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The Characterization and Biological Control Potential of an Endemic Entomopathogenic Nematode and Its Symbiotic Bacterium Through Behavioural, Molecular and Genomic Approaches

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DECLARATION

I, Lee-Anne Odelle Soobramoney, declare that this dissertation is my own unaided work. It is submitted in fulfillment of the Master of Science degree in Microbiology and Biotechnology at the University of the Witwatersrand, Johannesburg. The dissertation has not been submitted for any other degree at the University.

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ABSTRACT

The entomopathogenic nematodes (EPNs) have emerged as an important group of insect pests. The EPNs which comprise the *Steinernema* genus share symbiotic associations with *Xenorhabdus* bacteria. This research project focused on isolating and characterizing a novel and indigenous EPN isolate with its associated bacteria. The biological control potential of the nematode was investigated in the areas of host infectivity, infective juvenile recovery and progeny yield. These processes were investigated at three different factors. These included time, population size and temperature. The infectious abilities of the symbiotic bacteria were also evaluated without the contributions of the nematode partner at different bacterial doses, time intervals and temperature regimens. The genome of the bacteria was thereafter acquired through whole-genome sequencing and annotation techniques to elucidate the virulence mechanisms and genes involved in temperature adaptation.

The species isolated in this investigation was novel. The species shared an 85 % maximum identity to and taxonomically grouped with the species *Steinernema khoisanae*. The two species shared a common ancestor but the extended branch length of the species under investigation substantiated its novelty. The EPNs infected hosts at different time intervals, population densities and temperature regimens. However, the EPNs performed these processes to different extents. Host mortality significantly increased with time. The EPNs also infected insect hosts at the two experimental temperatures. However, host mortality was higher at the temperature regimen of 20° C and lower at 30° C. Host infections were not significantly different at two tested population densities of 500 and 1000 infective juveniles. The levels of interaction between temperature and time and temperature population density were not statistically significant. The subsequent biological process of recovery was evaluated.

The EPNs recovered at both population densities and temperature regimens. The infective juvenile recoveries were statistically insignificant at both population densities and temperature regimens. Since recovery was based on the mere presence of progeny infective juveniles, the percentages were high which contributed to the statistical insignificant findings. This also contributed to the non-significant interaction between population density and temperature. The last biological process investigated was the progeny yield of infective juveniles. The yields were

significantly different between both population densities of infective juveniles and temperature regimens. Higher yields were obtained at the temperature regimen of 20° C and 25° C. Lower yields were obtained at 30° C. The unexpected finding was higher progeny yields obtained from the lower population densities of infective juveniles. This contributed to the significant interaction present between population density and temperature. The bacteria were thereafter molecularly characterized.

The symbiotic bacteria shared a 99 % sequence similarity to the species *Xenorhabdus* sp. strain GDc328. It was interesting to observe the infectious abilities of the bacteria without contributions from the EPNs. This study was measured at different bacterial doses, time intervals and temperature regimens. Host mortality was achieved without contributions from the EPN. Host mortality significantly increased with bacterial dose and time. Host mortality was also significantly different between each temperature regimen. Higher mortalities were observed at 30° C and lower mortalities were observed at 20° C. The differences in the performance between the EPN-bacterial partnership and the bacteria alone were attributed to the manner in which adaptation occurred. Since the EPN-bacteria existed as a bi-partite entity, the partners evolved as a bi-partite complex. The bacteria were removed from the symbiosis and cultured individually. External factors may have re-shaped the performance of the bacteria at the different temperature regimens. To further understand the genetic mechanisms of temperature adaptation, host infectivity and symbiosis, the draft whole genome sequence of the bacteria was then acquired.

The genome of the bacteria comprised several genes which encoded the flagella system of the bacteria. Also pairs of co-localized toxin-antitoxin genes were discovered. Temperature acclimatization was performed through different cold and heat shock proteins and lastly several molecular chaperones. The studies showed that the species *Steinernema* spp. and its associated symbiotic bacteria *Xenorhabdus* sp. strain GDc328 were good bio-pesticide candidates for application against endemic insect pests.

DEDICATION

This thesis is dedicated to my loving parents Lenny and Lalitha Soobramoney. Thank you for all the selfless sacrifices made to ensure that I have all the resources necessary to achieve milestones such as these.

CONFERENCE AND RESEARCH OUTPUT

<u>Conference output:</u>

1. Poster presentation at the 7th Annual GDARD research symposium (2014) entitled: The isolation of antibiotic producing symbiotic bacteria from entomopathogenic nematodes.
2. Poster Presentation at the 8th Annual GDARD research symposium (2015) entitled: The isolation and characterization of a novel entomopathogenic nematode species from South African soil.

<u>Research output:</u>

1. Research publication described in the American Society of Microbiology Genome Announcements (2015): The Draft Whole-Genome Sequence of *Xenorhabdus* sp. Strain GDC 328, Isolated from the Indigenous South African Entomopathoegnic Nematode Species, *Steinernema khoisanae*. The contributing authors: Lee-Anne Soobramoney, Jonathan Featherston and Vincent Gray.

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CHAPTER 1: LITERATURE REVIEW

1.1 Nematodes

Nematodes are non-segmented, soft bodied invertebrates. They are multicellular organisms and are considered the most diverse group of animals on Earth (Stock, 2015). One cubic foot of soil may host thousands of nematode species each belonging to distinct taxonomic groups (Yeates and Bongers, 1999). Nematodes have evolved to occupy almost all conceivable ecological niches. Their prevalence has been documented in terrestrial and aquatic (fresh water and marine) ecosystems ranging from the tropical to Polar Regions, including the areas of high and low elevations (Yeates and Bongers, 1999).

The nematode community comprises different groups of nematodes. These groups include the species which feed on bacteria, algae and fungi. Also, this group encompasses species which are predators of small invertebrates and those that share internal and external associations with plants and animals (Powers et al., 2009). While only a minority of nematode species remain parasitic to humans, plants and domesticated animals, the majority of species exhibit beneficial attributes which are of great importance to agriculture. These species are categorized as insect pathogens (Stock, 2015).

One group has emerged as excellent biological control agents of insect pests. They are known as the entomopathogenic nematodes (EPNs) (Campos-Herrera et al., 2012). The term *entomon* actively describes the selective nature of EPNs for insect pests and the term pathogen highlights their detrimental nature.

1.1.1 Taxonomy of EPNs

EPNs are members of the kingdom Animalia and belong to phylum Nematoda. This phylum represents a portion of the superphylum Ecdysozoa. The two key characteristics of this superphylum include the presence of the cuticle structure which is periodically moulted and the absence of cilia in the adult stages of development (Blaxter et al., 1998). Furthermore, the EPNs

are classified within the Rhabditida order. Steinernematidae and Heterorhabditidae represent the two most widely studied families of EPNs (Stock and Goodrich-Blair, 2008).

1.1.2 Taxonomic status of Steinernematidae and Heterorhabditidae

Two genera of Steinernematidae have been established. The first includes a *Neosteinernema* genus which comprises one species, *Neosteinernema longicurvicauda*. The second includes the genus, *Steinernema* which comprises a large diversity of more than 70 species. However, Heterorhabditidae represents a monotypic family comprising a single genus, *Heterorhabditis* with more than 20 recognized species (Stock, 2015; Stock and Goodrich-Blair, 2008). The genera *Steinernema* and *Heterorhabditis* have become the subjects of extensive research in the discipline of entomopathogenic nematology (Stock, 2015).

1.2 Evolution of *Steinernema* and *Heterorhabditis*

Blaxter et al., (1998) established the phylogenetic framework for the phylum Nemtaoda. The phylogenetic analysis revealed that Heterorhabditidae was most closely related to Strongylida, an order which comprises vertebrate parasites. Nematodes within the Strongylida order shared the most recent common ancestor with *Pellioditis*, a free-living marine bacterivore (Stock, 2015). The taxonomic framework for Steinernematidae according to Blaxter et al., (1998) showed a close relation to Panagrolaimoidea which comprises free-living and insect associates. Also, they shared close relation to Strongyloididae which are a group of vertebrate parasites. This framework provided by Blaxter et al., (1998) showed the independent evolution of Steinernematidae and Heterorhabditidae. These two families however show large extents of similarities with regards to their lifestyles. Both families of EPNs independently acquired mutualistic symbiotic relationships with members of the Enterobacteriaceae family. Poinar (1993) suggested that these similarities were attributed to convergent evolution.

