



# Formulation of South African entomopathogenic nematodes using alginate beads and diatomaceous earth

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**Abstract** Large-scale use of entomopathogenic nematodes (EPNs) (Rhabditida: Steinernematidae and Heterorhabditidae) as biological control agents is impaired by their short shelf life. Three local South African EPN species, including *Steinernema yirgalemense*, *S. jeffreyense* and *Heterorhabditis bacteriophora*, were investigated for their role in formulations. Encapsulation of the infective juveniles (IJs) in alginate beads and diatomaceous earth (DE) was investigated. Survival of the IJs in the formulations was determined at 6 °C, 14 °C and 25 °C for four weeks. Of the IJs, 10–20% were observed to escape from the beads, depending on temperature, and readily survived the encapsulation process. DE did not cause the desiccation of the nematodes, with there still being a lower mortality rate by the 4th week of the study. 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. The alginate beads successfully retained most of the IJs and can be stored for a longer time.

**Keywords** Desiccation · Encapsulation · *Heterorhabditis bacteriophora* · *Steinernema yirgalemense* · *Steinernema jeffreyense* · Temperature

## Introduction

Entomopathogenic nematodes (EPN) of the genera *Heterorhabditis* Poinar (1975) and *Steinernema* Travassos (1927) and their associated symbiotic bacteria *Xenorhabdus* Thomas and Poinar (1983) (Enterobacteriales: Enterobacteriaceae) and *Photorhabdus* Boemare et al. (1993) (Enterobacteriales: Enterobacteriaceae) (Boemare et al. 1993; Akhurst et al. 1996; Ehlers 2007) are biocontrol agents used for the management of insect pests occurring in soil and cryptic environments worldwide. They have many advantages and for this reason many countries, with a few exceptions like South Africa (Hatting et al. 2019), have exempted EPNs from registration obligations (Akhurst and Smith 2002).

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 field use (Grewal 2000a, b). Their poor survival rate in storage at room temperature is a major hindrance to producers in their potential use as bioinsecticides (Grewal 2002). In addition, their ability to survive under such conditions

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is also highly compromised in terms of desiccation (Womersley 1990; Surrey and Wharton 1995).

The successful commercialisation of EPNs after mass production requires the development of storage and formulation techniques that reduce 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 diatomaceous earth (DE) is readily 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 et al. (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) have been made (Malan and 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*, *Ceratitis rosa*), (Diptera: Tephritidae), maize stalk borer (*B. fusca*), and sugarcane borer (*Eldana saccharina*) (Lepidoptera: Pyralidae) (Malan and Hatting 2015).

Nematodes encapsulated in sodium and calcium alginate gels by means of internal or external techniques (Kaya and Nelsen 1985; Kaya et al. 1987), and in other hydrophilic colloids (Patel and 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 and Patel 2013). Increasingly, beads and capsules are coated using such polyelectrolytes as xanthan gum, with altering charges (Hiltpold et al. 2012; Vemmer and Patel 2013; Kim et al. 2015) 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, 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<sup>2+</sup> 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<sup>2+</sup> 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<sub>2</sub>, 4% Al<sub>2</sub>O<sub>3</sub>, 1.7% Fe<sub>2</sub>O<sub>3</sub>, 1.4% CaO, > 1% MgO + K<sub>2</sub>O and 3% H<sub>2</sub>O, 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 against the insect pests of stored products, they vary in their 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 (Silver et al. 1995; Hiltpold et al. 2012).

The objectives of the 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 et al. (2016), *S. yirgalemense* and *Heterorhabditis bacteriophora* Poinar (1975), 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 and Hatting 2015; Malan et al. 2016).

*Galleria mellonella* (Lepidoptera: Pyralidae) larvae were cultured according to van Zyl and 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-diameter 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 and 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 (for aeration) 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 and 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. Ninety-eight thousand infective juveniles (IJs) 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<sup>2+</sup>-solution, containing 0.5% CaCl<sub>2</sub> H<sub>2</sub>O [Merck SA (Pty) Ltd], 10% glycerol and 0.075% formaldehyde in distilled water. The formation of the alginate beads was immediate. The Ca<sup>2+</sup> 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).

### Number of IJs escaping from alginate beads

The 22 beads used for storage and the eight 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 number of IJs 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 four weeks, to determine the survival percentage. Each bead was transferred to a new microcentrifuge tube. Two representative beads per sample (as a control) were crushed in a 1.5 ml microcentrifuge tube, using a disposable tissue grinder pestle (Axygen®, Axigen Biosciences, Union City, USA) and observed, using a stereomicroscope, in an embryo dish, to determine the

survival of the IJs that remained inside the beads. The process was repeated on a different test date, using a fresh batch of nematodes.

