixed manually, and set to 8000 µs (VNIR), or 3000 µs (SWIR). Images were recorded with a maximum frame rate of 100 frames per second (fps). Radiometric calibration was performed in the Breeze software package. A 50% grey reflectance standard Zenith Allucore diffuse (SphereOptics GmbH, Germany) was used as a white reference. Dark references were recorded, with both references being used to correct for uneven light intensity of different wavelength bands. Each sample, including DE alone, and *S. yirgalemense* and *S. jeffreyense* formulated in DE, was transferred to a glass Petri dish, and imaged four times with both cameras, with the imaging taking place either with, or without, the glass lid.

DATA ANALYSIS

For the FTIR analyses, statistical analyses were conducted using STATISTICA 13.2 software (StatSoft. Inc). During data exploration, the absorbance spectra were observed not to be normally distributed, and the results were subjected to Spearman's rank-order correlations, a non-parametric (rank-order) correlations equivalent of the paired t-test. All the variables differed significantly ($p < 0.05$) from each other. The above was done for the entire

spectrum, 4000-500 cm^{-1} and 1900-500 cm^{-1}. The results were further subjected to factor principal component analysis (PCA), with the varimax rotations being normalised. A varimax solution yields results, which facilitates the identifying of each variable with a single factor. The above-mentioned procedure was carried out on the entire FTIR spectra (regions 4000-500 cm^{-1}) and fingerprint regions (1900-500 cm^{-1}) that were established by means of visual comparison of the spectra, since many visible disparities were present in the fingerprint regions. Factor analysis, as a statistical method, is used to describe the variability among the observed, correlated variables in terms of a potentially relatively low number of unobserved variables, called factors, with it aiming to find independent unobserved latent variables. Unlike with the PCA variable reduction technique, factor analysis assumes the existence of an underlying model. Factor loadings (varimax-normalised, with marked loadings > 0.7) extraction yielded the two-factor principal components. Squared factor loading is the percentage of variance in that indicator variable, in terms of the specific factor. In the factor PCs used, the variance of the squared loadings of a factor (column) on all the tested variables in a factor matrix was differentiated by means of either large or small loadings of the variables in the next factor. Each variable could be identified with a single factor. Scatter plots of the PCAs case score showing which PCA had the largest variance explained between the variables were plotted.

For data on the EPN species, in addition to the factor analysis explained above, hierarchical cluster analysis (HCA) was performed on the entire FTIR spectra (regions 4000-500 cm^{-1}) and fingerprint regions (1900-500 cm^{-1}), using Ward`s method with 1-Pearson r. Ward's minimum variance criterion minimises the total within-cluster variance. Ward’s method says that the distance between two clusters, A and B, is how much the sum of the squares is likely to increase when merged. Ward’s method keeps such growth as small as possible. A distance metric for two variables, X and Y, which is known as Pearson's distance, can be defined from their correlation coefficient. The Pearson distance has been used in terms of cluster analysis (*de Amorim, 2015)*. The combination provides good correlation between different spectroscopic data.

For HSI data calibrated images were exported to Evince® processing software version 2.7.9 (Prediktera AB, Umea, Sweden). The images were automatically calibrated, using the white and dark references and converted to pseudo-absorbance. The HSI data were first normalised by means of mean centring, using standard normal variate (SNV) correction. A three-component PCA model (pixel and object-wise) was developed to allow for exploration of the data and to display the variation between the three sample sets obtained. The images were cleaned to remove the background and edge effects. Spectral preprocessing (SNV) was applied, which served to decrease the influence of physical properties and other artefacts.

Results

FTIR-ATR ANALYSIS OF EPN FORMULATED IN DE

The results show differences in the FTIR regions 4000-2750 cm^{-1} and 1900-500 cm^{-1} (Fig. 6.1). At certain points, the relative absorbances of the variables merge, and then later split. The trend was similar with all the variables, except for DE, which exhibits less absorbance in the FTIR region 4000-2750 cm^{-1} and a high peak (with the peaks of other variables, including DE, also being high) in the FTIR region of 1900-500 cm^{-1}.

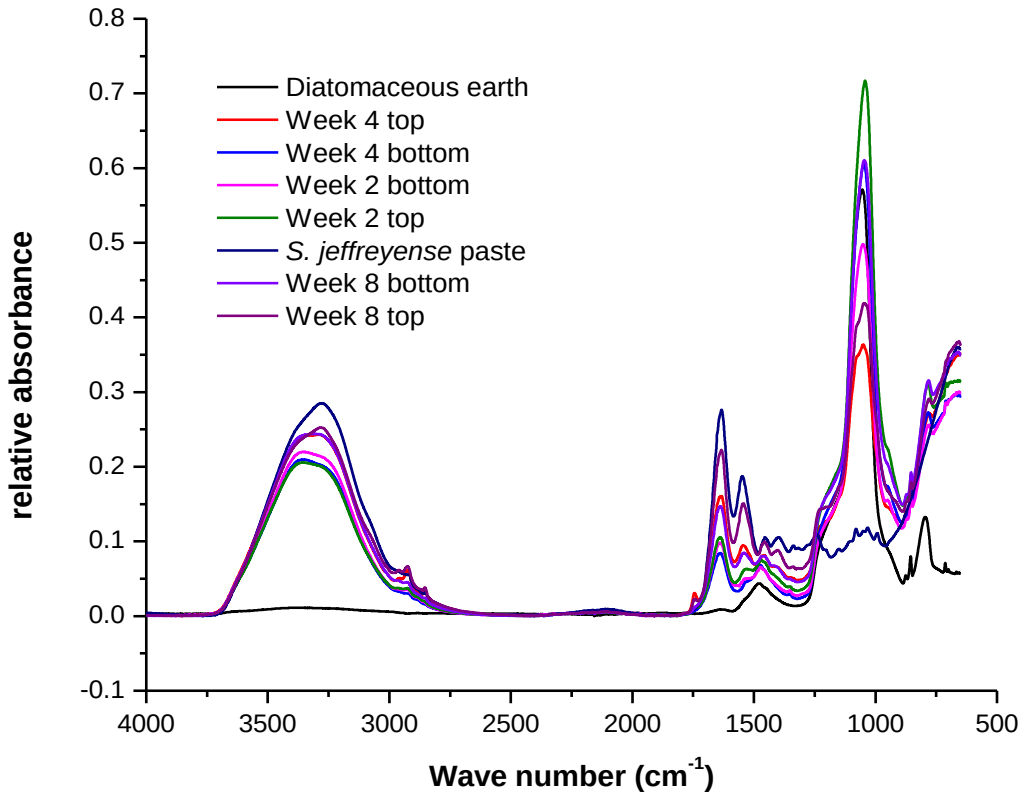


Fig. 6 1. FTIR spectra of *Steinernema jeffreyense* in diatomaceous earth (weeks 2, 4 and 8, top and bottom), S. jeffreyense paste and diatomaceous earth showing the differences in the region 4000-500 cm^{-1}.

Within the FTIR region 1900-500 cm^{-1}, the results show that each variable is different in its peak intensity, despite their parallelism. The highest peaks around FTIR region 1000 cm^{-1}, in their entirety, are due to the presence of DE, given that the peak for *S. jeffreyense* is farther down, and rises as that of the DE falls (Fig. 6.2). Most of the observed variations were found to occur in the fingerprint region. The spectral interpretations for the observed peaks in Fig. 6.1, 6.2, 6.5, and 6.6 are summarised in Table 6.1. Results were only given for the appearance of peaks, and not per variable. However, as seen from Fig. 6.1, 6.2, 6.5, and 6.6 and Table

6.1, new peaks appeared and disappeared between the variables, especially in weeks 2, 4 and 8 across the spectra. The trend is much more evident in region 3500-2200 cm^{-1}.

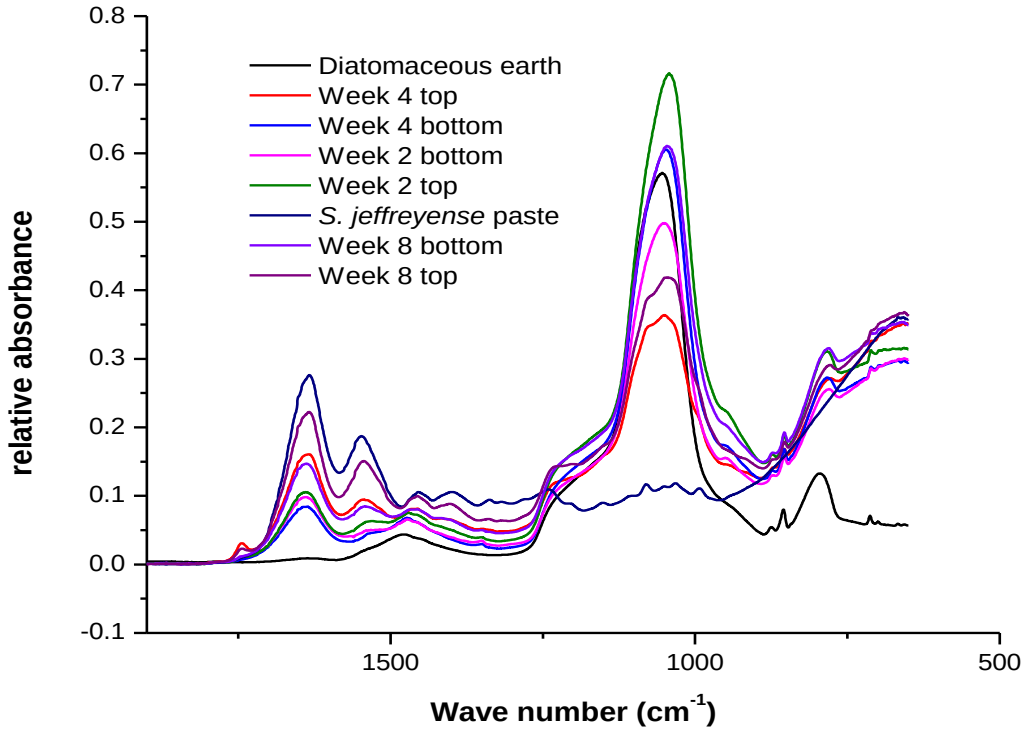


Fig. 6. 2. FTIR spectra of *Steinernema jeffreyense* in diatomaceous earth (weeks 2, 4 and 8, top and bottom), *S. jeffreyense* paste and diatomaceous earth showing the differences in the region 1900-500 cm^{-1}.

Table 6. 1. The spectral interpretations *for Steinernema jeffreyense* in diatomaceous earth over time.

Peak positions of formulated *S. jeffreyense* in DE scanned over time, together with the corresponding biomolecules						References
DE	*S. jeffreyense*	Week 2	Week 4	Week 8	Assignment	
3355					O-H, N-H, C-H, Stretching N-H asymmetric	(Dovbeshko *et al*., 2000, 2002; Movasaghi *et al.*, 2008)
		3348			Stretching N-H asymmetric	(Dovbeshko *et al.*, 2002)
			3295		Amid A (N-H stretching)	(Movasaghi *et al*., 2008)
				3282	C-H stretching	(Movasaghi *et al*., 2008; San-Blas *et al*., 2011, 2012)
	3278				Stretching O-H symmetric	(Movasaghi *et al*., 2008; San-Blas *et al*., 2011, 2012)
					C-H stretching (asymmetric) CH_3	(Holman *et al.*, 2008; Wu *et al*., 2001)
					Asymmetric stretching vibration of CH_3 of acyl chains	(Movasaghi *et al.*, 2008; San-Blas *et al*., 2011, 2012)
				2959		
			2924	2926	C-H stretching Asymmetric CH_2 lipids	(Fung *et al.*, 1996; Wu *et al*., 2001; Movasaghi *et al.,* 2008; San-Blas *et al*., 2012)
			2854	2854	C-H stretching, symmetric (CH_2, lipids, fatty acids)	(Dovbeshko *et al.*, 2002; Shetty *et al*., 2006)
				2166	Stretching N-H. A combination of hindered rotation and O-H bending (water)	(Fabian *et al*., 1995; Dovbeshko *et al.*, 2000; Movasaghi *et al.*, 2008)
				2107	Stretching N-H. A combination of hindered rotation and O-H bending (water)	(Fabian *et al*., 1995; Dovbeshko *et al.*, 2000; Movasaghi *et al.,* 2008)
			2123		Stretching N-H	(Dovbeshko *et al.*, 2000)
	2112				Stretching N-H. A combination of hindered rotation and O-H bending (water)	(Fabian *et al.* 1995; Dovbeshko *et al.*, 2000; Movasaghi *et al*., 2008)
			1744	1744	C=O stretching band mode of the fatty acid ester, lipids, ester group (C=O) vibration of triglycerides	(Yoshida *et al*., 1997; Movasaghi *et al.,* 2008)
		1640		1640	Amide I band of protein and H-O-H deformation of water	(Li *et al.,* 2005; Movasaghi *et al.,* 2008)
	1633		1633	1634	v(C=C), C=C uracyl, C=O	(Dovbeshko *et al*., 2000; Schulz & Baranska, 2007)
	1548				Ring base	(Dovbeshko *et al*., 2000; Movasaghi *et al.,* 2008)
			1544	1544	Amide II bands (arising from C-N stretching and & CHN bending vibrations)	(Huleihel *et al.,* 2002; Wood *et al.,* 1998; Movasaghi *et al.,* 2008)
				1541	Amide II absorption (primarily an N-H bending, coupled to a C-N stretching vibrational mode)	(Wood *et al.,* 1996; Chiriboga *et al.,* 1998; Wood *et al.,* 1998)

Peak positions of formulated *S. jeffreyense* in DE scanned over time, together with the corresponding biomolecules					References
1479				Amide II band in tissue proteins. Amide II mainly stems from the C-N stretching and C-N-H bending vibrations weakly coupled to the C=O stretching mode	(Eckel *et al.,* 2001; Shetty *et al*., 2006; Movasaghi *et al.,* 2008)
		1472		Amide II	(Eckel *et al.,* 2001)
				Asymmetric CH_3 bending modes of the methyl groups of proteins	(Fujioka *et al*., 2004)
	1455			CH_3 bending vibration (lipids and proteins)	(Fabian *et al*., 1995)
			1456	1456	
				1403 Symmetric CH_3 bending modes of the methyl groups of proteins	(Fujioka *et al*., 2004; Movasaghi *et al.,* 2008)
	1398			CH_3 symmetric deformation	(Agarwal *et al.,* 2006)
				Collagen	(Fung *et al*., 1996)
				1339 In-plane C-O stretching vibration, combined with the ring stretch of phenyl	(Schulz & Baranska, 2007; Movasaghi *et al.,* 2008)
	1241			Phosphate stretching bands from phosphodiester groups of cellular nucleic acids vibrating asymmetric phosphate	(Fung *et al*., 1996; Movasaghi *et al.,* 2008)
	1080			Symmetric $PO^{-}{}_2$ stretching phosphate vibration	
				Collagen and phosphodiester groups of nucleic acids	(Movasaghi *et al.,* 2008)
1054		1050	1050	Phosphate and oligosaccharides	
				P-O-C antisymmetric stretching mode of phosphate ester, and C-OH stretching of oligosaccharides	(Yoshida *et al*., 1997; Movasaghi *et al.,* 2008)
				1047 Phosphate and oligosaccharides	(Yoshida *et al*., 1997)
		1043		Phosphate and oligosaccharides	
				Symmetric phosphate group stretching in RNA and DNA	(Movasaghi *et al.,* 2008)
	1032			O-CH_3 stretching of methoxy groups	(Schulz & Baranska, 2007)
	993			C-O ribose, C-C	(Dovbeshko *et al.*, 2000)
855		854	853	853 Out-of-plane bending vibrations	(Schulz & Baranska, 2007; Movasaghi *et al.,* 2008)
796				Guanine	(Dovbeshko *et al.*, 2000)
		784		784 Guanine Out-of-plane bending vibrations	(Schulz & Baranska, 2007; Movasaghi *et al.,* 2008)
		780		779 Guanine Out-of-plane bending vibrations	(Schulz & Baranska, 2007; Movasaghi *et al.,* 2008)
713				711 Guanine Out-of-plane bending vibrations	(Schulz & Baranska, 2007; Movasaghi *et al.,* 2008)