1.3 The association of EPNs with insect pathogenic bacteria

Steinernematidae and Heterorhabditae share an obligate mutualistic association with members of the Enterobacteriaceae family. The genera *Xenorhabdus* and *Photorhabdus* are symbionts of *Steinernema* and *Heterorhabditis* respectively (Boemare and Akhurst, 2006). The *Xenorhabdus*

genus comprises a large diversity of more than 20 species known to be associated with EPNs in the genus, *Steinernema*. The *Photorhabdus* genus however comprises 4 taxonomically described species and more than 12 subspecies which are associated with EPNs in the genus, *Heterorhabditis* (Boemare and Akhurst, 2006; Stock, 2015). Poinar and Grewal (2012) stated that the acquisition of symbiotic bacteria by EPNs marked the turning point in the development of EPNs as biological control agents of insect pests. Since this acquisition, the bacteria have contributed significantly to the successes of EPNs with regards to their life cycles (figure 1.1).
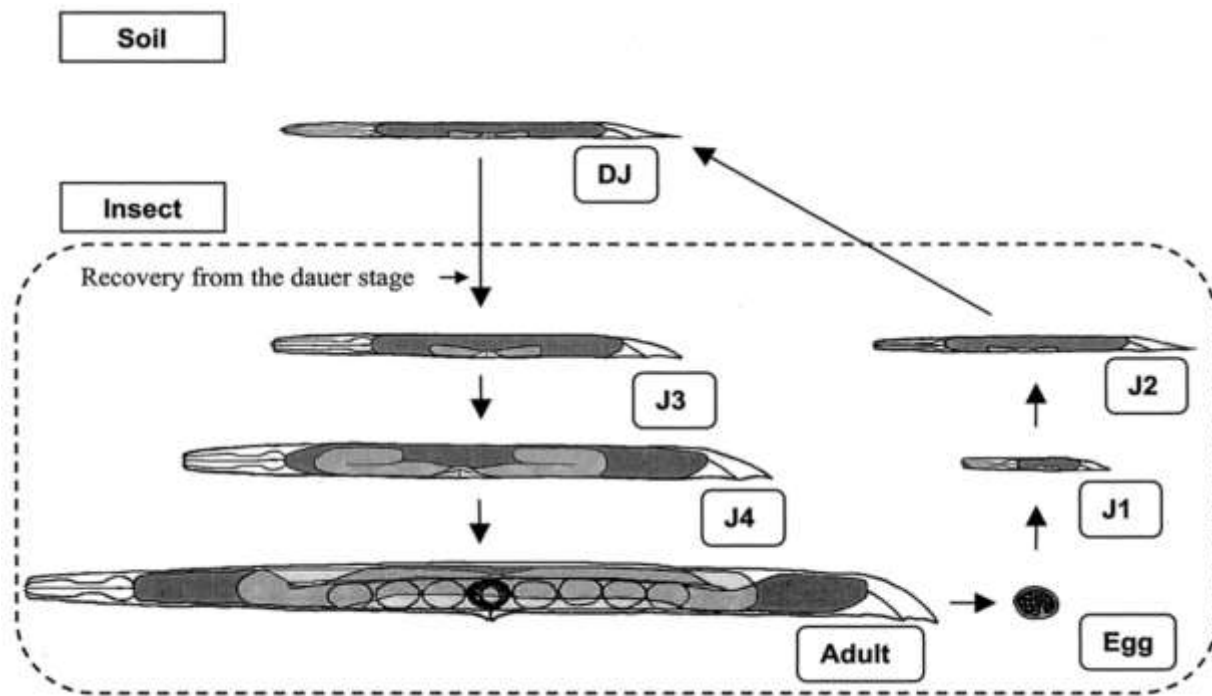
Figure 1.1: The general life cycle of EPNs reproduced from (Han and Ehlers, 2001).

1.4 Life cycle of *Steinernema* and *Heterorhabditis*

1.4.1 Infective Juvenile (J1)

The general life cycle of EPNs commences with the first stage of free-living infective juveniles (IJs). The term infective juvenile (IJ) or dauer juvenile (DJ) may be used interchangeably. At this stage, the EPNs are non-feeding and maintain a state of arrested of development. The IJs display excessive resistance to the different environmental stresses which are expressed from the abiotic components of the environment.

The infective juvenile stage of the nematode serves two crucial functions. The first includes dispersal within the soil as they actively seek and locate susceptible insect hosts through the production of signal cues. The second includes the strategies employed by the nematode to gain entry and parasitize the insect host (Stock, 2015; Grewal et al., 1996). Lastly, the IJs are responsible for vectoring and delivering their respective pathogenic bacteria from one insect host into another (Stock, 2015).

1.4.2   The retention of the enteric γ-Proteobacteria

Xenorhabdus bacteria are hosted in specialized intestinal structures called the receptacle by the different species which comprise the *Steinernema* genus. According to Stock (2015), the receptacle structure is a modification of the two anterior intestinal cells. This receptacle structure is absent in the *Heterorhabditis* genus. The *Photorhabdus* species of bacteria associated with the *Heterorhabditis* genus first bind to the esophago-intestinal valve of the IJ and thereafter migrate to the anterior portion of the intestinal lumen prior to the process of proliferation. The nature of the specificity which exists between EPNs and their associated bacteria may be attributed to the binding of surface receptors present on the gut epithelial cells of IJs to surface molecules located on each bacterial species (Forst and Clarke, 2002). The strategies employed by the two genera of EPNs regarding routes of entry into the insect host differ and are independent of their symbiotic bacteria (Boemare and Akhurst, 2006).

1.4.3   Routes of entry into insect hosts

The routes of entry into the insect host differ between the two genera of EPNs. The routes of entry which are utilized by the IJs which comprise the *Steinernema* genus include the natural openings of the host such as the mouth, anus and spiracles. Conversely, the IJs in the *Heterorhabditis* genus comprise tooth-like projections which enable direct entrance into the host through the penetration of the tegument structure of the insect. The cuticles of the IJs which are in the second stage of development (J2) undergo the process of shedding prior to entering the body cavity of the insect (Boemare and Akhurst, 2006).

1.4.4 Developmental transition of EPNs in the host

Following the process of entry into the insect host, the IJs migrate to the haemolymph where the delivery of their symbiotic bacteria and recovery of developmental arrest takes place (Stock, 2015). The bacteria rapidly proliferate and ensure timeous insect host mortality within 24-48 hours through lethal septicemia (Adams and Nguyen, 2002). The process of lethal septicemia encompasses the production of an arsenal of virulence factors. One key attribute regarding symbiosis is demonstrated by the bacteria. The bacteria execute a protective role for their entomopathogenic nematode (EPN) partners by creating an almost exclusive environment in the insect host which is strictly occupied by the two symbiotic partners. The bacteria perform this role through the production of antibiotics, bacteriocins and antimicrobial compounds which serve to eliminate competitive saprophytic and closely related microorganisms (Stock, 2015). A variety of compounds and enzymes are produced by the bacteria which convert the host into a nutrient plentiful source for EPN establishment (Webster et al., 2002).

1.4.5 The initial juvenile development

The life cycle of EPNs within the insect cadaver is largely dependent on the availability of nutrient resources. The bio-conversion of the insect host by the bacteria provides the nutrient resources for the establishment of EPNs. This process provides the signals for recovery from developmental arrest and the transition into actively feeding EPNs. The second stage infective juveniles (J2) feed and moult for two successive rounds to give rise to the third (J3) and fourth (J4) stage juveniles. The fourth stage juveniles feed on host nutrients and gradually moult into first generation adults (Adams and Nguyen, 2002).

1.4.5.1 Development of infective stages (*Heterorhabditis* species)

The members of the *Heterorhabditis* genus reinitiate their life cycles and mature into first generation self-fertilizing hermaphroditic adults. Subsequent to the process of self-fertilization, the eggs are retained and hatch into the first generation of stage 1 juveniles (J1) *in utero* of the parental hermaphrodite. The juveniles of the first developmental stage (J1) feed on the parental nematode and moult into second stage juveniles (J2). The nutrient availability in the parental nematode becomes limiting which ultimately serves as a signal for the development of infective juveniles. The second stage juveniles (J2) then transition into their infective stage of

development within the adult nematode. This process of intrauterine hatching and development within the body cavity of the parental nematode is deemed as *endotokia matricida*. The J3 infective juveniles thereafter burst through the cuticle of the parental nematode and emerge into the environment of the insect cadaver (Adams and Nguyen, 2002).