#### 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 live IJs in the dilutions were counted and tested for pathogenicity against the last-instar larvae of *G. mellonella*, using 24-well bioassay plates. Twelve alternate wells of a 24-well bioassay plate were used, fitted with a piece of filter paper, to which one *Galleria* larva was added. 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 to maintain high RH, 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 turned 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.

#### Survival of nematodes in diatomaceous earth

Nematodes were concentrated into a paste using a 32 µm sieve (Clear Edge Filtration SA (Pty) Ltd, South Africa). Each of the three EPN species were formulated in DE (Celite 209—Imerys Refractory Minerals SA (Pty) Ltd). The proportions of the ingredients used in the formulation were followed according to Grewal and 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) the final density of the formulation was 50,000 IJs g<sup>-1</sup>.

The formulated nematodes were weighed, with 10 g being added to the STC2P 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 high RH. The containers were stored for four weeks at 6 °C, 14 °C and 25 °C. The experiment was repeated for each nematode species on a different test date, 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 ten 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-measures ANOVAs were calculated accordingly. Where the results were not normally distributed, bootstraps were performed on the data for least significance difference (LSD) 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.

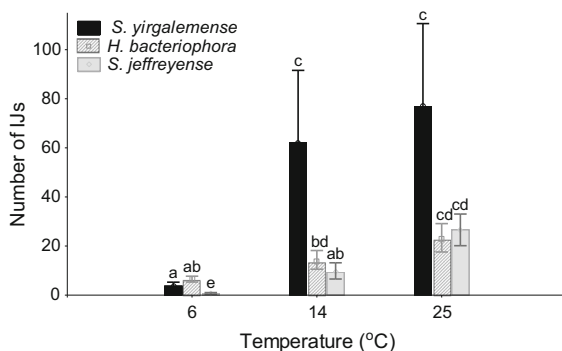
## Results

#### Rate of nematodes escape from alginate beads

A one-way ANOVA over batches indicated that there were no significant differences ( $F_{1,394} = 0.146$ ;

$p = 0.703$ ) between the total means of the two batches, thus the data were pooled for analysis. A two-way factorial ANOVA was done on the pooled data, on the number of nematodes moving out of the beads, relative to the factors temperature and species. The interaction was significant ( $F_{4,387} = 5.194$ ,  $p = 0.0004$ ). These interactions were investigated with LSD multiple comparisons which indicated temperature to significantly affect the number of nematodes moving out of the beads, but differently for each species. Fewer IJs moved out of the beads at 6 °C, followed by a slight 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. 1).

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* ( $p = 0.005$ ) and *S. jeffreyense* ( $p < 0.01$ ), which did not differ significantly ( $p = 0.13$ ) from each other. At 14 °C, 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* ( $p < 0.01$ ) and *S. jeffreyense*



**Fig. 1** Mean number of infective juveniles (IJs) (95% confidence interval) that moved out of the beads after four weeks in respect of *S. jeffreyense*, *H. bacteriophora* and *S. yirgalemense*, each at three different temperatures. Different letters above the bars indicate significant differences ( $p < 0.05$ ) in nematode numbers between species and temperature

( $p < 0.01$ ), 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. 1).

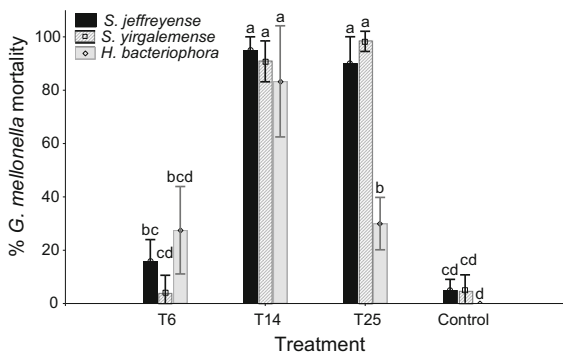
The mean number of IJs moving out of the beads for the nematode species at different temperatures increased, with an increase in temperature. The mean number of *Steinernema yirgalemense* IJs, at 6 °C, differed significantly from those at 14 °C and 25 °C ( $p = 0.01$ ). At 14 °C, the mean number did 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. 1).

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. 1).

#### Pathogenicity of IJ stored in alginate beads

A one-way ANOVA over batches indicated that there were no significant differences ( $F_{1,118} = 0.878$ ,  $p = 0.351$ ) between the percentage mortality of the two batches, thus the data from the two batches were pooled before analysis. A two-way factorial ANOVA on the pooled data indicated that the interaction between the treatments and the nematode species was significant ( $F_{6,108} = 22.164$ ,  $p < 0.001$ ) (Fig. 2). LSD multiple comparisons showed that for treatment T6 no differences were found between the nematode species, for T14 nematode species were again similar, while for T25, *S. yirgalemense* and *S. jeffreyense* did not differ, but differed from *H. bacteriophora* (Fig. 2).