The results show a wide spread of alcohol, proteins, carbohydrates, lipids, and fatty acids, among other molecules, across/along the FTIR spectra. For example, at peak 3355 cm^{-1}, functional groups O-H, N-H, C-H for alcohol, amine II and aromatic groups, respectively, are suspected. In the region 3500-2200 cm^{-1}, there are asymmetric lipids for the phospholipid bilayer, lipids, fatty acids and amine II for the secondary proteins. Peak 1744 cm^{-1} was observed during weeks 4 and 8, with it being assigned to the C=O stretching band mode of the fatty acid ester, lipids, ester group (C=O) vibration of triglycerides. Triglycerides are the main constituents of body fat among animals. The fatty acids in this section correspond to unsaturated fats, due to the observed double bond. More so, peak 1640 cm^{-1} was observed from weeks 2 to 8 of the study. The peak was assigned to the amide I band of protein (primary protein) and to the H-O-H deformation of water. Equally important, peak 1544 cm^{-1} appeared in weeks 4 and 8, and peak 1541 cm^{-1} in week 8. The peaks are assigned amide II absorption (primarily an N-H bending, coupled to a C-N stretching vibrational mode). Peak 1455/6cm^{-1} appeared in weeks 2, 4 and 8. They are assigned to asymmetric CH_3 bending modes of the methyl groups of proteins, and to the CH_3 bending vibration (lipids and proteins), whereas peak 1403 cm^{-1} for the symmetric CH_3 bending modes of the methyl groups of proteins was recorded in week 8. Furthermore, peak 1339 cm^{-1} was recorded in week 8, with it being assigned for the in-plane C-O stretching vibration, combined with the ring stretch of phenyl. Peaks 1241-1080 cm^{-1} were last recorded for *S. jeffreyense*, with their disappearance in weeks 2, 4 and 8. Such peaks are assigned to phosphate-stretching bands from phosphodiester groups of cellular nucleic acids vibrating asymmetric phosphate, as well as symmetric PO^{-}_2 stretching phosphate vibration for collagen and phosphodiester groups of nucleic acids respectively. Peak 1050 cm^{-1} in weeks 4 and 8 and peak 1047cm^{-1} in week 8 are assigned to phosphate and oligosaccharides. This is due to the P-O-C antisymmetric stretching mode of phosphate ester, and to the C-OH stretching of oligosaccharides, with peak 1043 cm^{-1}, in week 2, being assigned to phosphate and oligosaccharides. They form a symmetric phosphate group stretching in RNA and DNA. Peak 1032 cm^{-1} for *S. jeffreyense* is assigned to the O-CH_3 stretching of methoxy groups, which disappeared in weeks 2, 4 and 8. From peak 993 cm^{-1} onwards to 711 cm^{-1} only Guanine and out-of-plane bending vibrations are assigned (Fig. 6.1).

FACTOR ANALYSIS OF THE FTIR SPECTRUM IN ASSESSING THE QUALITY OF FORMULATED IJS

For the FTIR spectra in region 4000-500 cm^{-1}, factor analysis results on Eigenvalues (E) extraction gave rise to four principal components, with PC1, PC2, PC3 and PC4 measuring 87.36%, 11.89%, 0.58% and 0.14%, respectively, of the total variance of 99.95%. In this case, PC1 and PC2 measured most of the variance in all the variables accounted for by these factors. The above was further illustrated in the plot of the Eigenvalues, in which the curve made an elbow at factor PC2. Factor loadings (varimax-normalised, with marked loadings > 0.7) extraction yielded two-factor principal components. The squared factor loading is the percentage of variance in that indicator variable, explained by the factor. In the factor PCs, the variance of the squared loadings of a factor (column) on all the tested variables in a factor matrix was differentiated, by either large or small loadings of the variables in the next factor. The identification of each variable with a single factor was possible, as seen in Fig. 6.3.

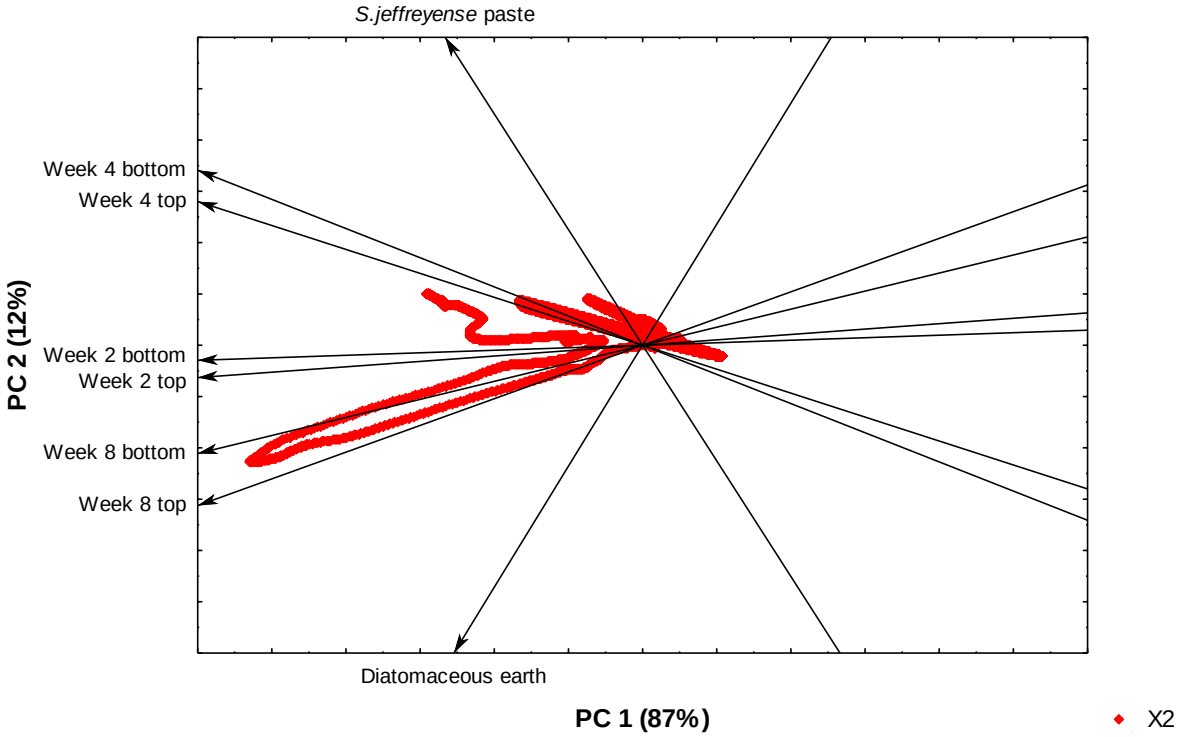


Fig. 6.3. PC1-PC2 scatter plots for the FTIR spectra of *Steinernema jeffreyense* in diatomaceous earth (weeks 2, 4 and 8, top and bottom), *S. jeffreyense* paste and diatomaceous earth in the region from 4000-500 cm^{-1}.

The factor analysis results indicated extreme variance of PCs with PC1 (87%), and PC2 (12%), giving 99% of the total variance of the system, which explained and specified that the PC1 expressively explained the utmost significant component within the system features. Only 1% of the information was lost, which could only be explained by other insignificant factors. The same analyses, as shown above, were carried out for the region 1900-500 cm^{-1} of the spectrum, in terms of which considerable variations in relation to the intensities of relative absorbances were observed among the variables. The variables were similarly significantly ($p < 0.05$) different from each other upon subjection to Spearman's rank-order correlations. Likewise, the factor analysis results yielded four principal components, with PC1, PC2, PC3 and PC4 measuring 85.00%, 14.13%, 0.63%, and 0.18%, respectively, of the total variance of 99.93%. In the same way, PC1 and PC2 measured most of the variance in all the variables accounted for by such factors. The factor loadings extraction yielded two-factor principal components. The factor analysis results indicated extreme variance of PCs with PC1 (85%), and PC2 (14%) giving 99% of the total variance concerned (Fig. 6.4). The factors clearly separated the main ingredients, *S. jeffreyense* paste and DE, on one side, along with other variables in between, as was reported in the preceding discussion of FTIR spectra in the current study.

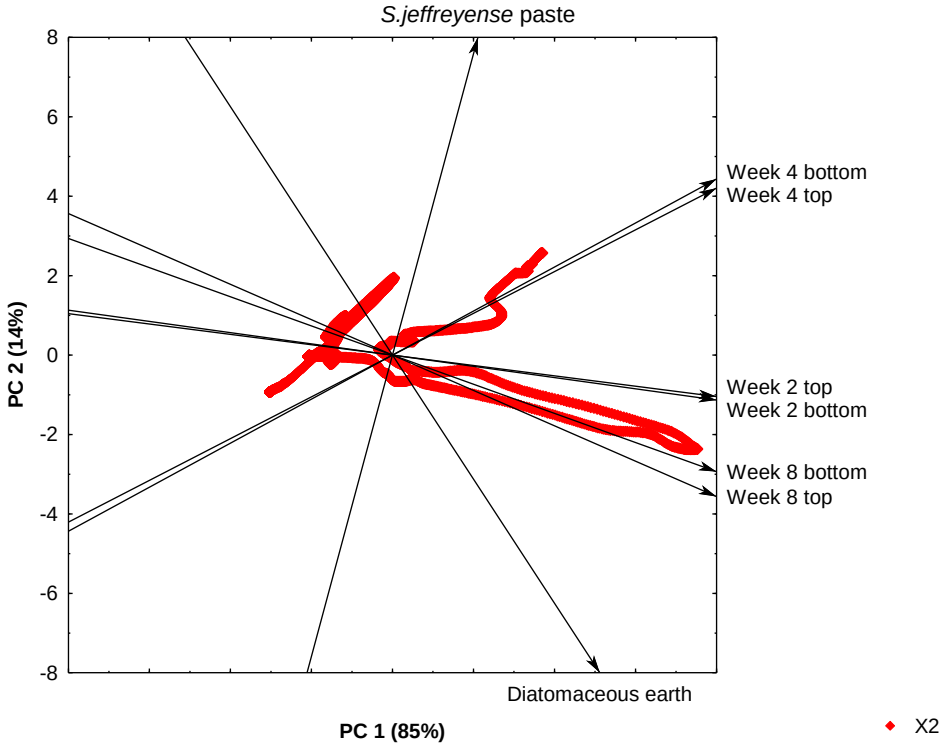


Fig. 6.4. PC1-PC2 scatter plots for the FTIR spectra of *Steinernema jeffreyense* in diatomaceous earth (weeks 2, 4 and 8, top and bottom), *S. jeffreyense* paste and diatomaceous earth in the region from 1900-500 cm^{-1}.

The scatter plots show slight shift in the position in the two plots of week 8, bottom and top, in the two plots of the same data. In addition, week 2 bottom and top variables almost merge. The PCs that explained most of the variance were neither decomposed nor deconstructed to obtain additional functional groups but used other tools to show differences in the variables in terms of their chemometric print. As has already been pointed out, detailed analysis of the results of the FTIR spectra, using formulated *S. jeffreyense* in DE that was scanned after weeks 2 and 4, respectively, was carried out to draw a cogent/logical conclusion.

ADDITIONAL FTIR-ATR ANALYSIS FOR WEEKS 2 AND 4

After separating the results for weeks 2 and 4 into two groups, including the entire spectrum and the region 1900-500 cm^{-1} of the spectrum, they were subjected to Wilcoxon matched-pairs signed-ranks testing in the form of a non-parametric test. The variables were found to be significantly ($p < 0.001$) different from each other for the entire spectrum, although not significantly ($p = 0.17$) different in the region 1900-500 cm^{-1} of the spectrum (Fig. 6.5 and 6.6, and Appendices 1a, 1b, 1c and 1d).

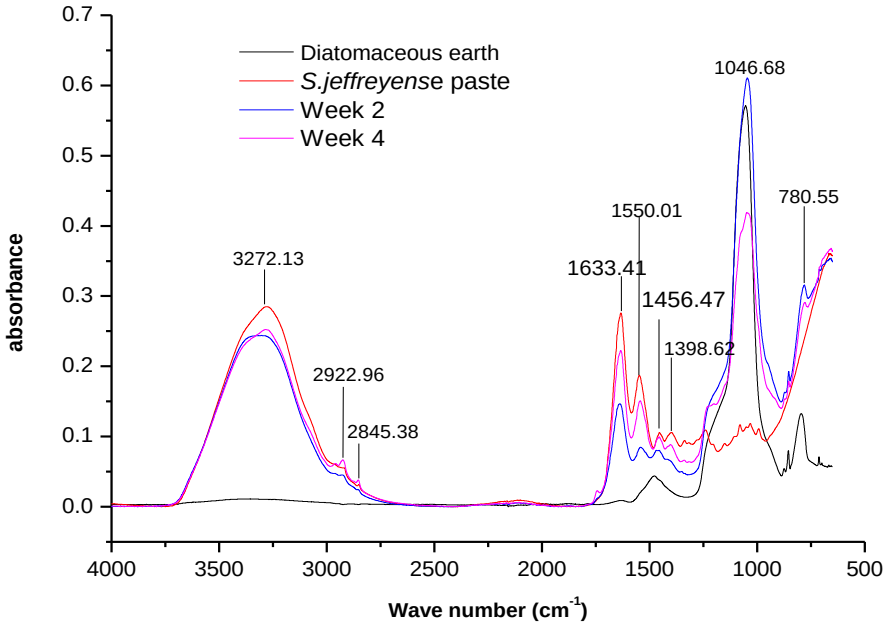


Fig. 6.5. FTIR spectra of *Steinernema jeffreyense* in diatomaceous earth (weeks 2 and 4), *S. jeffreyense* paste and diatomaceous earth showing the differences in the region from 4000-500 cm^{-1}.

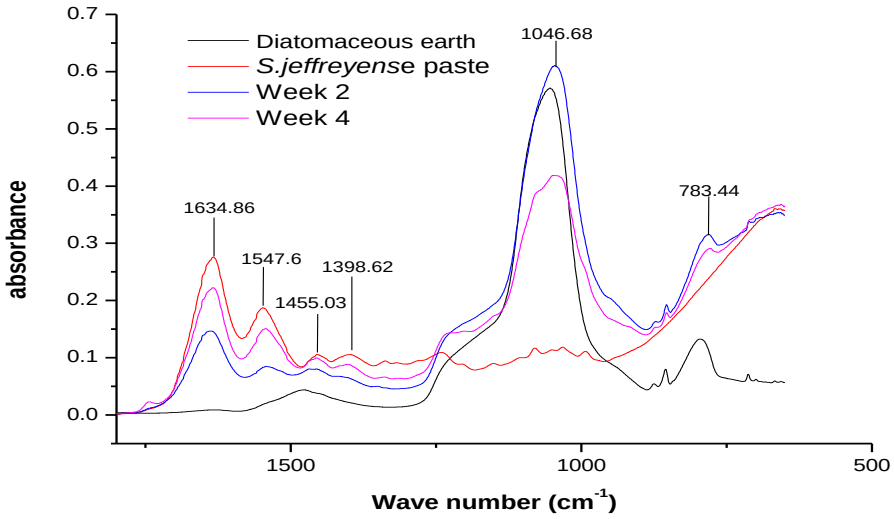


Fig. 6.6. FTIR spectra of *Steinernema jeffreyense* in diatomaceous earth (weeks 2 and 4), *S. jeffreyense* paste and diatomaceous earth showing the differences in the region from 1900-500 cm^{-1}.

EPN CHARACTERISATION USING FTIR-ATR

The results show differences in the FTIR regions 4000-2750 cm^{-1} and 1900-500 cm^{-1}. At certain points, the relative absorbances of the variables merge, and then later split. The trend was similar with all the variables. Mainly two regions of variation in relative absorbance exist in the FTIR regions 4000-2750 cm^{-1} and 1900-500 cm^{-1}. The fingerprint region (1900-500 cm^{-1}) shows that each EPN species is different in peak location and intensity of absorption. However, species are parallel to each other (Figs 6.7 and 6.8).