Depending on the availability of host nutrients, the first generation of IJs will develop into second generation adults which constitute distinct male and female sexes. The life cycle of EPNs are maintained until all nutrient reserves in the host are depleted which signal for the release of infective juveniles from the insect cadaver into the environment (Kooliyottil et al., 2013).

1.4.5.2   Development of infective stages (*Steinernema* species)

The life cycle of members which comprise the genus *Steinernema* may also be largely dependent on the availability of host nutrients. However, there are apparent differences present between the life cycles of *Steinernema* and *Heterorhabditis* species. The principal difference is highlighted by the form of reproduction. The phenomenon of amphimixis is observed within the *Steinernema* genus. This implies that the nature of reproduction is sexual and occurs between distinct first generation male and female adults. Subsequent to the process of copulation, the eggs are retained within the uterine structure of the female adult. The first generation juveniles hatch and develop into their respective juvenile stages (J1-J4) followed by the transition into second generation adults (Kooliyottil et al., 2013).

The second generation adults also adhere to the process of amphimixis. However, according to Kooliyottil et al., (2013), the eggs which are produced by the adults of the third generation develop into juveniles through the process of *endotokia matricida*. The juveniles however do not develop directly into the infective stages in the maternal adult as in the case of *Heterorhabditis* species. The transition of juveniles into the infective stages is obtained after their departure from the body cavity of the maternal adult. The IJs thereafter emerge from the host cadaver in the pursuit of new insect hosts. The entirety of this life cycle is known as the long cycle and is reliant on the surplus of host nutrients (Adams and Nguyen, 2002).

One other form of the life cycle may be observed. This form is known as the short life cycle which involves the production of infective juveniles from the eggs produced from first generation adults. This is attributed to nutrient deprivation. During this stage, second stage juveniles (J2) develop into the infective juveniles as an alternative to third stage juveniles (Adams and Nguyen, 2002).

1.5   The physiological and anatomical features of infective juveniles

The juveniles which are destined for infectivity undergo important physiological and anatomical changes. According to Chaston et al., (2013), three processes are obligatory. The first includes the colonization of the pharyngeal-intestinal valve (PIV) by the symbiotic bacteria. Secondly, the intestinal structure constricts during the process of bacterial retention in the PIV region. The third process involves the relaxation of the intestine which permits bacterial colonization of the receptacle structure. The infective stages of juveniles also encompass additional features. The mouth and anal structures close which imply the cessation of feeding. Lastly, prior to emergence, a doubled layered cuticle structure is formed (Stock, 2015). The emergence of IJs from the insect host is induced by the production of carbon dioxide and ammonia. The elevated levels of these products are attributed to the overcrowding of the IJ population within the insect host. The progeny of infective juveniles together with their respective enteric bacteria emerge into the environment to seek new hosts and ultimately recommence their life cycles (Kaya and Stock, 1997).

1.6   Entomopathogenic bacteria (Genus characteristics of *Xenorhabdus* and *Photorhabdus*)

The *Xenorhabdus* and *Photorhabdus* genera of bacteria belong to the gamma subclass of Proteobacteria. Both genera are members of the Enterobacteriaceae family (Boemare and Akhurst, 2006). These bacterial cells are asporogenous and are characterized by distinct rod-shaped morphologies. The sizes of cells are highly variable and comprise filamentous structures with lengths of 30-50 µm. The cells are Gram-negative and are facultative anaerobes with fermentative and respiratory forms of metabolism (Stock, 2015). Both species are nutritionally classified as chemoorganotrophic heterotrophs. One unique physiological feature of bacterial

cells in both genera includes their existence in two main phenotypic forms. This phenomenon is referred to as phase variation (Owuama, 2001).

1.6.1 Phenotypic phase variation of *Xenorhabdus* and *Photorhabdus*

Henderson et al., (1999) defined the concept of phase variation as the high frequency of on to off switching of phenotypic expression. The variant forms include phase I (primary) and II (secondary) cells. However, there are no differences in the DNA integrity of both phenotypic variants (Akhurst et al., 1992). Owuama (2001) suggested that phase shifting was attributed to an epigenetic regulatory mechanism. The phenotypic transition occurs spontaneously during the stationary phase of bacterial growth. The properties of phase I and II cells exhibit significant differences which are described in table (1) (Boemare and Akhurst, 2006).

Table 1.1: The phase variant characteristics of *Xenorhabdus* and *Photorhabdus* bacteria.

Characteristics	Phase Variant I	Phase variant II
Colonial properties		
Morphology	Mucoid	Smooth
Dye adsorption	+	-
Pigmentation	+	- or different
Motility	+	-
Swarming	+	-
Cytological properties		
Erythrocyte agglutination	+	-
Insect haemocyte agglutination	+	+
Structural properties		
Fimbriae	+	- or reduced
Glycocalyx thickening	++	+
Flagella	+	- or reduced
Polycrystalline inclusions	+	-
opnA and opnB proteins	+	-

Enzymatic activities		
Respiratory enzymes	+	+ +
Lecithinase	+ +	+
Protease	+ +	+
***In vivo* luminescence**	+ +	+

Symbols: (+ positive for the trait, ++ means that expression is greater, - negative for the trait)

References :(Boemare and Akhurst, 2006; Owuama, 2001).

The phenotypic traits exhibited by phase I variants becomes reduced or absent after the phenotypic switch into phase II variants cells. Boemare (2002) provided a rationale for the reduction in the number of phenotypic traits of secondary cells. The author postulated that phase II variant cells may represent the free-living ancestral strain of the bacteria. Smigielski et al., (1994) noticed higher respiratory enzyme activities and more efficient nutrient uptake abilities by phase II cells when compared to the cells of primary phase. The authors suggested that phase II variants represent a form of the bacteria which may survive outside of their nematode hosts. Owuama (2001) also concurred with this finding when he noticed that phase II cells adapted more efficiently as free-living microorganisms when compared to the primary cell variants. The mechanisms of phase shift induction are not entirely understood. However, Owuama (2001) postulated that the process could be a response to the changes in the environment.

1.6.2 The proposed mechanism of phase shifting

Owuama (2001) conducted an *in vitro* bacterial culture experiment and observed the induction of phase shifting by environmental factors. The author suggested that low salt ion concentrations and osmolarity were two environmental determinants that contributed to the phenotypic switching of primary cell variants into secondary cell variants. He then compared these findings to the *in vivo* conditions which are expressed in the gut region of the free-living infective juvenile. The author observed that low osmolarity and salt ion concentrations were expressed in the gut of the free-living IJ. Furthermore, Owuama (2001) observed that the gut region of the free-living infective juvenile was colonized by a small proportion of phase II phenotypic cell

variants. Stock (2015) described the phase shifting phenomenon as a process that remains reversible as significant frequencies.

1.6.3   The reversion of secondary cells into primary cell variants

Owuama (2001) observed the ability of reversion by phase II variant cells into phase I cells following the restoration of salt ion conditions and nutrient resources. He then postulated that high levels of nutrients and salt ion concentrations were required for the survival of phase I variant cells. These conditions were found to be expressed in the haemocoel of the insect. Remarkably, the haemocoel is regarded as the first site where primary phase cells are delivered by the infective juvenile (Owuama, 2001). The primary cells colonize this region of the insect host and are best suited to the conditions which are expressed in the haemocoel as the cells thrive through rapid proliferation. The instigation of insect host mortality by these cell variants elucidates the nature of their roles in symbiosis. According to Finlay and Falkow (1989) the pathogenic nature of primary cell variants are expressed through their primary cell constituents and physiological products.

1.6.4   Phase I variants (Physiology and cell composition)

The primary cells comprise cellular appendage structures which are known as fimbriae or alternatively pili. These structures are peritrichously arranged and comprise diameters of 6-7 nm. According to Forst and Clarke (2002), the fimbriae structures possess attachment properties which facilitate the specific binding of bacterial cells to the gut epithelia of the nematode. This interaction supports the rapid colonization and retention of bacterial cells within the gut of the infective juvenile (Forst and Clarke, 2002). One other critical constituent of primary cells are the flagella structures. These structures enable cell movement and largely contribute to the swimming and swarming motions of the bacterial cells (Forst and Clarke, 2002). Owuama (2001) suggested that the presence of these structures permits the coordinated migration of cells which may ultimately contribute to the effective colonization of the insect host. The surface components of the outer membrane comprise a crucial polysaccharide. This polysaccharide has been implicated in the initial stages of host infection.