For nematodes encapsulated in alginate beads and stored 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



**Fig. 2** Mean percentage mortality (95% confidence interval) at of *G. mellonella* larvae at 25 °C, inoculated with the infective juveniles of *Steinernema jeffreyense*, *Heterorhabditis bacteriophora* and *S. yirgalemense*, formulated and stored in alginate beads at three different temperatures after four weeks T6 (6 °C), T14 (14 °C) and T25 (25 °C). Different letters above the bars indicate significant differences ( $p < 0.05$ ) in insect mortality between nematode species and treatments

( $p = 0.22$ ) from each other. The *H. bacteriophora* IJs caused the highest mean percentage mortality of *Galleria* larvae, of  $27.50\% \pm 4.34\%$ , followed by *S. jeffreyense* and *S. yirgalemense*, with  $15.83\% \pm 4.34\%$  and  $4.17\% \pm 4.34\%$ , respectively (Fig. 2).

For nematodes encapsulated in alginate beads and stored 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 had the highest mean percentage *Galleria* mortality value of  $95.00\% \pm 4.34\%$ , followed by *S. yirgalemense* and *H. bacteriophora*, with  $90.83\% \pm 4.34\%$  and  $83.33\% \pm 4.34\%$ , respectively (Fig. 2). For nematodes encapsulated in alginate beads and stored 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 differed from the mean percentage obtained for the *S. yirgalemense* IJs. The *S. yirgalemense* IJs had the highest mean percentage *Galleria* mortality of  $98.33\% \pm 5.27\%$ , followed by *S. jeffreyense*, and by *H. bacteriophora* with  $90.00\% \pm 14.05\%$  and  $30.00\% \pm 13.72\%$ , respectively (Fig. 2). No significant differences were found between 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).

Within the nematode species, there were significant ( $p < 0.05$ ) and non-significant ( $p > 0.05$ ) differences per treatment. The *S. jeffreyense* IJs at 6 °C differed significantly from those at 14 °C ( $p < 0.001$ ) and at 25 °C ( $p < 0.001$ ) 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  $95.00\% \pm 4.34\%$  was observed at 14 °C, followed by  $90.00\% \pm 4.34\%$  and  $15.83\% \pm 4.34\%$ , at 25 °C and 14 °C, respectively (Fig. 2).

The *Galleria* mortality by the *S. yirgalemense* IJs, at 6 °C, differed significantly from that at 14 °C ( $p < 0.01$ ) and 25 °C ( $p < 0.01$ ). At 14 °C, *Galleria* mortality differed significantly from that at 25 °C ( $p = 0.006$ ). At 25 °C, *Galleria* mortality 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  $98.33\% \pm 4.34\%$  was observed at 25 °C, followed by  $90.83\% \pm 4.34\%$  at 14 °C, and by  $5.00\% \pm 4.34\%$  at 6 °C, (Fig. 2).

The *Galleria* mortality caused by *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, *Galleria* mortality differed significantly from those at 25 °C ( $p = 0.006$ ), and from the control ( $p = 0.0002$ ). At 25 °C, *Galleria* mortality 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  $30.00\% \pm 4.34\%$  at 27 °C, and the  $27.50\% \pm 4.34\%$  at 6 °C (Fig. 2).

#### Survival in diatomaceous earth

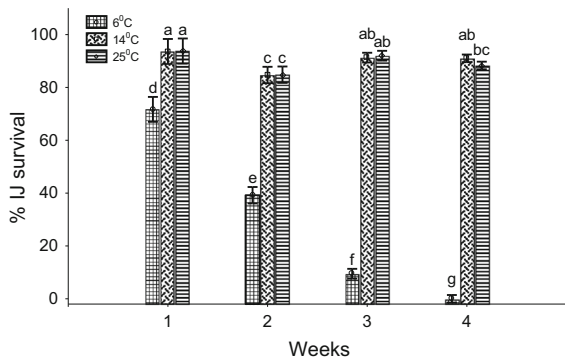
##### *Combined effect of temperature on the survival of three EPN species*

The data for survival of *S. yirgalemense*, *H. bacteriophora*, and *S. jeffreyense* were combined and analysed. The analysis showed higher temperatures (14 °C and 25 °C) having no significant difference between the two batches while the lowered survival trend at 6 °C were generally the same. No significant differences