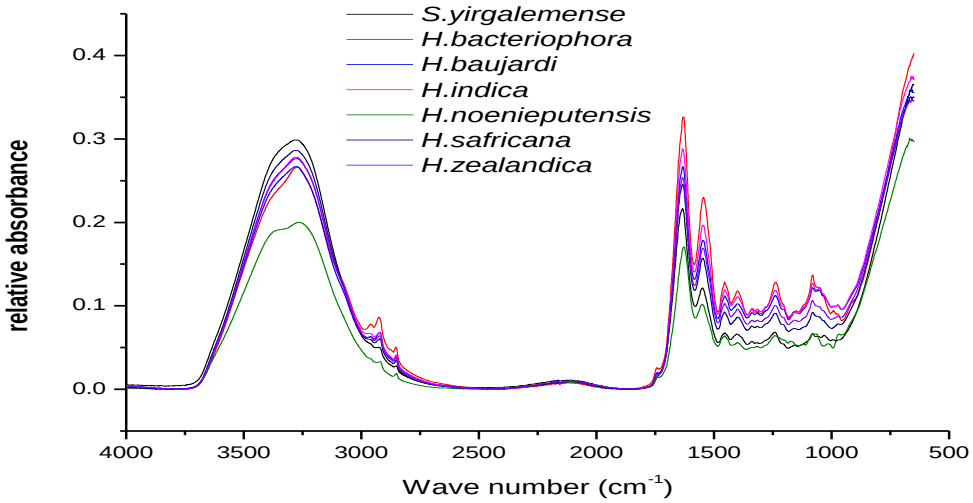


Fig. 6.7. FTIR spectra of *Steinernema yirgalemense, Heterorhabditis bacteriophora, H. baujardi, H. indica, H. noenieputensis, H. safricana,* and *H. zealandica*, showing the differences in the region from 4000-2750 cm^{-1} and 1900-500 cm^{-1}.

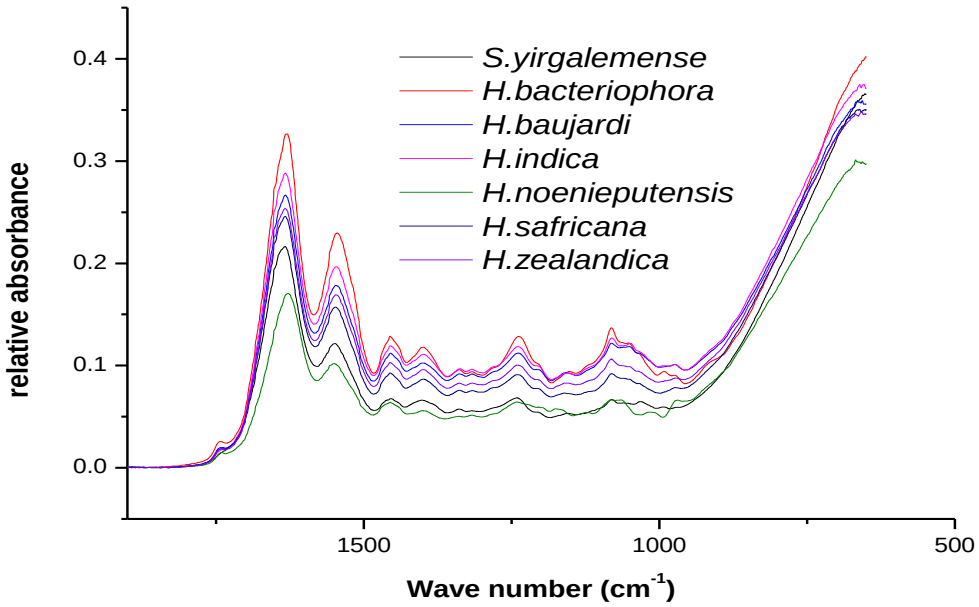


Fig. 6 8. FTIR spectra of *Steinernema yirgalemense, Heterorhabditis bacteriophora, H. baujardi, H. indica, H. noenieputensis, H. safricana,* and *H. zealandica, showing the differences in the region 1900-500 cm^{-1}.*

FACTOR ANALYSIS OF THE FTIR SPECTRA IN ASSESSING EPNS

For the FTIR spectra in region 4000-500 cm^{-1}, factor analysis results, on eigenvalues (E) extraction, gave rise to two principal components, with PC1 (99%), and PC2 (1%) explaining the entire total variance of the system, although PC1 meaningfully explained the utmost significant component within the system features (Fig. 6.9). The region 1900-500 cm^{-1} of the FTIR spectra was no exception from the FTIR spectra in region 4000-500 cm^{-1}, given that the factor analysis results were similar (Fig 6.10). Furthermore, the factors separating the EPNs and the scatter plots show proximity of species, unlike in Figs 6.3 and 6.4, where *S. jeffreyense* was widely separated from the DE. The scatter plots show slight orientation of the species in the two plots. The species originally plotted in an upper position in one plot ended up in a lower position in another plot.

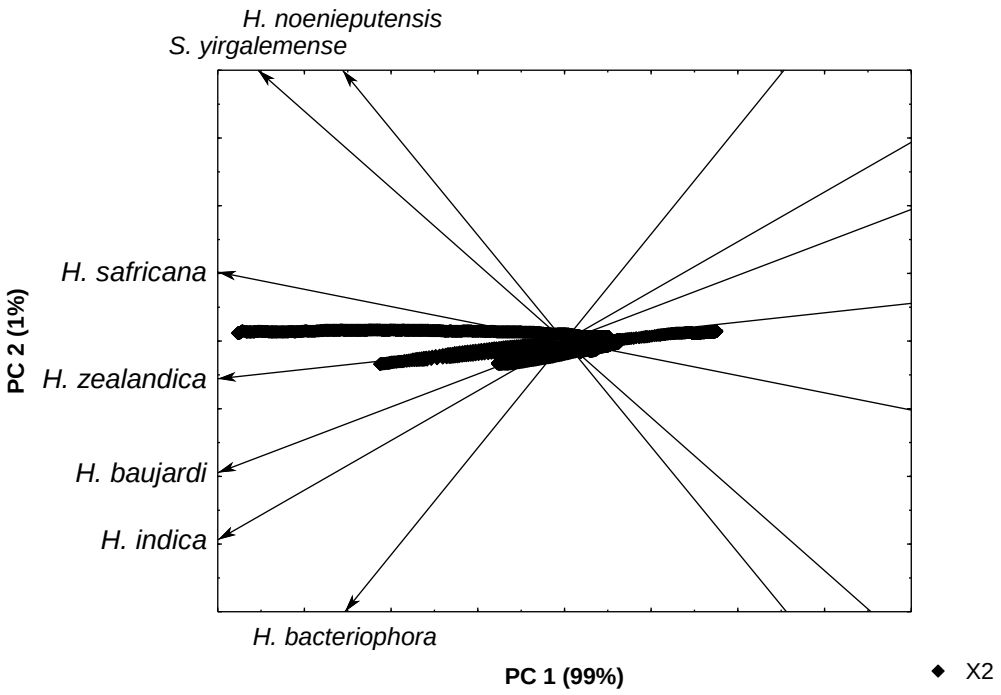


Fig. 6.9. PC1-PC2 scatter plots for the FTIR spectra of *Steinernema yirgalemense, Heterorhabditis bacteriophora, H. baujardi, H. indica, H. noenieputensis, H. safricana,* and *H. zealandica* in the region from 4000-500 cm^{-1}.

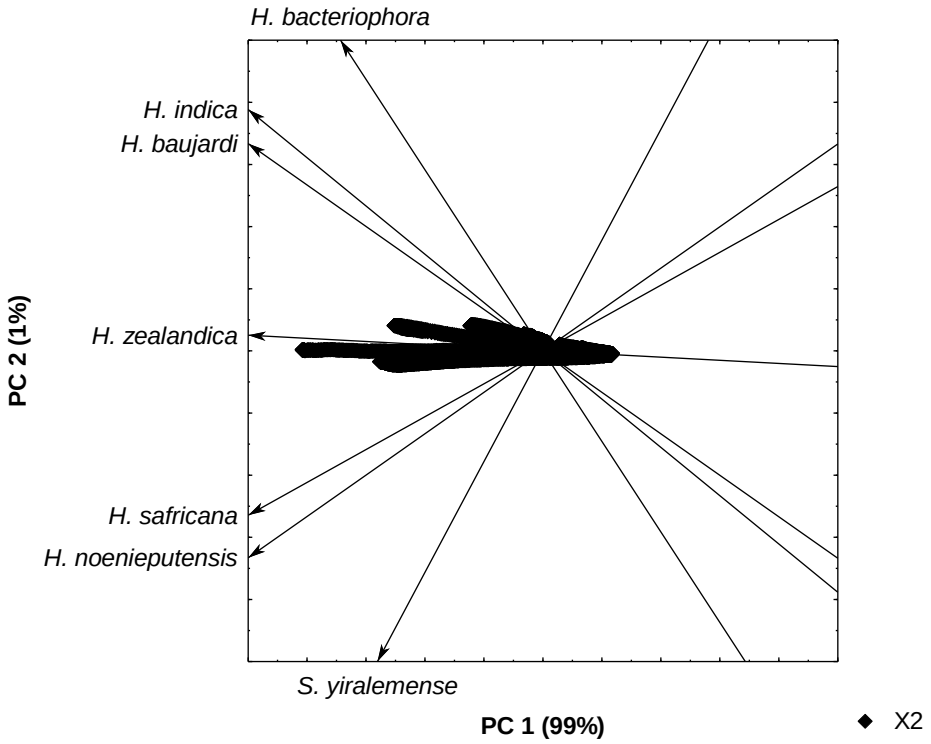


Fig. 6.10. PC1-PssC2 scatter plots for the FTIR spectra *of Steinernema yirgalemense, Heterorhabditis bacteriophora, H. baujardi, H. indica, H. noenieputensis, H. safricana,* and *H. zealandica* in the region from 1900-500 cm^{-1}.

CLUSTER ANALYSIS OF EPN SPECIES

The cluster analyses were drawn from FTIR spectra regions 4000-500 cm^{-1} and 1900-500 cm^{-1}, respectively. The tree clusters depict strong similarity of the different EPN species (Figs 6.11 and 6.12).

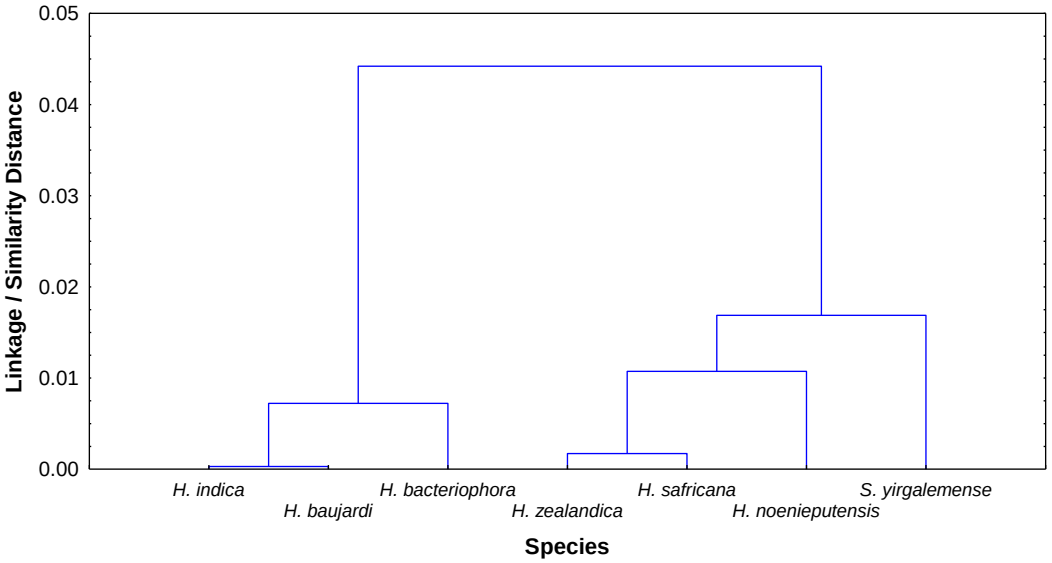


Fig. 6.1. Tree diagram for the FTIR spectra of *Steinernema yirgalemense, Heterorhabditis bacteriophora, H. baujardi, H. indica, H. noenieputensis, H. safricana,* and *H. zealandica* in the region from 4000-500 cm^{-1}, according to Ward`s method and 1-Pearson r.

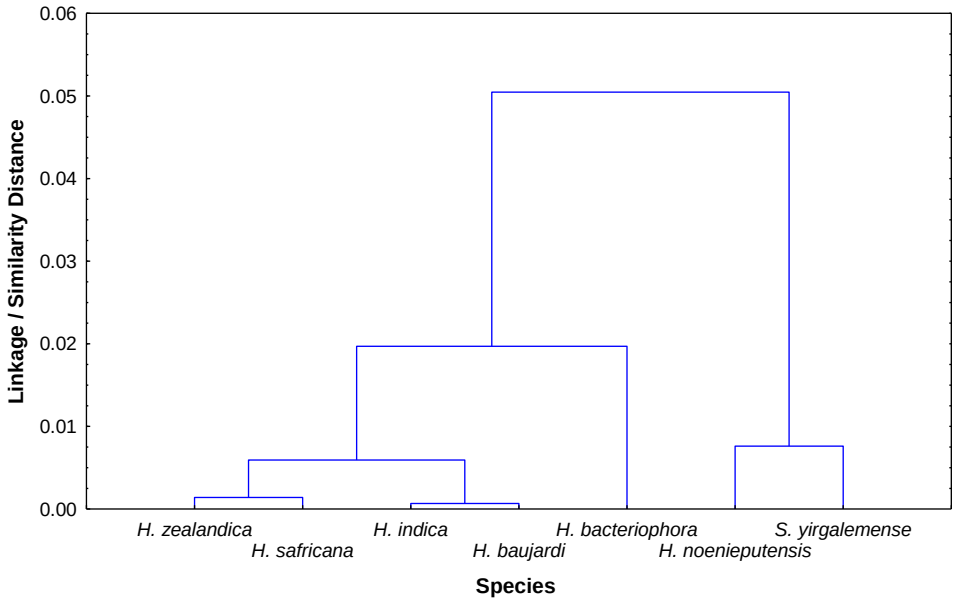


Fig. 6.2. Tree diagram for the FTIR spectra of *Steinernema yirgalemense, Heterorhabditis bacteriophora, H. baujardi, H. indica, H. noenieputensis, H. safricana,* and *H. zealandica* in the region from 1900-500 cm^{-1}, according to Ward`s method and 1-Pearson r.

SWIR – no glass with SNV correction and PCA model pixel-wise analysis

Data analysed with the PCA model pixel-wise analysis showed that PC1 contributed 77.4%, PC2, 12% and PC3, 1.97% explaining a total of 91.37%, with 8.63 of the information lost (Fig. 6.13). The results in Fig. 6.13 obtained by PCA shows two principal components explaining a variance of 91.37% with 8.63% of the information lost. The PCA score plot shows clear variation along the first principal component, which lies between *S. jeffreyense* and DE, whereas *S. yirgalemense* and DE show low variation, being explained only by the second principal component in the PCA score plot (Fig. 6.13). Given that the peaks in the PC loadings were unclear (Fig. 6.14), they were not further analysed.