1.6.5 Lipopolysaccharides

Lipopolysaccharides (LPS) are endotoxins which are produced by the bacteria. These molecules are considered as the immunodominant antigens of *Xenorhabdus* and *Photorhabdus* species. The LPS molecules are expressed as haemocytotoxins in the insect host. Their roles have been elucidated in the avoidance of cellular elimination (Owuama, 2001). The molecules perform this function by evoking damage to the insect haemocytes. The LPS molecules suppress the phenoloxidase pathway of the host and trigger the degradation of the lipid moieties of the insect which serves as the primary source of antimicrobial peptides (Dowds and Peters, 2002).The primary cell variants also synthesize toxic physiological products which are essential for host infection. Amongst the different physiological products produced by the primary bacterial cells, the exoenzyme protease expresses the highest extents of virulence against the insect host (Owuama, 2001).

1.6.6 Proteinases

The primary bacterial cells express elevated levels of proteases for 48 hours following initial infection. The activity of the exoenenzyme contributes to lethal toxemia which ultimately ensures insect host mortality. The symptoms of toxemia include insect paralysis, atony and the cessation of feeding. The metamorphosis of the larvae becomes disturbed at the pupae stage of development. This is attributed to the activity of the exoenzyme which prevents cocoon synthesis. Owuama (2001) postulated that the disruption of metamorphosis may also affect the hormonal system of the insect. Lastly, the activity of proteases creates histological lesions in the insect host. The lesions may appear as dark tissue debris in the thorax region of the host and irregular necrotic spots in the extra and intra cellular regions of the hind gut.

Forst and Clarke (2002) suggested that multiple protease enzymes may be expressed by primary cells and function in the hydrolysis of insect proteins. The authors stated that the hydrolysis process provides the nutrient resources that are required for the development of the nematode and bacteria. Apart from proteinases, additional exoenzymes may be required for the bio-conversion of the insect host.

1.6.7 Lecithinase and Lipases

The enzyme lecithinase is produced by the primary cells of *Xenorhabdus* and *Photorhabdus* species of bacteria. According to Forst and Clarke (2002), this enzyme functions in the breakdown process of insect phospholipids which ultimately supplies the lipid sources that are required for nematode growth. The lipase enzymes are extracellular proteins which are produced by the bacteria. This enzyme is expressed in high levels during the stationary phase of bacterial growth and possesses entomotoxic activity toward the insect larvae. These physiological products which are produced by the primary cell variants provide suitable growth conditions for nematode establishment and reproduction (Boemare, 2002).

Akhurst (1980) observed a notable difference in the reproductive ability of nematodes with both cell variants. During an *in vitro* experiment, Akhurst (1980) noticed that the infective juvenile production increased by 3 and 7 folds following the *in vitro* culture experiment on the primary cells of *Photorhabdus* and *Xenorhabdus* species respectively. The establishment and reproduction of EPNs may be supported by one other fundamental characteristic which is only expressed by primary cell variants. This characteristic comprises the production of secondary metabolites.

1.6.8 Secondary metabolite production

A study conducted by Webster et al., (2002) showed that over 30 bioactive secondary metabolites are produced by the primary cells of *Xenorhabdus* and *Photorhabdus* species. These metabolites are structurally and chemically diverse compounds. The metabolites which are produced by *Xenorhabdus* species are categorized into one of the large groups which include the xenorhabdins, xenocoumacins xenorxides and indoles. The two main metabolite groups of *Photorhabdus* species include the anthraquinones and hydroxystilbenes. The bioactivities of the metabolites produced from both species include antimicrobial and antimycotic activities. The metabolites function mainly through the inhibition of the ribonucleic acid (RNA) and protein synthesis pathways of target microorganisms. This process ensures the preservation of the insect cadaver which ultimately prevents the putrefaction of the host (Webster et al., 2002). Apart from the production of secondary metabolites by primary phase cells, the virulent nature of the

bacteria may also be endorsed by their capabilities of overcoming a well-established immune system of the insect host (Owuama, 2001; Dowds and Peters, 2002).

1.7   Immunological status of the insect

The insect comprises two lines of defense which functions co-operatively in the removal of invasive bacterial cells from the circulation system of the host. The defense mechanisms of the insect host comprise the cellular and humoral responses. The cellular response occurs by the mediation of circulating haemocytes. The insect haemocytes comprises two main classes which includes the plasmatocytes and granular cells (Owuama, 2001). These cells function in the recognition of alterations to self and foreign targets. The recognition of non-self occurs by the direct interaction with haemocytes or alternatively through a binding event which includes surface receptors of haemocytes and the molecules of the foreign target. The haemocyte-mediated defense becomes activated. This process may be regulated by the signaling molecules and effector proteins which controls the events of cell adhesion and cytotoxicity (Dowds and Peters, 2002; Lavine and Strand, 2002).

The forms of cellular responses include phagocytosis, encapsulation and nodule formation (Dowds and Peters, 2002; Lavine and Strand, 2002). The process of phagocytosis becomes induced by a series of intercellular and intracellular signaling events. The detection of low bacterial colony forming units induces the phagocytosis form of the cellular response. However, a large bacterial cell count induces one other expeditious form of the cellular response. This form is recognized as nodulation. The process of nodulation comprises the entrapment and sequestration of foreign targets by the haemocytes of the insect into structures which are termed as nodules (Lavine and Strand, 2002). The mechanism of nodulation entails the lengthening of the cell surface of haemocytes into actin rich membrane projections. These structures are designated the term, filopodia and express strong adhesive properties which enable efficient attachments to the bacterial cells. The haemocytes together with their entrapped foreign microorganisms aggregate into nodules. These nodules then attach to the fat tissue of the host which enables departure from the general circulation (Owuama, 2001). Alternatively, the humoral response may be activated.

1.7.1 The humoral response of the insect

The humoral response involves a sequence of successive enzyme reactions which holistically contributes to the activation of the melanization process. During the humoral response, the circulating haemocytes trigger an enzymatic cascade which induces the prophenoloxidase pathway. The activation of this pathway subsequently triggers melanin deposition. The deposition of the melanin compound functions in the entrapment of pathogens around the sites of infection. Furthermore, the activation of melanization stimulates the production of microbicidal reactive oxygen species (ROS) which functions interactively with the antimicrobial peptides produced by the insect host. Together, all these factors function in the elimination of the invasive bacterial cells (Tokura et al., 2014; Lavine and Strand, 2002). The study conducted by Owuama (2001) showed that 92 % of the bacterial cell population was removed by the immune system of the insect host after 6 hours of entrance. However, the author observed that after a period of 2 hours, the bacteria re-entered and the population escalated to 44 % in the haemolymph of the insect.

1.7.2 The re-emergence of the bacteria

According to Dowds and Peters (2002), a proportion of the bacteria proliferate within the nodule structures of the host. The authors inferred that the multiplication of the bacteria within these structures served as a re-entry approach into the haemolymph of the insect. The process of bacterial proliferation proceeds after the re-emergence of the cells which eventually increases the size of the population. The bacterial load overwhelms the immune system of the insect host thereby resulting in host susceptibility and eventually mortality (Dowds and Peters, 2002). A broad range of susceptible insects are host targets for EPNs and their symbiotic bacteria.

1.8 The diverse insect orders targeted by EPNs

The majority of insect species which are host targets for EPNs belong to one of the five insect orders. These orders include the Coleopteran, Lepidopteran, Dipteran and Orthopteran orders (Dowds and Peters, 2002). According to Hazir et al., (2004), the diverse target host ranges of natural enemies are considered to be a desirable trait in biological control.

1.8.1 Biological control

Hazir et al., (2004) defined the concept of biological control as the maintenance of the host population in low numbers by the action of natural enemies. The authors divided this concept into two categories which includes natural and applied biological control. The first category involves the reduction of the native host population by native or co-evolved natural enemies. The second category which is applied biological control involves human intervention. This intervention serves to enhance the activities of the natural enemies. Applied biological control is subdivided into classical and augmentative biological control.