were obtained between batches over survival, ( $F_{1,58} = 0.045$ ,  $p = 0.83$ ), thus the batches were pooled. The repeated-measures analysis of the pooled data over weeks 1 to 4 showed significant interaction ( $F_{6,171} = 171.89$ ,  $p < 0.01$ ) between the temperatures and time regarding the survival of EPNs. Such interaction was interpreted with LSD multiple comparisons. A steep decline occurred in the mean percentage survival of the EPNs over time at 6 °C, whereas a near-stable condition of mean percentage survival was maintained at 14 °C and at 25 °C, respectively (Fig. 3). In week 1, the mean percentage survival of EPNs at 6 °C differed significantly from that at 14 °C and at 25 °C ( $p < 0.01$ ). In week 1, an almost equal mean percentage survival rate was obtained at 25 °C ( $93.81\% \pm 2.36\%$ ), and at 14 °C ( $93.63\% \pm 2.36\%$ ), followed by that which was obtained at 6 °C ( $71.70\% \pm 2.36\%$ ). The mean percentage survival rate of EPNs remained high between weeks 1 and 4 respectively at 14 °C ( $93.63\% \pm 2.36\%$  to  $91.01\% \pm 0.72\%$ ), and at 25 °C ( $93.81\% \pm 2.36\%$  to  $88.31\% \pm 0.72\%$ ) (Fig. 3).

*Combined desiccative effect of diatomaceous earth on the survival of EPNs*

No significant differences ( $F_{1,178} = 0.0089$ ,  $p = 0.766$ ) were obtained between the batches and survival using a repeated-measures ANOVA, with one

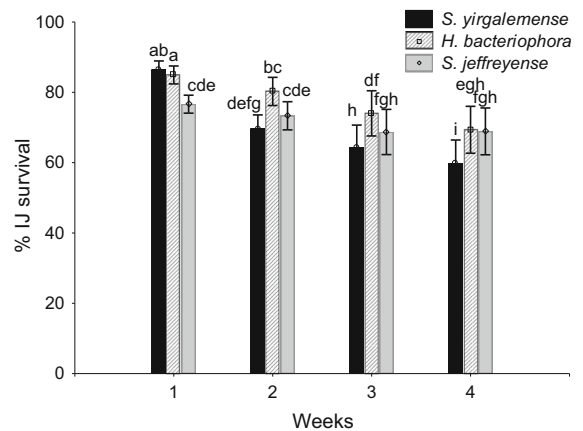


**Fig. 3** Mean percentage survival rate (95% confidence interval) of combined results of infective juveniles (IJs) of *Steinernema yirgalemense*, *Heterorhabditis bacteriophora*, and *S. jeffreyense* formulated and stored in diatomaceous earth at three different temperatures after a period of four weeks. Different letters above the bars indicate significant differences ( $p < 0.05$ ) in IJ survival between temperature and weeks

categorical variable namely the batch, thus enabling the data from the two batches to be pooled and analysed. The analysis of the data, using repeated-measures ANOVA, from weeks 1 to 4, showed significant interaction ( $F_{6,531} = 8.462$ ,  $p < 0.0001$ ) between the different nematode species regarding their survival over time. Such interaction was interpreted with LSD multiple comparisons. A general decline in the mean percentage survival rate of the nematodes occurred over time (Fig. 4).

**Discussion**

The current study reports on suitability of 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 (Hiltbold 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. In addition, 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).



**Fig. 4** Mean percentage survival (95% confidence interval) infective juveniles of *Steinernema yirgalemense*, *Heterorhabditis bacteriophora*, and *S. jeffreyense* formulated in diatomaceous earth and stored for a period of four weeks (data from different temperatures pooled). Different letters above the bars indicate significant differences ( $p < 0.05$ ) in IJ survival between species and time

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 four weeks was relatively low, indicating that alginate beads can release IJs at a relatively slow rate, and thus improve their long-term room temperature shelf stability.

Temperature had a significant effect on the different EPN species tested, with regard to 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 survival of IJs. Furthermore, Addis et al. (2016), in their study of the storage temperatures of *S. yirgalemense*, 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 of *S. yirgalemense* survived for a period of up to 66 days. The *S. yirgalemense* was the same strain 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 and 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 low temperatures between 4 and 15 °C (Grewal and 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 after storage at low temperatures. Therefore, the three formulated nematodes should be stored at temperatures ranging between 14 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*.

DE is widely used in insect management, 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 given the desiccative and hygroscopic nature of DE. 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 lower mortality rate by the 4th 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 (Silver et al. 1995; Hiltbold et al. 2012; Matadamas-Ortiz et al. 2014). 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 *Oryza-epihilus surinamensis* (L.), with increasing exposure intervals and concentration levels. The low density of IJs  $\text{g}^{-1}$  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, this study indicates that the properties of alginate beads can be adjusted to enable them to release IJs on a regular basis. Like in other tropical and subtropical EPN species and strains, a loss in virulence and survival is equally reported as occurring at relatively low temperatures for the three EPNs stored at 6 °C. The high survival of the studied species obtained at room temperature in both alginate beads and DE formulations 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  $\text{g}^{-1}$ ) 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.

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#### Compliance with ethical standards

**Conflict of interest** The authors declare that they have no conflict of interest.

**Research involving human and animal participants** The research does not involve human participant or animals.

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