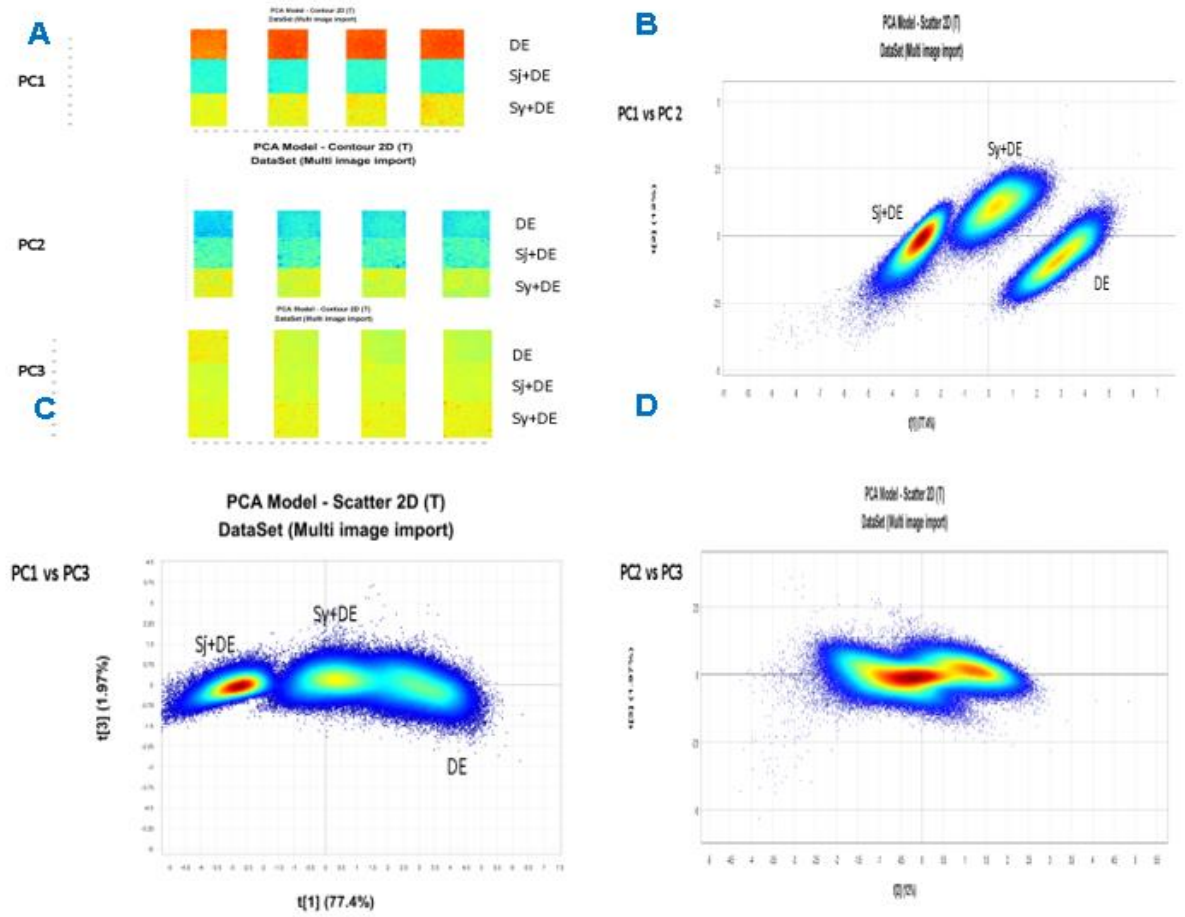


Fig. 6.3. A: Near-infrared (NIR) hyperspectral imaging (HIS) SWIR-384 (short-wave infrared) spectra PCA model – contour 2D (T) data set (multiple image import); **B**: PC1-PC2; **C:** PC1-PC3; and **D**: PC2-PC3 scatter 2D (T) plot values for SWIR-384 spectra (from 780-2500 nm), showing distribution of *Steinernema yirgalemense* and *S. jeffreyense* in diatomaceous earth and diatomaceous earth separately, as control.

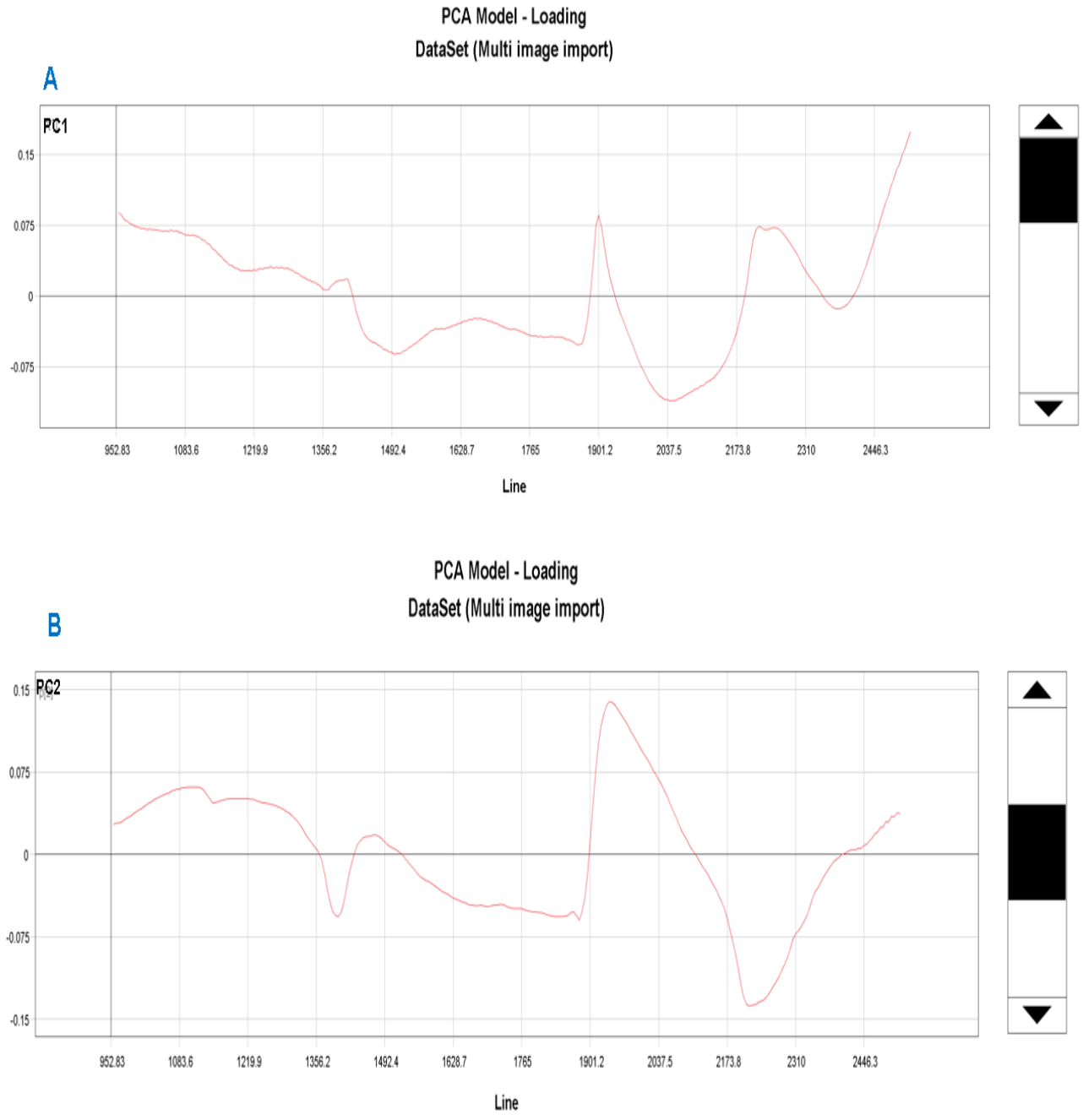


Fig. 6.4. Short-wave infrared (SWIR)-384 spectra hyperspectral imaging (HSI) PCA model –loading data set (multiple image import): **A**. PC1, and **B**. PC2 for *Steinernema yirgalemense* and *S. jeffreyense* in diatomaceous earth and diatomaceous earth separately, as control, with no glass / SNV correction.

SWIR – no glass with SNV correction and PCA model object-wise analysis

Data analysed using the PCA model object-wise, showed that PC1 contributed 89.6% and PC2, 9.9%, explaining a total variance of 99.5%, with only 0.5% of the information having been lost. The PCA score plot, just as in Fig. 6.13, shows a clear variation along the first principal component, which is between *S. jeffreyense* and DE, whereas *S. yirgalemense* and DE show low variation, which is only explainable by the second principal component in the PCA score plot (Fig. 6.15). Similarly, the peaks in the PC loadings were just as unclear (Fig. 6.16), and, hence, were not sufficiently decomposed to enable a logical conclusion to be drawn regarding the corresponding functional groups.

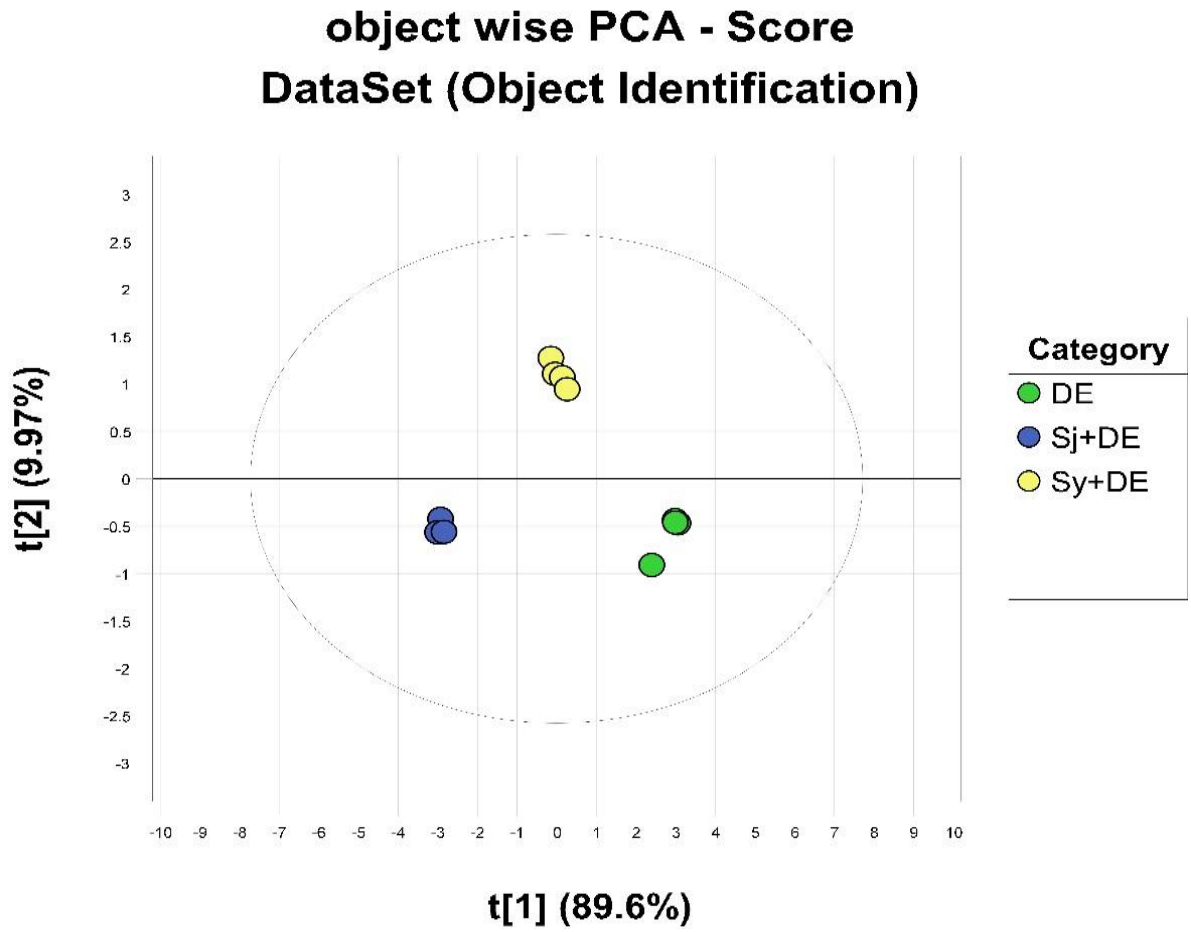


Fig. 6.5. Short-wave infrared (SWIR)-384 spectra hyperspectral imaging (HSI) object-wise PCA – score data set (object identification), PC1-PC2 score values for SWIR-384 spectra (from 780-2500 nm), showing distribution of *Steinernema yirgalemense* and *S. jeffreyense* in diatomaceous earth, and diatomaceous earth separately as control.

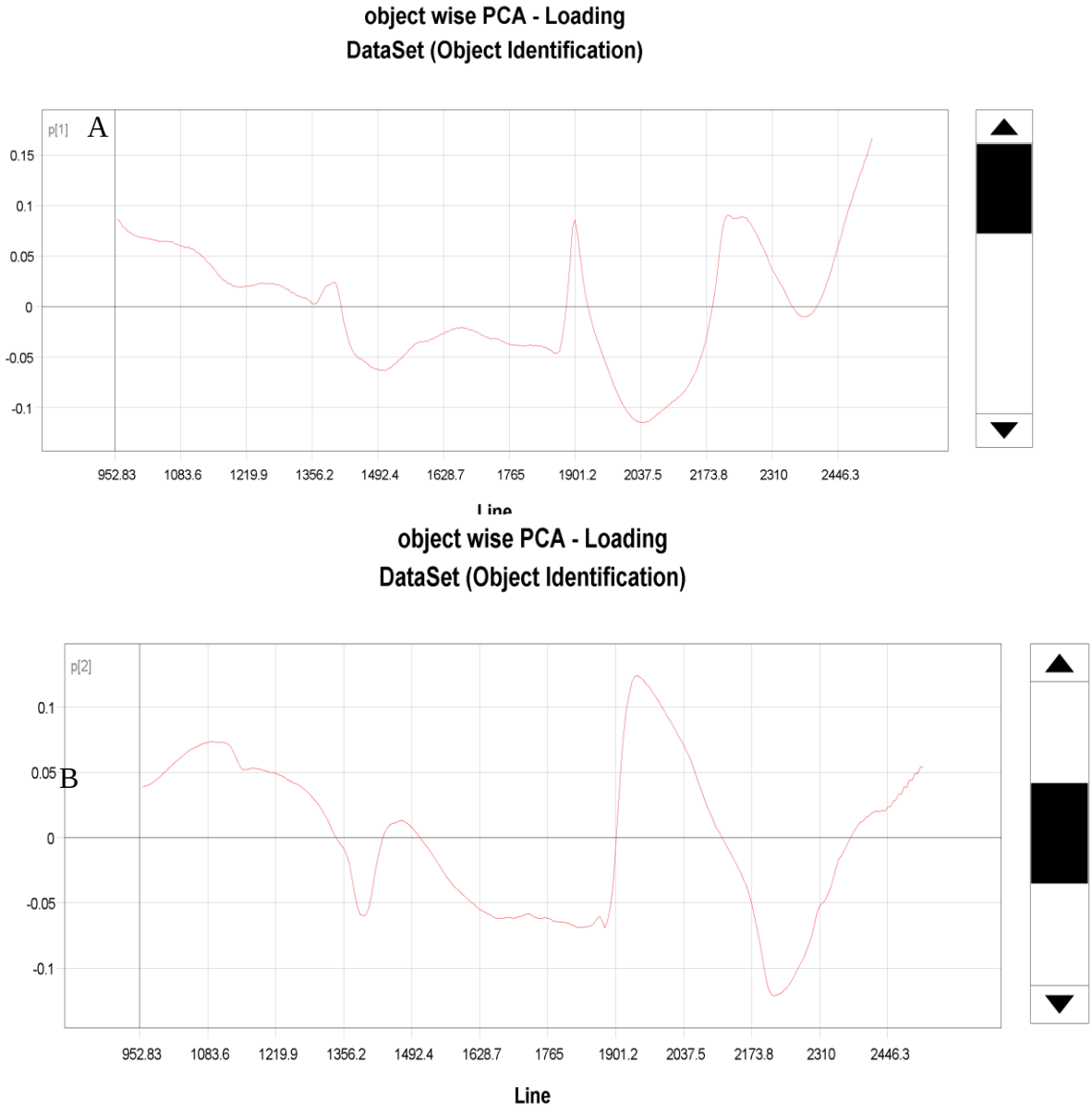


Fig. 6.6. Short-wave infrared (SWIR)-384 spectra hyperspectral imaging (HSI) PCA model –loading data set (multiple image import): **A.** PC1, and **B**. PC2 for *Steinernema yirgalemense* and *S. jeffreyense* in diatomaceous earth and with diatomaceous earth separately as control, with no glass / SNV correction.

The results for SWIR-384 spectra with glass were no different from those that were obtained for the spectra with glass and were not reported. Equally important, the results for VNIR, with or without glass, were obscure and could not be interpreted, leading to them not being reported.