1.8.2 Classical and augmentative biological control

Classical biological control encompasses the regulation of exotic or native host populations by the introduction of exotic natural enemies. The augmentative approach of applied biological control involves maximizing the effectiveness of the natural enemies. This process is facilitated by human intervention which may entail either the manipulation of the environment or the natural enemies. The manipulation of the natural enemies may be achieved by two approaches. These are the inoculative and inundative releases of the natural enemies into the environment (Hazir et al., 2004).

1.8.3 Inoculative and Inundative releases

The inoculative approach encompasses the seasonal release of natural enemies in small numbers around the pest infected sites. This process commences when the pest population levels are low. In this way, a long-term control measure may be achieved as the host population is prevented from advancing to levels which may impose economic threats. Contrastingly, the inundative approach entails the release of the natural enemies in overwhelming numbers. This method is accompanied by the expectation of an immediate reduction within the pest population (Hazir et al., 2004).

The EPNs have been utilized in both the classical and augmentative forms of biological control programs. However, the prospects of EPNs as inundative biological control agents have become the focus of applied research. A study conducted by Hazir et al., (2004) showed that the EPNs in

the genera, *Steinernema* and *Heterorhabditis* have been highly effective especially as inundatively applied biological control agents of soil borne insect pests.

1.9   The attributes of EPNs as biological control agents of insect pests

According to Hazir et al., (2004), the soil environment may be regarded as one of the most challenging environments to achieve successful biological control. The EPNs however, are naturally occurring inhabitants of the soil which ultimately contributes to their effectiveness as biological control agents of soil borne insect pests (Koppenhöfer, 2000). The activities of the EPNs are extended beyond the surface of the soil into the foliar, subterranean and cryptic habitats.

The foliar and subterranean habitats comprise the regions which are above and below the surface of the soil. The cryptic environments include the regions of the plant which are infected by insect-boring pests (van Zyl and Malan, 2014; Hazir et al., 2004). The inundative application of EPNs has negligible effects on non-target hosts which implied that the safety of vertebrates and plants remained uncompromised (Koppenhöffer, 2000). Also, the EPNs recycle within the environment and the majority of species have demonstrated compatibility with many insecticides, herbicides and fungicides. Lastly, the mass propagation of the EPNs occurs by simplistic methods (Hazir et al., 2004). The traditional approach of chemical pesticide usage played a pivotal role in regulating the key insect pests of horticultural and agricultural crops. However, the long term application of chemical pesticides gradually introduces threats which may have detrimental effects on the natural environment (van Zyl and Malan, 2014).

1.9.1   The consequences chemical pesticide usage

The long-term implementation of chemical pesticides may lead to the development of insect host resistance. Two possible outcomes may occur in this regard. The first outcome includes the resurgence of the target insect pests and the second involves the outbreak of secondary pests. Furthermore, the persistence of pesticide residues on agricultural crops may contribute to the contamination of the natural resources of the environment which include the soil, ground water and air. This imposes further health risks onto humans and animals. A reduction in the

availability of chemical pesticides has been achieved due to the stringent safety regulation requirements. The reduction was attained by the prohibition of many chemical pesticide products from the world markets (van Zyl and Malan, 2014). The integrated pest management programs encourage the use of natural enemies as first line defense strategies against the target insect pests (Ehlers, 2006). Since the discovery of the first EPN species in 1923, applied researchers focused on the potential of EPNs as biological control agents with their ultimate goal to formulate and commercialize different EPN products (Poinar and Grewal, 2012).

1.10   The historical advancements in commercialization

The first account of EPNs as biological control agents was documented in New Jersey. According to Poinar and Grewal (2012), Glaser and colleagues investigated the species, *Steinernema glaseri* against the Japanese beetles (*Popillia japonica*) in New Jersey. The success of this investigation led to the mass propagation and inundative release of this species into the field by tanks which were motor driven. By the year, 1981, different EPN species were propagated within the model insect host organism, *Galleria mellonella* and commercialized against the different garden insect pests. A year later, the BR supply company raised the species *Steinernema carpocapsae* against mole crickets. Furthermore, this company produced a product called Neocide which targeted the carpenter worm. In the year 1983, the company Biotechnology Australia commercialized the product Otinem which targeted black vine beetles in the European and Australian countries (Poinar and Grewal, 2012).The two other countries where the EPNs have been commercialized include Japan and China (van Zyl and Malan, 2014). The progresses that were made in the development of EPN formulations have also been substantial (Shapiro-Ilan et al., 2010).

1.10.1 The advances in the formulation technology

The first EPN formulation strategy was implemented in 1979. This strategy encompassed the placement of EPNs in or on inert solid and liquid carriers. This type of formulation ensured that the activity and mobility of the EPNs remained unrestricted in or on their respective substrates. These carriers included poly-urethane sponges, peat and vermiculite (Grewal, 2002). The progressive series of investigative studies within this research discipline led to a comprehensive

understanding of the storage requirements for each individual EPN species (van Zyl and Malan, 2014).

The formulations were then modified to optimize the temperature, pH and osmolarity requirements of the EPNs which comprised the different genera. It was then discovered that the slow rate of water loss resulted in the conservation of lipid reserves within the EPNs. According to van Zyl and Malan (2014), these reserves influenced the viability and pathogenicity of the EPNs. The temperature tolerance thresholds of the EPNs were also enhanced. Consequently, these findings led to the development of dry or partially desiccated EPN formulations. These products include the polyacrylamide gels, water-dispersible granules and powders (Grewal, 2002). These EPN products were marketed within the different sectors of agriculture.

1.10.2 The application of EPN products in the different areas of agriculture

Over the past three decades, extensive progresses in research and development have made EPNs available for the application in agriculture/horticulture. The different sectors in agriculture where EPNs have been applied include the cranberry bogs, citrus groves and mushroom plantations. Also, the applications of EPNs have been extended to ornamental plants, lawns and turf (Lacey and Georgis, 2012). The table below describes the insect pests which have been biologically controlled by the different commercially available products of EPNs.

Table 1.2: The list of insect pests which targeted different agricultural crops and were biologically controlled by application of different EPN species.

Targeted crops	Common name of insect pest	Scientific name of insect pest	Entomopathogenic nematode species [1]
Ornamentals (Nurseries and greenhouses)	Black vine weevil	*Otiorhynchus sulcatus*	Sc Sg
	Diaprepes root weevil	*Diaprepes abbreviatus*	Sr
	Leaf miner	*Liriomyza spp.*	Sc and Sf
	Billbug	*Sphenophorus* spp.	Sc and Hb
	Black cutworm	*Agrotis ipsilon*	Sc

Lawns and Turf	Mole cricket	*Scapteriscus* spp	Sscap, Sc and Sr
	Scarab grub	Coleoptera: Scarabaeidae	Sc, Sg, Ss, Hz and Hb
Cranberry bogs	Cranberry girdler	*Chrysoteuchia topiaria*	Sc
Citrus groves	Citrus root weevil	*Pachnaeus* spp	Hb and Sr
Mushrooms	Fungus gnats	Diptera: Sciaridae	Hb and Sf

Reproduced from Shapiro-Ilan, et al., (2014) and Lacey & Georgis, (2012)

[1]The nematode species were abbreviated by the following: S = *Steinernema*, H = *Heterorhabditis*.

Sc = *S. carpocapsae*, Sg = *S. glaseri*, Sr = *S. riborave*, Sf = *S. feltiae*, Sscap = *S. scapterisci*, Ss = *S. scarabaei*.

Hb = *H. bacteriophora*, Hz = *H. zelandica*.

1.10.3 The efficacies of EPNs

The EPNs infect their target hosts with different efficacies. According to a study conducted by Shapiro et al., (2014), the EPNs suppressed their target hosts by at least 75 % in field trials and greenhouse experiments. According to Koppenhöffer (2000), some experiments demonstrated little or no impact on their target pest populations whereas other experiments produced high efficacious results. The author explained that the efficacies of EPNs are species specific. Furthermore, the efficacies of the EPNs are affected by the target host species as the levels of susceptibility differ between the five insect orders. Kaya and Koppenhofer (2004) stated that understanding the match between the biology and ecology of both the target host and the EPN is crucial to obtain significant reductions within the pest population. Also, to achieve optimum efficacy, the application of the EPNs must be co-ordinated with the appropriate developmental stages of the insect. The abiotic components of the environment may also influence the success of EPN application (Shapiro-Ilan et al., 2012).