Discussion

The study reports on the ability of ATR-FTIR spectral analysis to detect chemometric changes in the EPN formulated product over time. This is the first time, to the current researchers' knowledge, that FTIR has been utilised in accessing the quality control of EPNs, in terms of the chemical changes occurring in formulated nematodes, opposed to their virulence. In short, the results have coincidentally revealed the appearance and disappearance of peaks in the spectra of formulated nematodes as the time of storage increases. This trend was much more evident in region 3500-2200 cm^{-1}. The trend could probably be attributed to methyl changes in the lipids, confirming biomolecular chemical changes in the formulated nematode. In addition, peak 1744 cm^{-1}, for example, was observed in weeks 4 and 8, which is assigned to the C=O stretching band mode of the fatty acid ester, lipids, and ester group (C=O) vibration of triglycerides (Movasaghi *et al.*, 2008). Triglycerides are the main constituents of body fat in animals. The fatty acids in this section correspond to unsaturated fats, due to the observed double bond. Such bonding clearly shows the breakdown of triglycerides for conversion into trehalose during the glyoxylate cycle (Barrett *et al*., 1970). Trehalose is important in preserving the structures of nematodes under harsh environmental conditions and in the present case, under the desiccative effect of DE (Womersley, 1990; Grewal *et al.*, 2006, 2011; Perry *et al.*, 2012). According to Grewal *et al.* (2011), the ability of anhydrobiotic organisms to tolerate desiccation is largely associated with the accumulation of carbohydrates, including trehalose and water stress-related proteins. Trehalose protects membranes and proteins from desiccation injury, by means of: (1) substituting the structural water that is associated with the phospholipid bilayer; (2) maintaining membrane fluidity; (3) retaining the duo layer in the liquid crystalline state; and (4) forming glass (vitrification) to stabilise the cell content.

In addition, the observed peak at 3355 cm^{-1} corresponds to the functional groups O-H, N-H, C-H for alcohol, amine II and aromatic groups, respectively (Dovbeshko *et al*., 2000, 2002; Movasaghi *et al.*, 2008). In region 3500-2200 cm^{-1}, asymmetric lipids are present for the phospholipid bilayer, as well as are lipids, fatty acids and amine II for the secondary proteins (Movasaghi *et al.*, 2008). Peak 1640 cm^{-1} was observed from weeks 2 to 8 of the study, yet it corresponds to the amide I band of protein (primary protein) and to the H-O-H deformation of water (Li *et al.,* 2005). During desiccation, trehalose equally safeguards proteins by replacing the 'bound water' (on that note, the likely deformation of water), in addition to decreasing the 'browning' or Maillard reaction. The deformation of water molecules alone is evident enough to emphasise the fact that stress in the nematode could have caused the observed change. Most of the observed peaks were in the formulated *S. jeffreyense*, rather than in the *S.*

jeffreyense paste and the DE, further confirming the presence of biomolecular chemical changes, preparing the nematodes for survival during formulation.

Results have further showed that the different tested variables have different intensities of absorption in the FTIR spectra, implying that they differ from each other. Though some of the differences were visible even without analysis, the different analytical tools, like multivariate data analyses, are not limited to the factor PCA, and HCA further confirmed the close similarity among the variables, especially in the case of the EPN species studied. Some peaks are difficult to discern, as attested by San-Blas *et al*. (2012). The observed disparities in the cluster of EPNs, as with *S. yirgalemense*, clustering within heterorhabditid nematodes should not be confused with the molecular phylogenetic trees of these nematodes, where *S. yirgalemense* cluster among steinernematid nematodes, which are based on molecular DNA composition in their constructs, they are viewed in terms of their chemical composition. Equally important, performing a secondary derivative on the data is likely to correct such an erroneous appearance. Previous studies employed secondary derivatives for proper analysis and reported on the exact functional group, or molecule, that was responsible for the observed or summarised relative absorbance peaks. However, the current study followed an alternative trend, by utilising other statistical means, like multivariate analysis, using factor and cluster analyses to draw inferences. The results obtained are like those by Virágh *et al*. (2003), who used FTIR, while analysing the role of polar (phospholipid) membranes, polyunsaturated fatty acids and sterols in the thermo-adaptation of the *Steinernema* species. The differences in the intensity of spectral peaks of the two strains of nematode *S. feltiae*, *S. feltiae* VIJE (from Norway) and *S. feltiae* IS6 (from Israel), were relied upon in inference, regardless of the culture temperature of the nematodes. The concept was equally tested in the present study in terms of the scanning of the FTIR spectra of *S. jeffreyense* in DE, after weeks 2 and 4 of storage in formulation; the subjection of the spectra to the Wilcoxon paired test; and the confirmation of the observed differences in the intensities of the spectra.

The study, again for the first time, has been successful in harnessing the application of HSI in the quality control of EPN products. Although the results of the HSI were not analysed beyond the principal component analysis PC loading examination, the PC score plots revealed the variances between the EPN species and the DE tested. Given the small size of the nematodes, and the height at which the VNIR and SWIR cameras were mounted, obtaining such results creates hope for the advancement of the research along similar lines. As the peaks in the PC loadings SWIR were unclear, they were not decomposed to assign the relevant peaks to the corresponding functional groups.

The limitations regarding the use of HSI in the quality control of EPNs include the height of the microscope. The cameras are high and yet nematodes are microscopic. It is probably due

to this that the results for the VNIR, with or without glass, were obscure and could not be resolved. In addition, both the FTIR and the HSI would not require use with a homogeneous product, as they only detect impurities. This fact has been elaborated on by Su & Sun (2017), in relation to FTIR, Raman and HSI techniques, in the quality determination of powdery foods. The researchers asserted that, in principle, spectral imaging techniques were irrelevant for checking the quality attribute of homogeneous samples, and that the degree of HSI accuracy attained would depend on whether the powdered sample was representative and heterogeneous in respect to bulk. Being adapted to particulate data acquisition, they are not suited to dealing with such homogeneous samples as the formulated EPN product. The above held true in the case of the current study, whose earlier hypothesis involved introducing entomopathogenic fungi into one of the formulations to create a heterogenous sample, but which was restricted in application, as the spores involved would have contaminated the laboratory. Instead, a lower density of *S. yirgalemense* and a higher density of *S. jeffreyense* in the DE provided changes detectable by the HSI SWIR camera. The principal component score plots suggested that *S. yirgalemense* was closer to DE than was to *S. jeffreyense*.

Furthermore, many different sample scans are required to create a model. However, the above would require having an unlimited number of nematodes that could only be supplied by means of *in vitro* culture. Both ATR-FTIR and HSI require modelling to be easily utilised in nematology research. Using focal plane array (FPA) imaging detectors in the FTIR will probably replace use of the HSI in future nematology research, aimed at assuring the quality of formulated EPNs. More so, the HSI also generates such large images that they can even crash computers with small random-access memory and storage space (Su & Sun, 2017). Therefore, the images presented in the current study were of poor quality when presented as screen shots. However, the ability for gaining rapid information about food chemical components, while using these techniques, remains commendable.

In conclusion, the present study reports the first-time application of ATR-FTIR in the quality control of formulated EPNs. The researchers were able to observe the changes occurring in the functional groups of the spectra, as the time of storage increased. The ability to make such observations further compels those who are involved in the commercial application of EPNs to use them within the designated amount of time, so as to avoid deterioration in their quality. The results have further confirmed the notion, that EPNs have a short shelf life, as they could be easily traced in the appearance of new peaks during the second week of storage in formulation. Future research into the use of FTIR should create a standard model for the quality control of formulated EPNs, based on the results obtained in the present study. Modelling would necessitate correlating the spectral data with other variables such as pathogenicity, virulence, survival as already covered in the previous chapters above.

Of the two techniques studied, preference is given to FTIR rather than to HSI, since the former's data require minimal the use of pre-analytical processes, unlike with HSI. More so, the authors recommend substituting HSI with FPA imaging detectors on FTIR in future research, aimed at the quality control of formulated EPNs. Furthermore, HSI will, most likely, only detect impurities like microbial growth in the formulated EPN product, unlike with FTIR, which can detect changes within a single point. The results achieved with the spectral interpretation of formulated *S. jeffreyense* and the desiccative effect of DE are comparable to those that were attained with the scanning electron micrographs, which depicted the immense effect that DE had on IJs within a short space of time. The HSI is, however, still useful in other research fields of biology, however, for application in the quality control of formulated EPN products, further research in this direction is necessitated. A limitation of HSI is that it requires many samples to create a meaningful model as a reference for future quality assurance, unlike in the case of the FTIR, which is easily obtainable with the future *in vitro* culture of nematodes.

References

Abusharkh, S.E., Erkut, C., Oertel, J., Kurzchalia, T.V. & Fahmy, K. (2014). The role of phospholipid headgroup composition and trehalose in the desiccation tolerance of *Caenorhabditis elegans*. *Langmuir* 30, 12897-12906. DOI: 10.1021/la502654j.

Agarwal, R., Tandon, P. & Gupta, V.D. (2006). Phonon dispersion in poly (dimethylsilane). *Journal of Organometallic Chemistry* 691, 2902-2908. DOI: 10.1016/J.JORGANCHEM.2006.02.032.

Ami, D., Natalello, A., Zullini, A. & Doglia, S.M. (2004). Fourier transform infrared microspectroscopy as a new tool for nematode studies. *FEBS Letters* 576, 297-300. DOI: 10.1016/J.FEBSLET.2004.09.022.

Barrett, J., Ward, C., Fairbairn, D. (1970). The glyoxylate cycle and the conversion of triglycerides to carbohydrates in developing eggs of *Ascaris lumbricoides*. *Comparative Biochemistry and Physiology* 35, 577-586. DOI:10.1016/0010-406X(70)90974-6

Bouyanfif, A., Liyanage, S., Hewitt, J.E., Vanapalli, S.A., Moustaid-Moussa, N., Hequet, E. & Abidi, N. (2017). FTIR imaging detects diet and genotype-dependent chemical composition changes in wild type and mutant *C. elegans* strains. *The Analyst* 142, 4727-4736. DOI: 10.1039/C7AN01432E.

Bouyanfif, A., Liyanage, S., Hequet, E., Moustaid-Moussa, N. & Abidi, N. (2018). Review of FTIR microspectroscopy applications to investigate biochemical changes in *C. elegans*. *Vibrational Spectroscopy* 96, 74-82. DOI: 10.1016/J.VIBSPEC.2018.03.001.

Chiriboga, L., Xie, P., Yee, H., Vigorita, V., Zarou, D., Zakim, D. & Diem, M. (1998). Infrared spectroscopy of human tissue. I. Differentiation and maturation of epithelial cells in the human cervix. *Biospectroscopy* 4, 47-53. DOI: 10.1002/(SICI)1520-6343(1998)4:1<47::AID-BSPY5>3.0.CO;2-P.

De Amorim, R.C. (2015). Feature relevance in Ward's hierarchical clustering using the L p norm. *Journal of Classification* 32, 46-62. DOI: 10.1007/s00357-015-9167-1.

Dovbeshko, G.I., Gridina, N.Y., Kruglova, E.B. & Pashchuk, O.P. (2000). FTIR spectroscopy studies of nucleic acid damage. *Talanta* 53, 233-246. DOI: 10.1016/S0039-9140(00)00462-8.

Dovbeshko, G.I., Chegel, V.I., Gridina, N.Y., Repnytska, O.P., Shirshov, Y.M., Tryndiak, V.P., Todor, I.M. & Solyanik, G.I. (2002). Surface enhanced IR absorption of nucleic acids from tumor cells: FTIR reflectance study. *Biopolymers* 67, 470-486. DOI: 10.1002/bip.10165.

Dunn, D., Belhur, P.D. & Malan, A.P. (2018). Production optimization of a novel entomopathogenic nematode, *Steinernema jeffreyense*, in shake flasks. Applied Microbiology and Technology (in prep.).

Eckel, R., Huo, H., Guan, H.-W., Hu, X., Che, X. & Huang, W.-D. (2001). Characteristic infrared spectroscopic patterns in the protein bands of human breast cancer tissue. *Vibrational Spectroscopy* 27, 165-173. DOI: 10.1016/S0924-2031(01)00134-5.

Fabian, H., Jackson, M., Murphy, L., Watson, P.H., Fichtner, I. & Mantsch, H.H. (1995). A comparative infrared spectroscopic study of human breast tumors and breast tumor cell xenografts. *Biospectroscopy* 1, 37-45. DOI: 10.1002/bspy.350010106.

Ferreira, T., Addison, M.F. & Malan, A.P. (2014). *In vitro* liquid culture of a South African isolate of *Heterorhabditis zealandica* for the control of insect pests. *African Entomology* 22, 80-92. DOI: 10.4001/003.022.0114.

Ferreira, T., Van Reenen, C., Tailliez, P., Pagès, S., Malan, A.P. & Dicks, L.M.T. (2016). First report of the symbiotic bacterium *Xenorhabdus indica* associated with the entomopathogenic nematode *Steinernema yirgalemense*. *Journal of Helminthology* 90, 108-112. DOI: 10.1017/S0022149X14000583.

Fujioka, N., Morimoto, Y., Arai, T. & Kikuchi, M. (2004). Discrimination between normal and malignant human gastric tissues by Fourier transform infrared spectroscopy. *Cancer Detection and Prevention* 28, 32-36. DOI: 10.1016/J.CDP.2003.11.004.

Fung, M.F.K., Senterman, M.K., Mikhael, N.Z., Lacelle, S. & Wong, P.T.T. (1996). Pressure-tuning Fourier transform infrared spectroscopic study of carcinogenesis in human endometrium. *Biospectroscopy* 2, 155-165. DOI: 10.1002/(SICI)1520-6343(1996)2:3<155::AID-BSPY2>3.0.CO;2-7.

Grewal, P.S. (2002). Formulation and application technology. In: Gaugler, R. (Ed.). *Entomopathogenic nematology*. Wallingford, UK, CABI Publishing, pp. 265-287. DOI: 10.1079/9780851995670.0265.

Grewal, P.S. & Peters, A. (2005). Formulation and quality. In: Grewal, P.S., Ehlers, R.-U. & Shapiro-Ilan, D.I. (Eds). *Nematodes as biocontrol agents.* Wallingford, UK, CABI Publishing, pp. 79-89. DOI: 10.1079/9780851990170.0079.

Grewal, P., Bai, X. & Jagdale, G. (2011). Longevity and stress tolerance of entomopathogenic nematodes. In: Perry, R. & Wharton, D. (Eds). *Molecular and physiological basis of nematodes*. Wallingford, UK, CABI Publishing, pp. 157-181. DOI: 10.1079/9781845936877.0157.

Grewal, P.S., Bornstein-Forst, S., Burnell, A.M., Glazer, I. & Jagdale, G.B. (2006). Physiological, genetic, and molecular mechanisms of chemoreception, thermobiosis, and anhydrobiosis in entomopathogenic nematodes. *Biological Control* 38, 54-65.

Holman, H.-Y.N., Bjornstad, K.A., Martin, M.C., Mckinney, W.R., Blakely, E.A. & Blankenberg, F.G. (2008). Mid-infrared reflectivity of experimental atheromas. *Journal of Biomedical Optics* 13, 030503. DOI: 10.1117/1.2937469.

Huleihel, M., Salman, A., Erukhimovitch, V., Ramesh, J., Hammody, Z. & Mordechai, S. (2002). Novel spectral method for the study of viral carcinogenesis in vitro. *Journal of Biochemical and Biophysical Methods* 50, 111-21. DOI: 10.1016/S0165-022X(01)00177-4.

Kagimu, N., Ferreira, T. & Malan, A.P. (2017). The attributes of survival in the formulation of entomopathogenic nematodes utilised as insect biocontrol agents. *African Entomology* 25, 275-291. DOI: 10.4001/003.025.0275.

Kaya, H.K. & Stock, S.P. (1997). Techniques in insect nematology. In: Lacey, L. (Ed.). *Manual of techniques in insect pathology*. San Diego, CA, Academic Press, pp. 281-324. DOI: 10.1016/B978-012432555-5/50016-6.

Li, Q.-B., Sun, X.-J., Xu, Y.-Z., Yang, L.-M., Zhang, Y.-F., Weng, S.-F., Shi, J.-S. & Wu, J.-G. (2005). Diagnosis of gastric inflammation and malignancy in endoscopic biopsies based on Fourier transform infrared spectroscopy. *Clinical Chemistry* 51, 346-350. DOI: 10.1373/clinchem.2004.037986.

Liu, D., Zeng, X.-A. & Sun, D.-W. (2013). NIR spectroscopy and imaging techniques for evaluation of fish quality − A review. *Applied Spectroscopy Reviews* 48, 609-628. DOI: 10.1080/05704928.2013.775579.

Malan, A.P., Knoetze, R. & Tiedt, L. (2014). *Heterorhabditis noenieputensis* n. sp. (Rhabditida: Heterorhabditidae), a new entomopathogenic nematode from South Africa. *Journal of Helminthology* 88, 139-151. DOI: 10.1017/S0022149X12000806.

Malan, A.P., Knoetze, R. & Tiedt, L.R. (2016). *Steinernema jeffreyense* n. sp. (Rhabditida: Steinernematidae), a new entomopathogenic nematode from South Africa. *Journal of Helminthology* 90, 262-278. DOI: 10.1017/S0022149X15000097.

Malan, A.P., Knoetze, R. & Moore, S.D. (2011). Isolation and identification of entomopathogenic nematodes from citrus orchards in South Africa and their biocontrol potential against false codling moth. *Journal of Invertebrate Pathology* 108, 115-125. DOI: 10.1016/J.JIP.2011.07.006.

Malan, A.P. & Ferreira, T. (2017). Entomopathogenic nematodes. In: Fourie, H., Spaull, V.W., Jones, R.K., Daneel, M.S. & De Wale, D. (Eds). *Nematology in South Africa: A view from the 21st century*.

Cham, Switzerland, Springer International, pp. 459-480. DOI: 10.1007/978-3-319-44210-5_22.

Movasaghi, Z., Rehman, S. & ur Rehman, D.I. (2008). Fourier transform infrared (FTIR) spectroscopy of biological tissues. *Applied Spectroscopy Reviews* 43, 134-179. DOI: 10.1080/05704920701829043.

Nguyen, K., Malan, A., De Waal, J. & Tiedt, L. (2008). *Heterorhabditis safricana* n. sp. (Rhabditida: Heterorhabditidae), a new entomopathogenic nematode from South Africa. *Nematology* 10, 381-396. DOI: 10.1163/156854108783900258.

Perry, R.N., Ehlers, R.-U. & Glazer, I. (2012). A realistic appraisal of methods to enhance desiccation tolerance of entomopathogenic nematodes. *Journal of Nematology* 44, 185-190. PMID: 23482912.

San-Blas, E., Guerra, M., Portillo, E., Esteves, I., Cubillán, N. & Alvarado, Y. (2011). ATR/FTIR characterization of *Steinernema glaseri* and *Heterorhabditis indica*. *Vibrational Spectroscopy* 57, 220-228. DOI: 10.1016/j.vibspec.2011.07.008.

San-Blas, E., Cubillán, N., Guerra, M., Portillo, E. & Esteves, I. (2012). Characterization of *Xenorhabdus* and *Photorhabdus* bacteria by Fourier transform mid-infrared spectroscopy with attenuated total reflection (FT-IR/ATR). *Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy* 93, 58-62. DOI: 10.1016/J.SAA.2012.03.006.

Schulz, H. & Baranska, M. (2007). Identification and quantification of valuable plant substances by IR and Raman spectroscopy. *Vibrational Spectroscopy* 43, 13-25. DOI: 10.1016/J.VIBSPEC.2006.06.001.

Shetty, G., Kendall, C., Shepherd, N., Stone, N. & Barr, H. (2006). Raman spectroscopy: Elucidation of biochemical changes in carcinogenesis of oesophagus. *British Journal of Cancer* 94, 1460-1464. DOI: 10.1038/sj.bjc.6603102.

Su, W.-H. & Sun, D.-W. (2017). Fourier transform infrared and Raman and hyperspectral imaging techniques for quality determinations of powdery foods: A review. *Comprehensive Reviews in Food Science and Food Safety* 17, 104-122. DOI: 10.1111/1541-4337.12314.

Van Zyl, C. & Malan, A.P. (2015). Cost-effective culturing of *Galleria mellonella* and *Tenebrio molitor* and entomopathogenic nematode production in various hosts. *African Entomology* 23, 361-375. DOI: 10.4001/003.023.0232.

Virágh, E., Fodor, E., Chitwood, D., Farkas, T. & Fodor, A. (2003). An analysis of the role of polar (phospholipid) membranes, polyunsaturated fatty acids and sterols in the thermo adaptation of entomopathogenic nematode. *Citeseer*. [Online] Available: http://citeseerx.ist.psu.edu/viewdoc/download?doi=10.1.1.540.9529&rep=rep1&type=pdf [2018, August 08].

Wharton, D.A., Petrone, L., Duncan, A. & McQuillan, A.J. (2008). A surface lipid may control the permeability slump associated with entry into anhydrobiosis in the plant parasitic nematode Ditylenchus dipsaci. *Journal of Experimental Biology* 211, 2901-2908. DOI: 10.1242/jeb.020743.

Womersley, C.Z. (1990). Dehydration survival and anhydrobiotic potential. In: Gaugler, R. & Kaya, H.K. (Eds). *Entomopathogenic nematodes in biological control.* Boca Raton, FL, USA, CRC Press Inc, pp. 117-137.

Wood, B.R., Quinn, M.A., Burden, F.R. & McNaughton, D. (1996). An investigation into FTIR spectroscopy as a biodiagnostic tool for cervical cancer. *Biospectroscopy* 2,143-153.

Wood, B.R., Quinn, M.A., Tait, B., Ashdown, M., Hislop, T., Romeo, M. & McNaughton, D. (1998). FTIR microspectroscopic study of cell types and potential confounding variables in screening for cervical malignancies. *Biospectroscopy* 4, 75-91.

Wu, J.-G., Xu, Y.-Z., Sun, C.-W., Soloway, R.D., Xu, D.-F., Wu, Q.-G., Sun, K.-H., Weng, S.-F., Xu, G.-X. (2001). Distinguishing malignant from normal oral tissues using FTIR fibre-optic techniques. *Biopolymers* 62, 185-192. DOI: 10.1002/bip.1013.

Yoshida, S., Miyazaki, M., Sakai, K., Takeshita, M., Yuasa, S., Sato, A., Kobayashi, T., Watanabe, S., Okuyama, H. (1997). Fourier transform infrared spectroscopic analysis of rat brain microsomal membranes modified by dietary fatty acids: Possible correlation with altered learning behavior. *Biospectroscopy* 3, 281-290. DOI:10.1002/(SICI)1520-6343(1997)3:4<281::AID-BSPY3>3.0.CO;2-7.

Zhang, X., Nansen, C., Boussaid, F., Yan, G. & Aryamanesh, N. (2015). Importance of spatial and spectral data reduction in the detection of internal defects in food products. *Applied Spectroscopy*, 69(4), 473-480.

Chapter 7

General discussion and conclusion

Worldwide restrictions exist on the use of agrochemical pesticides, with alternatives for biocontrol being advocated and lobbied for by conservationists. Among the biologicals of much interest are the entomopathogenic nematodes (EPNs) of the genera *Steinernema* and *Heterorhabditis*, and their associated symbiotic bacteria *Xenorhabdus* and *Photorhabdus*. In view of the above, 12 novel local species of EPNs have been isolated from South African soil to date, with numerous local studies having been undertaken into nematode efficacy, indicating outstanding success in using certain nematode-insect combinations, such as false codling moth, weevil, codling moth, and fruit fly.

Furthermore, studies are under way for the *in vitro* mass culture of EPNs in large fermenters for commercial application in South Africa. However, the large-scale commercial utilisation of EPNs as biological control agent, in integrated pest management programmes is limited by their finite shelf life, both in storage and formulations. Thus, attaining a suitable formulation is a crucial final step in transferring the nematodes from the laboratory to the field. Not only is the nature of such formulations of key importance to the successful storage and transport of mass-cultured nematodes, but the formulation used should also be able to maintain nematodes of high quality. The development of such a formulation to enable both medium-sized and large enterprises to disperse and apply nematodes successfully against key insect pests in both under-cover and large-scale commercial application was of focus in the current study.

The study investigated a suitable formulation for EPNs, regarding their survival and infectivity under varying conditions of temperature, oxygen consumption, dehydration/desiccation, water activity (a_w), and energy reserves. In other words, the researchers involved aimed at becoming fully cognisant of the survival mechanisms of infective juveniles (IJs) (the survival stage of EPNs), employed in commercially applicable formulations, with the intention of attaining an acceptable shelf life, by means of the objectives elaborated on below.

The study evaluated *Steinernema yirgalemense*, *S. jeffreyense* and *Heterorhabditis bacteriophora* for their suitability in formulations and for their long-term storability at different temperatures. The formulations included the encapsulation of the infective juveniles (IJs) in alginate beads, as well as the use of diatomaceous earth (DE) at 6°C, 14°C and 25°C for 4 weeks. The beads, which successfully retained most of the IJs, can be stored for longer

periods of time, while in the case of DE they showed a high survival rate (80%) by the fourth week. The study of EPN species revealed poor survival and the loss of virulence at the low temperatures of a refrigerator, in both formulations. The present report is the first on the successful formulation of local South African EPNs. The usefulness of alginate beads was found to be limited in terms of scale-up production, thus requiring further research in mass production. If this impediment is alleviated, they will be used in all the sectors and levels of production, such as under cover, and in-home gardens. The use of DE, on the other hand, can easily be upscaled, with the application involving the dissolving of the product in water tanks and sprays, using available farm equipment.

Incidentally, in both formulations, the results showed a decline in the survival and pathogenicity of the EPNs at low temperatures. This is an important finding, due to the phenomenon being of benefit in terms of commercial application in relation to South African producers, as refrigeration costs during the storage and transportation of EPN products can, thus, be eliminated. Such is not the case for the European and American markets, for which EPNs require refrigeration. The EPN products in this case are in concurrence with those produced by the Volcani Institute – Agriculture Research Organization-Negev Israel, where EPN products can even be dispatched using slow mail, without additional cooling. Never the less, the study recommends conducting similar future research investigations at the higher temperatures of 8°C and 10°C to widen the market scope.

In addition, the study recommended optimising the formulating of EPNs in DE, given the associated advantages. The optimisation was the second objective of the study, which aimed at maximising the quality of DE formulations by means of improving their efficiency and productivity for purposes of long-term room temperature storability. The optimisation process involved considering the viability of *S. yirgalemense* at room temperature, and at higher density in DE after 4 weeks. The direct effect of antifungal agents on the efficacy of *S. yirgalemense* was equally investigated. This is because microbial contamination often occurs in EPNs, with such contamination tending to lower the quality and shelf life in formulations. Therefore, peroxyacetic acid (PAA), trans-cinnamic acid (TCA) and nipagin were investigated as antifungal agents in the study. A decline in the survival rate and pathogenicity of *S. yirgalemense*, due to the addition of PAA, was reported. Contrastingly, TCA and nipagin had no effect on the survival rate and pathogenicity of *S. yirgalemense.* The shelf life of IJs stored in DE formulation at room temperature improved, when measured against the 80% mean survival rate of *S. yirgalemense* in week 4, at 25°C. The improved room temperature shelf life confirms the observations recorded in relation to the first objective. Again, this is another first report of room temperature long term shelf life of South African EPNs. Equally important, nipagin and TCA have been identified as potential antifungal agents for use during the

commercialisation of EPNs. Since nipagin is cheaper than TCA, it is recommended for future use. More so, the study has confirmed PAA as being a potent nematicide against plant-parasitic nematodes.

More still, the study investigated the oxygen consumption rates of *S. yirgalemense*, *S. jeffreyense* and *H. bacteriophora* as there is limited information, on the respiratory physiology of the nematode/bacterium complex of EPNs during production, storage, and formulation. Furthermore, low oxygen supply jeopardises EPN survival. Therefore, the present study determined, by means of basal measurement, the specific oxygen consumption rate (OCR) of the IJs of the three EPN species, using fibre-optic sensors. The results showed that nematode size inversely influences its OCR, with smaller nematodes having a higher surface-area-to-volume ratio than do larger nematodes, with a higher OCR. *Steinernema jeffreyense* and *S. yirgalemense* did not significantly differ from each other in terms of the results obtained, which is ascribed to their proximity in size, with the former being slightly larger than the latter, but they differed significantly from *H. bacteriophora*. The study reports on the first-known use of fibre-optic sensors in determining the OCR of EPNs, although the technique is already being used in other fields of biology. However, the above only happened in 1 out of 4 batches. The study therefore recommends further studies of EPNs, with the same tool, using different EPN species. Also, improving the protocol to get homogeneous results in future research is needed.

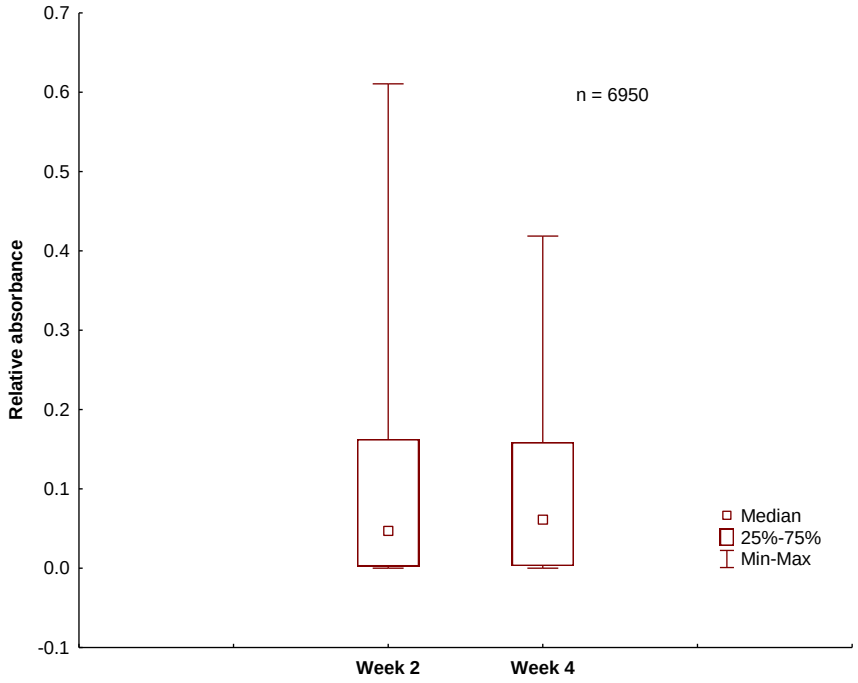
The next objective was aimed at determining the water activity (a_w) of the IJs of *Steinernema yirgalemense* at 25 °C. The determination was achieved by investigating a_w as a deciding factor in terms of microbial contamination. The aspect of desiccation was further examined in relation to the quality and shelf life of EPNs in formulation. In the current study, the concept of determining the moisture content at the corresponding a_w-values, using the Guggenheim-Anderson-Boer (GAB) isotherm model, has been studied, with regards to DE, as well as in terms of the survival of *S. yirgalemense*. Scanning electron microscopy was employed to determine the effect of DE on *S. jeffreyense* during storage in formulation. A decline in the survival rate of *S. yirgalemense* at high a_w-values, due to bacterial contamination and toxin production, was reported. Of equal importance is the recommendation that the moisture content at different a_w-values for each of the ingredients used in the formulation should be investigated separately, to enable stabilising of the formulation and improvement of the shelf life. Equally, the challenge of controlling a_w with the subsequent sampling was considered, in line with the difficulty experienced with simultaneously assessing both the moisture/water content and the survival of IJs. Scanning micrographs depicted the strongly desiccative effect of DE on *S. jeffreyense*, which proved to be beyond rejuvenation on the addition of water. Such effects were found to be random, and not widely distributed throughout

the sample. The above-mentioned shortcoming can be mitigated by increasing the density of IJs/g in formulation in future research, reported as being relatively stable in the current study.