1.11 The abiotic factors

The abiotic components of the environment include the pH, moisture and texture of the soil. The other components include relative humidity, ultra violet (UV) radiation and temperature. The optimum pH requirements of the soil range from pH levels of 4-8. Shapiro-Il et al., (2012) stated that strong alkaline pH levels may be detrimental to the EPNs. An adequate level of moisture is

required for the persistence, pathogenicity and movement of the EPNs in the soil. However, the restriction of EPN movement and oxygen depravity may be found in soils which are saturated with high levels of water. The texture and structure of the soil may also determine the survival and migratory abilities of the EPNs. Glazer (2002) stated that dense soil types which include the soils that comprise high organic matter and clay contents, limit oxygen availability. Consequently, the reduction in the aeration capacity of the soil affects the survival of the EPNs as they are facultative aerobic organisms (van Zyl and Malan, 2014). Lastly, the optimum temperature requirements for the reproduction and infection of the EPNs are species or strain specific (Shapiro-Ilan et al., 2012). Although the successes of applied EPNs are influenced by the different abiotic components, the EPNs have also demonstrated potential to adapt to new environmental conditions. The isolation of the different strains and species of EPNs from exotic geographic locations suggest their adaptive nature. The adaptive nature or survival strategies of the EPNs are related to their outstanding behavioral traits and physiological responses (Stock, 2015).

1.12   Behavior (Desiccation tolerance and avoidance)

According to van Zyl and Malan (2014), the behaviors of the EPNs are complex and may be influenced by the voluntary or external chemical and physical stimuli. One stimulus includes the desiccation of the EPNs. The concept of desiccation may be defined as the gradual loss of water from the cuticle structure of the infective juveniles. The differences which are present in the cuticle structure of each nematode species and strain permits varied extents of survival (Grewal, 2002). Two strategies are employed by the EPNs which enable their survival during the period of desiccation. The EPNs may adopt one of the two strategies to survive the period of desiccation. These strategies involve desiccation avoidance and tolerance. For the purposes of my research project, I will discuss the EPNs which comprise the genus *Steinernema*.

The desiccation avoidance behavior is demonstrated by the aggregation of conspecific EPN populations or by migration into the deeper parts of the soil. The moisture of the soil is positively correlated to the soil depth. The study conducted by Grewal (2002) showed that the species *S. feltiae* and *S. riborave* exhibited desiccation avoidance strategies. Other EPN species may exhibit the desiccation tolerance strategy. This strategy involves the tight coil formation of the individual

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EPNs. This strategy limits the areas of the cuticle which are exposed to air thereby reducing significant water loss (Glazer, 2002). During the process of desiccation tolerance, the metabolism of the EPNs cease. This state is known as anhydrobiosis (van Zyl and Malan, 2014).

1.12.1 Anhydrobiosis

The anhydrobiosis process involves a biochemical response to desiccation. This process is accompanied by the metabolic arrest of the EPNs which ultimately aids in the conservation of the energy reserves (van Zyl and Malan, 2014). Consequently, the process enhances the longevity of the EPNs. Glazer (2002) observed the elevated production of polyols and sugars in anhydrobiotic species. The author postulated that the production of these compounds served as protectants for the intracellular proteins and membranes of the EPNs. These findings were coherent with the study which was conducted by Grewal et al., (2006). The authors investigated the ability of the EPNs to retain their infective nature after desiccation and rehydration. The infectivity of the nematodes was conserved and in addition to the production of sugars and polyols, increased levels of trehalose were also obtained. The authors elucidated the role of this molecule in membrane stability. The stabilization process occurred by the reaction which involved the hydroxyl group of the trehalose molecule and the phosphate group of the phospholipid layer. This molecule displaced the original water molecule. The initial reaction ensured that the membranes maintained a gel-like composition. Conversely, the reaction of trehalose ensured that the biological membranes of the EPNs were maintained in a fluid state thereby ultimately conserving the integrity of the membranes. A series of studies investigated the lengths to which the EPNs within the *Steinernema* genus survived desiccated conditions. The authors inferred that these lengths were substantial. The temperature component of the environment may be regarded as one other crucial factor which affects the efficacy of the applied EPNs.

1.13   Temperature

The temperature component of the abiotic environment is regarded as one of the key aspects which affect the activity and survival of the EPNs (Selvan et al., 1996; Hill et al., 2015). The persistence of the EPNs occurs at diverse temperature ranges. Conversely, the temperature requirements for the different biological processes of EPNs are well-defined. These processes

include the infectivity, establishment and reproduction of the EPNs. The individual EPN species has unique temperature activity range requirements for each biological process. This concept is regarded as the thermal niche breadth. According to Grewal et al., (2006), the thermal niche breadths of different EPN species were conserved.

1.13.1 The infective and reproductive niche breadths

The study conducted by Grewal et al., (1994) investigated the thermal niche breadths for the infectivity, establishment and reproduction of 6 different EPN species. The table below summarizes the findings of the study.

Table 1.3: The infective, reproductive and establishment thermal niche breadths for the different EPNs in the genus, *Steinernema*.

EPN species[1]	Thermal niche breadth (ºC)		
	Infection	**Establishment**	**Reproduction**
S. arenarium	10-35º C	10-32º C	12-32º C
S. feltiae	08-30º C	08-30º C	10-25º C
S. carpocapsae	10-32º C	12-32º C	12-32º C
S.glaseri	10-37º C	10-37º C	12-32º C
S. riborave	10-39º C	10-39º C	20-35º C
S. scapterisci	10-35º C	20-32º C	20-32º C

Adapted from Grewal et al., (1994)

[1]The EPN species were abbreviated as follows: S = *Steinernema*

According to Molyneux (1986), the thermal niche breadths of the EPNs were conserved and reflected the conditions of their original geographical location. This claim was supported by Grewal et al., (1994) and Hazir et al., (2001). The study which was conducted by Grewal et al., (1994) investigated isolates of three species from different geographical locations. The authors observed a difference in the reproductive potential of the isolates but the temperature range activities (thermal niche breadths) for reproduction, infection and establishment were the same. Furthermore, this finding was coherent with Hazir et al., (2001). The author observed the infective and reproductive thermal niche breadths for 5 isolates of the species *S. feltiae*. These

isolates were also obtained from different geographical regions. The author observed no differences in the reproductive and infective thermal niche breadths of the isolates. These findings led the authors to believe that EPNs maintained defined thermal niche breadths which was unaffected by their localities. However, this finding was challenged by comprehensive investigations. Grewal et al., (2014) stated that the temperature tolerances within the thermal niche breadths and temperature activity ranges of the EPNs for each biological process may be modified.

The study conducted by Grewal et al., (1996) demonstrated that thermal tolerance and thermal activity ranges were malleable. The authors investigated the temperature shifts in tolerance and activity of two individual EPN species after genetically selecting the strains at the two temperature extremes. The warm and cold temperature extreme values were 30º C and 15º C respectively. An extension in thermal limits for infection was observed from both warm and cold temperature selected strains. Also, an extension in thermal tolerance for establishment was observed from the strains that were propagated at cold temperatures. The strains which were selected at warm temperatures demonstrated improvements in the establishment of their population within the original thermal niche breadth. The reproductive thermal niche breadth was also extended. The authors observed that the improvements in performance and the extension in the thermal niche breadths occurred simultaneously when the strains were propagated at their respective selection temperatures. The findings of Grewal et al., (1996) were coherent with the study conducted by Jagdale and Gordon (1998).

Jagdale and Gordon (1998) observed that thermal limits were affected by temperature at which the EPNs were propagated. The authors observed that the propagation of the EPNs at warm temperature regimes, improved the survival at upper thermal limits whilst simultaneously reducing their tolerance to the lower temperature thresholds. Conversely, the propagation of the EPNs at cold temperature regimes improved their tolerances to low temperatures whilst diminishing their tolerance to warm temperature regimes. These authors then inferred that the survival and infectivity of the EPNs was modified by their propagation temperatures. Together, these findings suggested that propagating the EPNs at constant temperatures influenced their thermal tolerances and extended the thermal niche breadths for each biological process. Grewal

et al., (2006) also concurred with these findings and suggested that temperature tolerances of the EPNs may be modified through genetic selection by propagating the species at constant temperatures. Grewal et al., (1996) postulated four outcomes to the genetic adaptation of the EPNs.

1.13.2 Four outcomes to genetic adaptation

Grewal et al., (1996) stated that the EPN population may adapt genetically to novel temperatures provided that the propagation time is sufficient and genetic variability is present. The authors presented four outcomes to genetic adaptation. The first outcome includes the shift in thermal tolerances towards the temperature which was selected for propagation. The second outcome involves a non-shift in thermal tolerance but improvements in the performance at novel temperatures. This outcome is accompanied by a decline in the performance of the adapted population in ancestral environments. The third outcome suggested the non-shift in the thermal tolerance of the EPNs and improved performances at novel temperature without decreased efficacies in the ancestral environments. The last outcome involved the shift in the thermal niche breadths of the EPNs. This outcome ensures that the performance of the EPNs at experimental temperatures remains unaffected. Three outcomes have been demonstrated in the studies mentioned above. Grewal et al., (1996) stated that the improvements in the performance of the EPNs following the process of novel temperature selection may be attributed to either genetic adaptation or phenotypic acclimation.

1.13.3 Physiological mechanisms to adaptation (unsaturated fatty acids)

The EPNs employ different physiological mechanisms to adapt to thermal changes in the environment. One of the physiological mechanisms involves the shift in the ratios of unsaturated and saturated fatty acids of storage and phospholipids. The maintenance of membrane fluidity at low temperatures is achieved by an increase in the proportion of unsaturated fatty acids in the phospholipid layer. A study conducted by Fodor et al., (1994) compared the proportion of unsaturated fatty acids in phospholipids at 18º C and 25º C. The authors observed increased amounts of the unsaturated fatty acids in phospholipids at 18º C in contrast to the proportions which were present at 25º C. Furthermore, the arrangement of the phospholipid molecules within the cell membrane composition was less ordered at 18º C. Jagdale and Gordon (1997) observed

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that the index for unsaturation in phospholipids increased with a decline in environmental temperatures. The authors attributed this finding to the increase in polyunsaturated fatty acids and decrease in saturated fatty acids such as palmitic and stearic acids. The authors also observed the production of neutral lipids during low temperatures which served as energy substrates (Grewal et al., 2006). Also different metabolic enzymes may be highly expressed during seasonal changes.

1.13.4 Metabolic enzymes

Two enzymes which influence two major pathways in metabolism are expressed in elevated levels during cold temperatures. These enzymes include the hexokinase and glucose-6-phosphate dehydrogenases which functions in the initiation of glycolysis and the pentose phosphate pathway respectively. The elevated enzyme expression may contribute to increased energy metabolism and lipid synthesis of the EPNs. Grewal et al., (2006) observed increased expression of these two enzymes in the species *S. feltiae*, *S. riborave* and *S. carpocapsae* during propagation at low temperatures. Furthermore, the authors observed that the Michaelis-Menten (K_m) constant values for both enzymes were the lowest. This finding led the authors to believe that the modification of the kinetic properties of metabolically significant enzymes may be one strategy used by the EPNs to adapt to seasonal changes. Furthermore, the EPNs synthesize isoenzymes of metabolic enzymes which facilitate the adaptation to temperature changes. The adaptation process of the EPNs during adverse temperature conditions may also be facilitated by the production of the global stress response factor. The global stress response factor is known as the trehalose molecule (Jain and Roy, 2008).

1.13.5 The global stress responsive factor

The production of the trehalose molecule was observed in different nematode species during the process of acclimation to warm and cold temperatures. The accumulation of trehalose was dependent on the temperatures at which the EPNs were propagated and the individual species which were investigated. Jagdale and Grewal (2003) observed rapid increases in trehalose production by 1.5-fold, 3.5-fold and 0.3-fold for *S. carpocapsae, S. feltiae and S. riborave* respectively during acclimation at 5º C. Trehalose accumulation increased significantly from basal production levels during acclimation at 35º C. During warm temperature acclimation, the

accumulation of trehalose was correlated with improvements in thermal tolerance and virulence of *S. carpocapsae*. Furthermore, the heat tolerance of *S. feltiae* was enhanced with significant improvements in the virulence at warm temperatures when compared to the non-acclimatized species. Lastly, the warm temperature acclimation of the species *S. riborave* was correlated with increased trehalose production. This molecule positively enhanced the freeze tolerant abilities of the species and improved the virulent efficiencies of the EPNs. The heat and cold shock responses induces the process of trehalose metabolism by the activation of two enzymes. These include the trehalose-6-phosphate-synthase (T6PS) and the trehalase enzymes. Jagdale et al., (2005) observed that the expression of these enzymes decreased significantly when the culture temperatures of the EPNs were restored. These findings suggested that the production of trehalose was a general response to thermal stresses. These forms of EPN plasticity allows adverse environmental conditions to be buffered against (Hill et al., 2015).

1.14 Climatic conditions of South Africa

South Africa has experienced extreme temperature fluctuations over the past few years. During the early summer in 2015, the region of Gauteng experienced three consecutive heat waves. Furthermore, spring thunderstorms and droughts were reported in different parts of the country (Wright et al., 2015). The EPNs have not yet been formulated as commercial products for the South African agricultural market. However, naturally occurring EPN populations have been previously isolated and identified (van Zyl and Malan, 2014). Establishing the groundwork for the commercialization of endemic strains remains one of the long term goals in this research discipline. One fundamental principle of EPN product formulation involves understanding the responses of the EPNs to temperatures. Another good characteristic of the formulation products involves the maximization of EPN activity at the target sites. For the past few years, the conventional EPN techniques and behavioral studies have contributed significantly to recognizing the potential of the EPNs as biological control agents. However more recently, a powerful tool has been introduced into this research discipline by molecular geneticists. This technique is called whole genome sequencing.

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1.15   Genomic sequencing

The whole genome sequence of an organism defines the framework which organizes and directs the genetic content of an individual. This information may be considered as one of the most valuable forms of knowledge which may be obtained for any life form. The whole genome of an organism provides information which pertains to the ecology, organismal biology, life history traits and evolution. One major advantage of this technique involves harboring the complete variety of genes from which the organism is derived. Furthermore, knowledge about the structural and regulatory elements which constitute the genome may be acquired (Dillman et al., 2012). The variation which exists within and amongst different species and strains may be obtained. This information permits comparative genomic studies which may shed insight into the mechanisms of gene acquisition. The whole genome sequencing technique has unlocked new avenues of research which may contribute to the improvements of the EPN-bacterial partnership in the biological control agents of insect pests. Improvements may be obtained in the areas of bacterial virulence, infective juvenile longevity, resistance of insect immunity, trait stability and EPN colonization and persistence and lastly tolerance to temperature extremes (Dillman et al., 2012).

1.16   Aims and Objectives

1.16.1 Research Rationale

Different endemic species and strains of EPNs occur naturally within the different regions of South Africa. The environmental temperatures often fluctuate rapidly in different regions of the country especially within the region of Gauteng. The effect of rapid temperature changes on the biological processes of the EPNs requires comprehensive evaluation. The biological processes include the infection, recovery and yield of the EPN population. These processes are considered as key determinants which influences the efficacy of the EPNs in the field application. Over the past years, the EPNs remained the prime focus of local and global temperature-related investigations. Only a minority of research investigations concentrated on evaluating the role(s) of the bacteria during temperature adaptation. Grewal et al., (1996) suggested that adaptation to temperature regimes may occur in both symbiotic partners. These roles may elucidated by the application of conventional EPN techniques, however to ascertain the full potential of the bacteria as insect pathogens, novel genomic techniques are required.

1.16.2 Research aims

1. To evaluate the plasticity of a selected species EPNs in the areas of host infection, infective juvenile recovery and yield in response to selected temperatures relative to the control temperature at which the EPN had been maintained in culture in the laboratory.

2. To ascertain the virulence of the bacteria against the insect host model, *G. mellonella* without the contributions of the EPN partner and to determine whether the virulence of the bacteria is maintained across the two experimental temperature regimes.

3. The third research aim involved the sequencing, assembly and annotation of the insect pathogenic bacteria associated with the EPN for the purpose of establishing the existence of genes which may be involved in temperature adaptation.

1.16.3 Research Objectives

1.1

- To successfully isolate and propagate a selected endemic EPN species from South African soils using the soil larval baiting and White trap techniques and the sand based pathogenicity confirmation assays respectively.