The final objective was to assess the effect of formulation on nematode quality over time, as well as to devise alternative methods of quality control. Since virulence, which remains the most important standard for the measurement of nematode quality, is often determined by using either one-on-one or sand-well bioassays, which are costly in terms of laboratory consumables and time, new alternatives have been investigated. The potential for the quality control of formulated *S. jeffreyense* and *S. yirgalemense* in DE, and the characterisation of different species using attenuated total reflectance (ATR), in conjunction with Fourier-transform infrared spectroscopy (FTIR) and hyperspectral imaging (HSI), tools have been investigated. The results reported noted, for the first time, the use of ATR-FTIR spectral analysis in detecting chemometric changes in the formulated EPN product and changes occurring over time, during storage. The changes are connected to nematode survival, due to environmental stresses. The HSI tools were able to differentiate between variables, in terms of differences in nematode densities, in the formulated sample. For EPN characterisation, the study reports close similarities among the different species investigated, as detected by the ATR-FTIR, which is a significant finding. Despite ATR-FTIR being widely used in other nematode-related research, it had not previously been used in the quality control of nematodes, with the current report being the first to record their successful utilisation in this regard. Modelling of the application, and miniaturisation of the set-up, using existing FTIR systems, need to be further investigated. This can be a real turning point in the quality control of EPN products. Use of the hyperspectral tool, in contrast, requires additional research at high nematode densities to attain meaningful conclusions in future studies.

The main challenge experienced during the present study lay in obtaining the required number of nematodes for the setting up of experiments by way of *in vivo* culturing. Since such studies require the use of relatively high numbers of nematodes, *in vitro* culturing is recommended, as such a process would save valuable research time. The objectives of the study have been met, with novel findings that have broadened the scientific understanding of the subject in question. The results of the study have also led to the making availability of an effective formulation for commercial application.

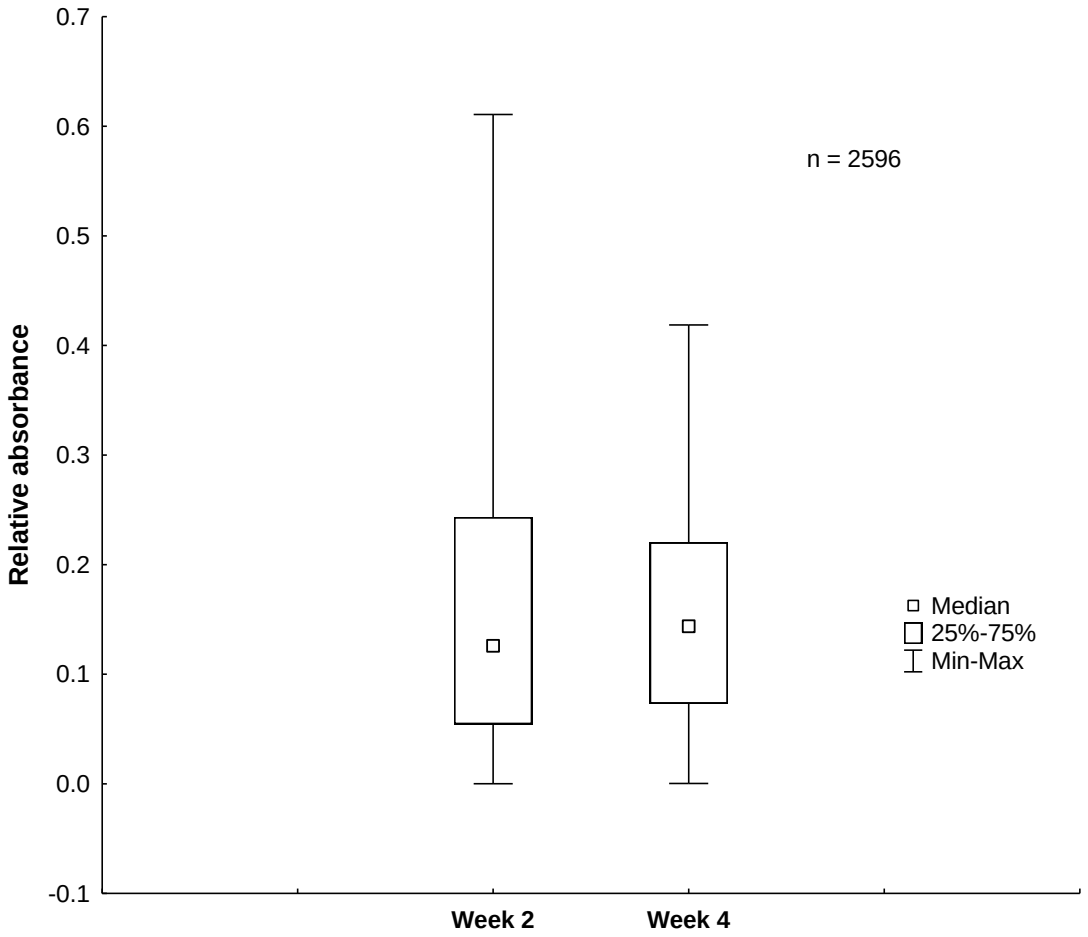
Appendix 1



Appendix 1A. Box-and-whisker plots of the region 4000-500 cm^{-1} of the FTIR spectra for *Steinernema jeffreyense*, formulated in diatomaceous earth and scanned in both weeks 2 and 4, respectively. Each big box contains the middle 50% of the data, with the small box within each box representing the median, and the lower and upper ends of the box representing the first (25%) and third (75%) quartiles, respectively, with whiskers extending out to the minimum and maximum.

Appendix 1B. Descriptive statistics (relative absorbance of the entire FTIR spectra for *Steinernema jeffreyense* formulated in diatomaceous earth and scanned in both weeks 2 and 4).

Variable	Valid N	Mean	Median	Min	Max	Lower quartile	Upper quartile	Std dev
Week 2	6950	0.095319	0.044356	0.00	0.610752	0.002493	0.162656	0.123198
Week 4	6950	0.094019	0.061433	0.00	0.418725	0.002918	0.158784	0.106628



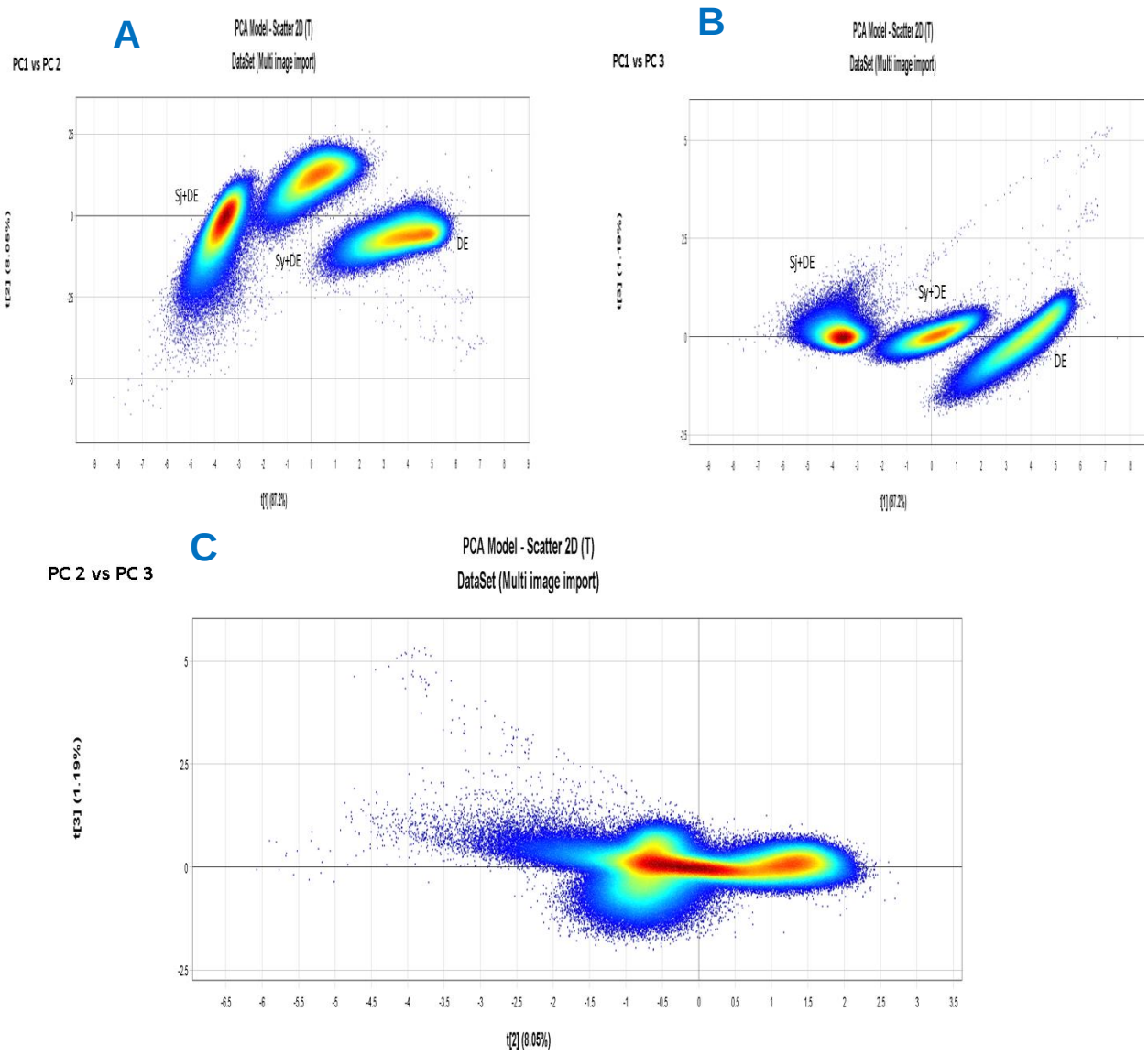
Appendix 1C. Box-and-whisker plots of the region 1900-500 cm^{-1} of the FTIR spectra for *Steinernema jeffreyense*, formulated in diatomaceous earth and scanned in both weeks 2 and 4, respectively. Each big box contains the middle 50% of the data, with the small box within each box representing the median, and the lower and upper ends of the box representing the first (25%) and third (75%) quartiles, respectively, with whiskers extending out to the minimum and maximum.

Appendix 1D. Descriptive statistics (relative absorbance of the region 1900-500 cm^{-1} of the FTIR spectra for *S. jeffreyense* formulated in diatomaceous earth and scanned in both weeks 2 and 4.

Variable	Valid N	Mean	Median	Min	Max	Lower quartile	Upper quartile	Std dev
Week 2	2596	0.164215	0.125903	0.000071	0.610752	0.054340	0.243370	0.148972
Week 4	2596	0.157286	0.143677	0.000328	0.418725	0.073189	0.220365	0.114345

SWIR – with glass/SNV correction

PCA model pixel-wise analysis

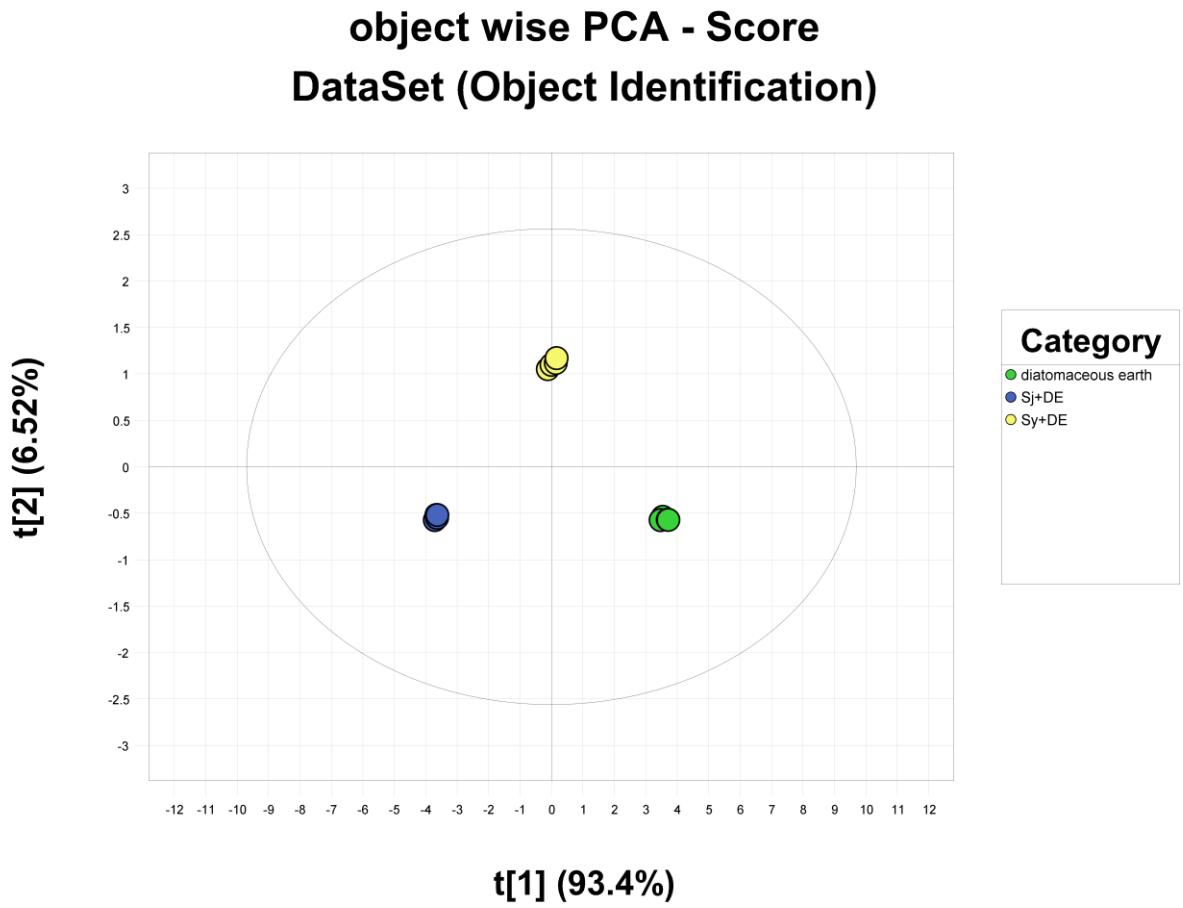


Appendix 1E. A. PC1-PC2; **B**. PC1-PC3; **C**. PC2-PC3 scatter 2D (T) plot values for SWIR-384 spectra (from 780-2500 nm), showing the distribution of *Steinernema yirgalemense* and *S. jeffreyense* in diatomaceous earth and separately in diatomaceous earth, as control.

The PC1 contributed 87.2%, PC2, 8.05% and PC3, 1.19%, explaining a total variance of 97.15%, with only 2.85% of the information having been lost.

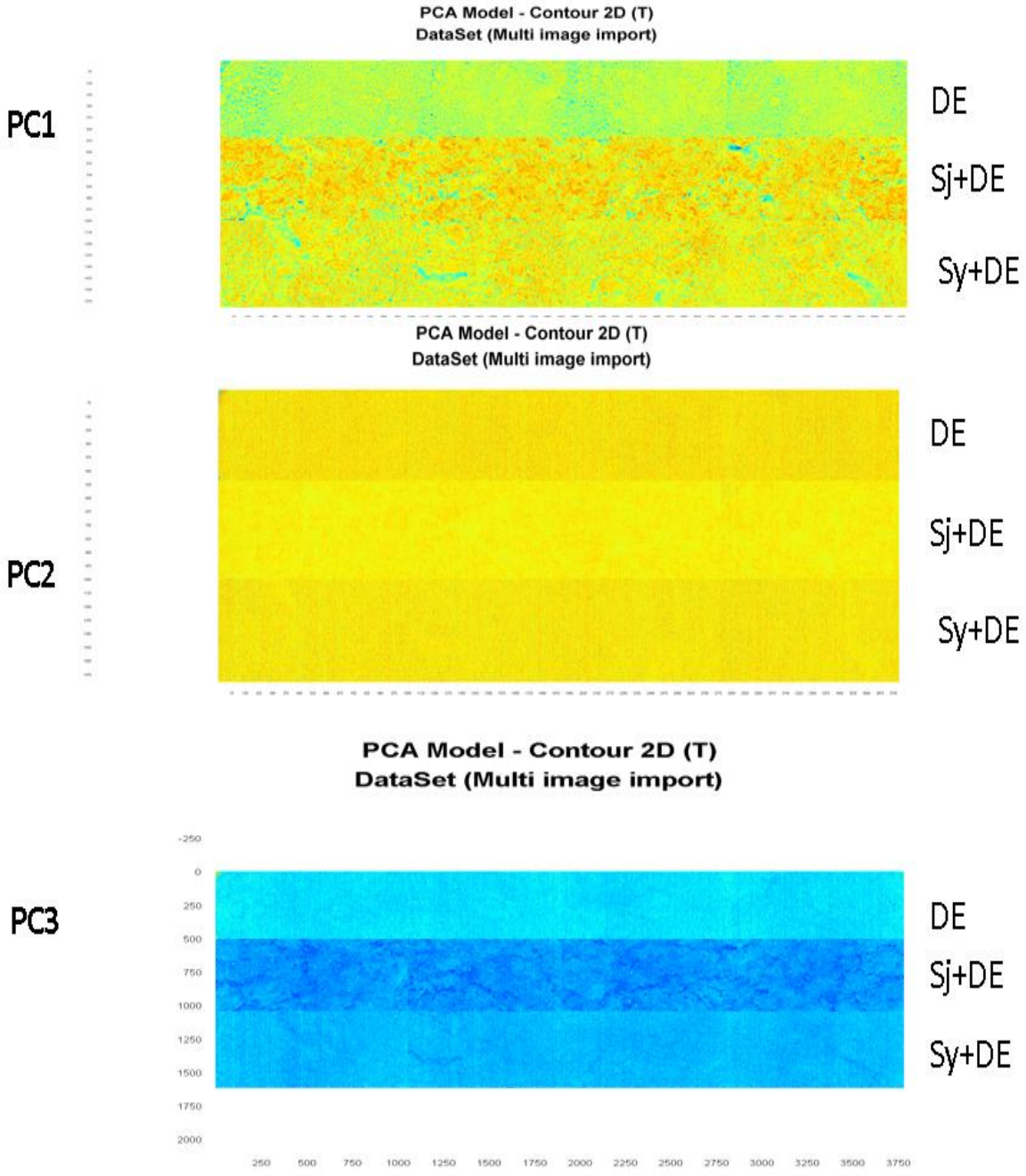
SWIR – with glass/SNV correction

PCA model object-wise analysis

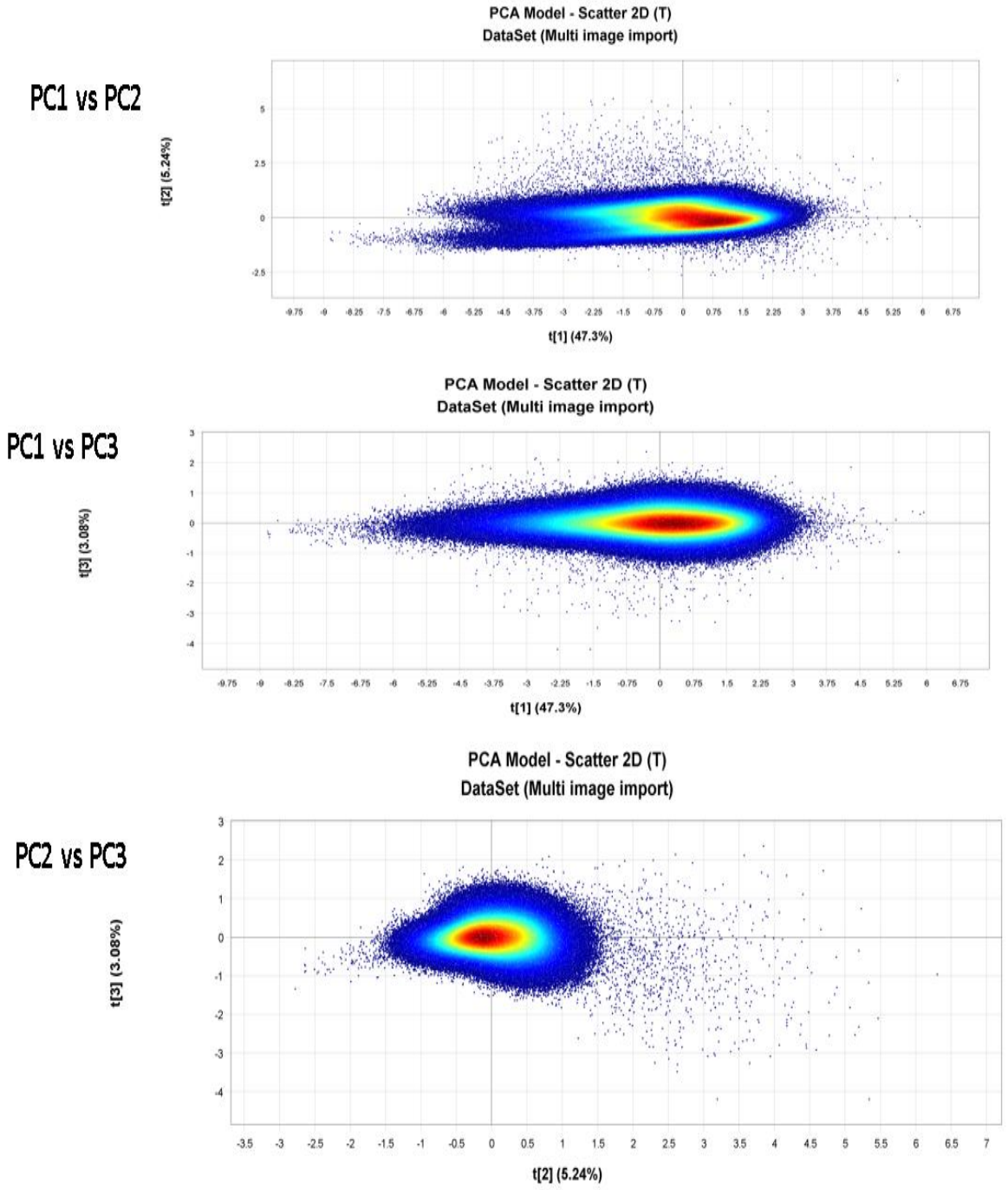


Appendix 1F. PC1-PC2, scatter 2D (T) plot values for SWIR-384 spectra (from 780-2500 nm), showing the distribution of *Steinernema yirgalemense* and *S. jeffreyense* in diatomaceous earth and separately in diatomaceous earth, as control. The PC1 contributed 93.4% and PC2, 6.52% explaining a total variance of 99.92%, with only 0.08% of the information having been lost. In conclusion, the entire variance was explained by means of the system.

VNIR – with glass/SNV correction

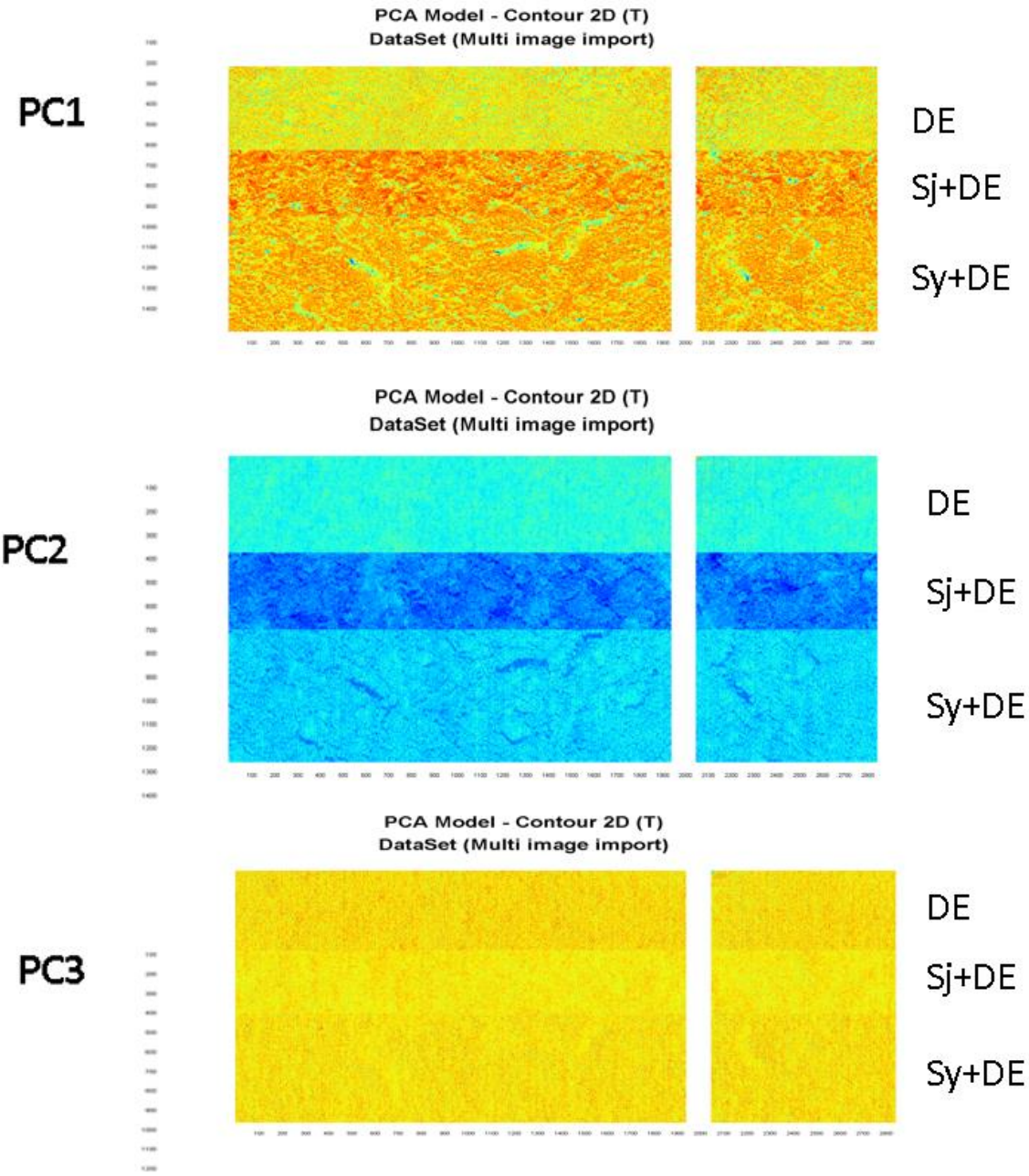


Appendix 1G. VNIR-1800 spectra PCA model – contour 2D (T) data set (multiple image import), showing distribution of *Steinernema yirgalemense* and *S. jeffreyense* in diatomaceous earth and separately in diatomaceous earth, as control. The scans were not fully separated on completion of the cleaning-up process of the data.



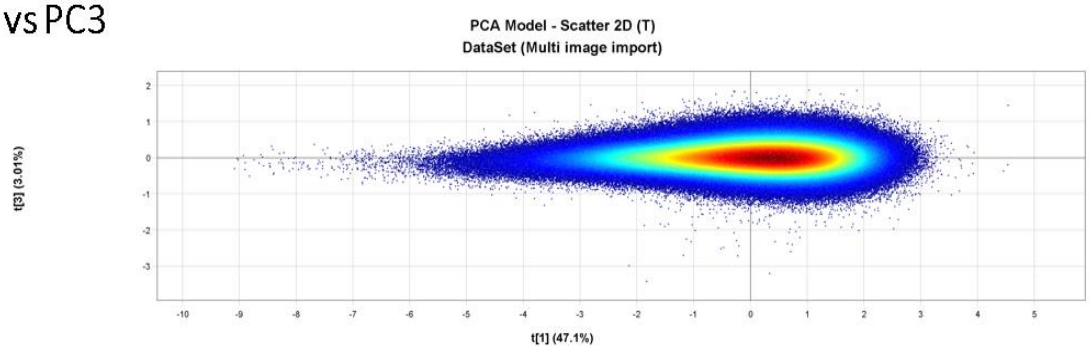
Appendix 1H. PC1-PC2; PC1-PC3; and PC2-PC3 scatter 2D (T) plot values for VNIR-1800 spectra (from 780-2500 nm), showing distribution of *Steinernema yirgalemense* and *S. jeffreyense* in diatomaceous earth and separately in diatomaceous earth, as control. The PC1 contributed 47.3%, the PC2, 5.24% and the PC3, 3.08% explaining a total variance of 55.62%, with only 44.38% of the information having been lost.

VNIR – with no glass/SNV correction

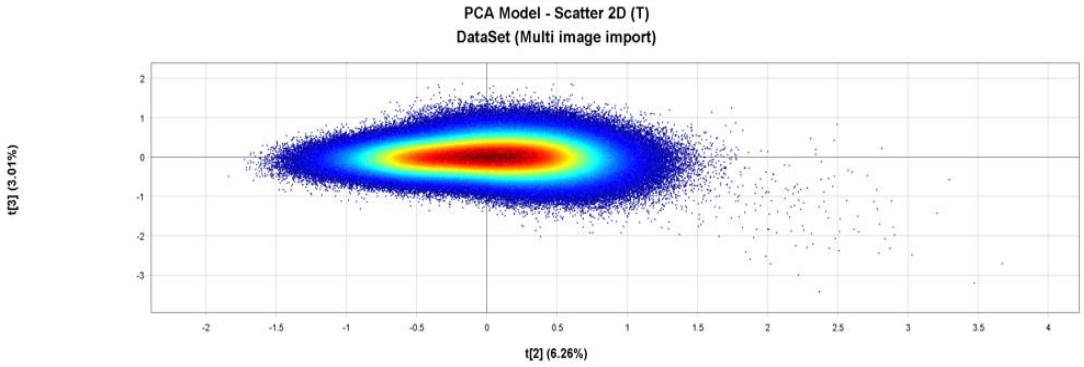


Appendix 1l. VNIR-1800 spectra PCA model – contour 2D (T) data set (multiple image import), showing distribution of *Steinernema yirgalemense* and *S. jeffreyense* in diatomaceous earth and separately in diatomaceous earth, as control. The scans were not fully separated on completion of the cleaning-up process of the data.

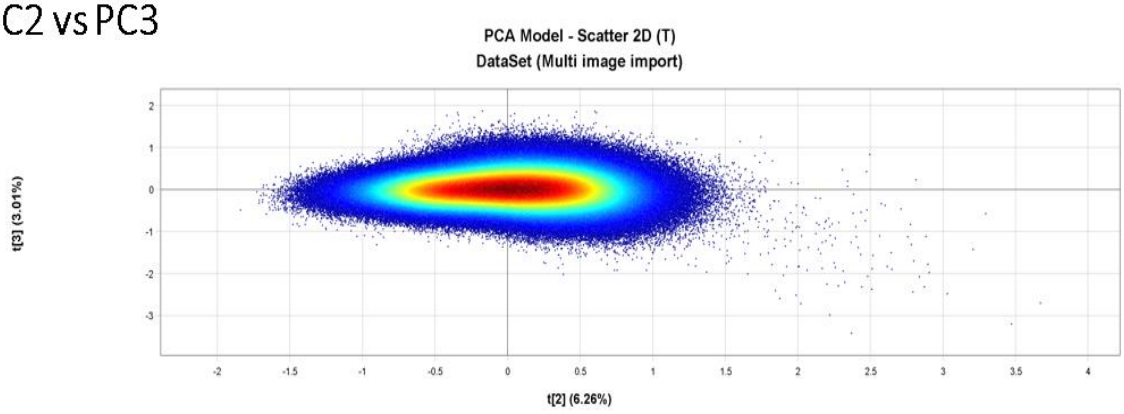
PC1 vs PC3



PC2 vs PC3



PC2 vs PC3



Appendix 1J. VNIR-1800 spectra PCA model − contour 2D (T) data set (multiple image import); PC1-PC2; PC1-PC3; and PC2-PC3 scatter 2D (T) plot values for VNIR-1800 spectra (from 780-2500 nm), showing the distribution of *Steinernema yirgalemense* and *S. jeffreyense* in diatomaceous earth and separately diatomaceous earth, as control. The PC1 contributed 47.1%, PC2, 6.26% and PC3, 3.01%, explaining a total variance of 56.37%, with only 43.63% of the information having been lost.