- To morphologically and molecularly characterize the taxonomic and phylogenetic affinities of the selected EPN species using Microscopy techniques, gene amplification of the small eukaryotic ribosomal sub-unit deoxyribonucleic acid (18S rDNA) region and phylogenetic computational methods respectively.

- To perform dose dependent sand based larval infection and mortality assays, White trap IJ recovery and IJ production enumeration techniques for assessing the plasticity of the EPN life cycle responses to acute temperature acclimation.

2.1

- To establish in cultures on selected culture media the phase I phenotypic variants of the bacteria isolated from the larval haemolymph extraction and using the streak plate techniques.

- To molecularly characterize the taxonomic and phylogenetic affinities of the bacterial colonies using the small prokaryotic ribosomal sub-unit deoxyribonucleic acid (16S rDNA) region for amplification and phylogenetic computational procedures respectively.

- To perform dose response assays by the intravenous administration of the bacterial inoculum into host insect larvae at the selected temperatures.

3.1

- To successfully extract genomic bacterial deoxyribonucleic acid (DNA) and perform high throughput paired end sequencing techniques.

- To perform quality control assessments of the sequence reads by using the FastQC algorithm.

- To trim and assemble the prokaryotic sequence reads using the Trimmomatic and SPAdes v 3.5 genome algorithms.

- To perform a functional genome annotation on the enteric bacteria using the National Center for Biotechnological Information / Prokaryotic Genome Annotation Pipeline (NCBI / PGAP).

CHAPTER 2: THE CHARACTERIZATION OF AN ENDEMIC EPN SPECIES AND ITS SYMBIOTIC BACTERIA.

2.1   INTRODUCTION

The first entomopathogenic nematode (EPN) species in South Africa was discovered and in Grahamstown within the Eastern Cape Province in 1953. Since this discovery, considerable research surveys were performed in the provinces of the Free State, Western and Eastern Cape, Kwa-Zulu Natal, Mpumalanga and Gauteng. Two novel endemic species which belong the genus *Steinernema* have been isolated and classified in South Africa. These include the species *Steinernema khoisanae* and *Steinernema citrae* (van Zyl and Malan, 2014). Van Zyl and Malan (2014) expressed the need for the identification of novel and endemic EPN species. The authors stated that the enotmopathogenic nematodes (EPNs) have not yet been formulated and commercialized as products for the South African market. One factor which limited the application of exotic EPN species and products in South Africa included the stringent regulation acts of the agricultural sector. This act required safety permits and complete impact studies for the importation of exotic species. The authors stated that the exotic species may not be suitable for local application since the species may not be acclimatized to the ecology of South Africa. Furthermore threats may be imposed to the native EPN populations which exist within the soil. Consequently, the identification of endemic EPN species remained critical especially when considering product formulation for agricultural application. The efficacies of EPNs regarding host infection may be regarded as species specific.

In South Africa, local surveys have identified different *Steinernema* species which have demonstrated infectivity against pests of deciduous fruit. These insect pests include the mealybug, the false codling moth, the banded fruit weevil and the codling moth. As stated in the literature review, the symbiotic bacteria contribute significantly to insect host mortality. The EPNs which comprise the genus *Steinernema* are symbiotically associated with *Xenorhabdus* species of bacteria. The bacterial species has evolved as a mutualistic symbiotic partner of the EPN and a pathogen of insect hosts. Good-rich-Blair and Clarke (2007) stated that the transition between these two roles may be stringently regulated. *Xenorhabdus* species of bacteria adapt and

exploit different host environments due to their sensitivity and rapid responsive abilities to changes in nutrient levels, temperature, pH and oxygen. The taxonomy of the genus was highlighted in the literature review (see section 1.3). According to Boemare and Akhurst (2006), the *Xenorhabdus* genus was the first to be described and all known species associated with the EPNs were classified into this genus. However, over time, the phenetic, fatty acid and chemotaxonomic differences between species gave rise to one other genus.

The conventional criterion for EPN and bacterial identification included morphological diagnostic techniques. However, the accuracy of the technique became limited due to the species specific phenotypic similarities. Supplementary techniques were required to discover the variation which existed between the different species (Darissa and Iraki, 2013). Molecular approaches have been widely adopted for the efficient and rapid characterization of the EPNs and bacteria. These techniques include the use of nucleotide sequence data. The data may not only serve as a diagnostic tool for taxonomic level classification but also possesses important information for phylogenetic inference (Stock, 2015).

2.2   METHODS AND MATERIALS

2.2.1   Collection of soil samples

The soil samples were collected from the Britz region within the North West Province (25°46'46.3"S 27°57'26.4"E). This grassland habitat hosted wild fig tree plants. Each soil sample was collected from a depth of 15 cm from the surface of the ground using a post-hole digger. 2 L ice cream tubs were filled with each soil sample. The volume of soil collected measured to 1000 cm^3. At this particular site, 6 samples were collected at random over an area of 2-4 m^2 (Kaya and Stock, 1997). Subsequently, the soil larval baiting technique was employed to extract the infective juvenile species from the soil samples.

2.2.2   The baiting technique

This technique was a modification of the original protocol which was founded by (Bedding and Akhurst, 1975). The technique involved elevating the moisture content of the soil to 8 % (w/v) by the additional of tap water. The addition of water enabled the viability and movement of the EPNs. Thereafter, 10 freshly reared last instar *G. mellonella* larvae were placed on the soil surface and the ice cream tubs were sealed, inverted and set aside on the bench top for 48 hours at 25º C. Subsequently, the dead insect larvae were removed from the soil tubs and the soil samples were replenished with fresh last instar *G. mellonella* to increase the IJ extraction efficiencies. This technique is regarded as one simplistic method which is cost efficient and possesses high EPN extraction efficiencies (Kopenhöffer, 2000).The dead larvae which showed symptoms of EPN infection were prepared for the White trap technique by surface sterilization. The sterilization process involved rinsing the cadavers with sterile distilled water and spraying the cadavers with 70 % (see appendix) ethanol.

2.2.3   The White trap technique

The apparatus for the White trap technique consisted of an inverted Petri dish lid (watch glass) which was positioned within a large Petri dish (100 × 15 mm). The watch glass was lined with a thin sheet of Whatman filter paper (55 mm) and the large Petri dish was filled with 10-15 ml of tap water. The water was added to ensure that the sheet of filter paper remained moistened. The dead larvae were then transferred onto the filter paper and incubated at 25º C for a period of 5 days. The infective juveniles (IJs) emerged from the insect cadavers and migrated into the film of

water. The IJs were then collected into 50 ml Falcon tubes for the successive experiments (Kaya and Stock, 1997).

2.2.4   Koch's postulates

The IJs were used to complete Koch's postulates to verify their role as the causative agents of larval mortality. For this technique, two components of the White trap were discarded. These components were the watch glass and the filter paper which contained the decayed larva. Thereafter, 40 g of autoclaved river sand was added into the Petri dish which contained the IJs which were submerged in the water film. These components were mixed gently with a sterile Pasteur pipette. 10 freshly reared last instar *G. mellonella* were transferred onto the surface of the sand and the Petri dish was sealed, inverted and incubated at 25º C for 72 hours. Thereafter, the dead larvae were removed using sterile forceps, surface sterilized by the methods mentioned in the baiting section (2.2.2.) and set onto White traps for subsequent infective juvenile (IJ) emergence. After the IJs emerged from the insect cadavers, they were propagated for several generations through the last instar *G. melonella*. The progeny of IJs were collected in 50 ml Falcon tubes for the subsequent characterization studies.

2.2.5   Microscopic analysis

The IJs were cultured *in vitro* on solid lipid agar into the respective adult stages. First and second generation males and females were separated based on characteristic features which were visualized with the aid of the dissecting light microscope. One characteristic feature involved the size of the EPNs. The body lengths of the adult females were larger than the males. Also, the posterior tail end of the male EPN resembled the shape of a hook whereas the tail morphologies of the females were elongated. Once the EPNs were separated according to their respective sexes, the nematodes were transferred into 50 ml Falcon tubes for the subsequent processes. The preparation for microscopy involved three key processes. These were, heat killing, fixing and cooling of the EPN specimens. The heat killing process was performed by placing the tube filled with nematodes into a 60º C water bath for 2 minutes. For the fixing process, 5 ml of the Triethanolamine Formalin (TAF) fixative (see appendix) was mixed with an equal volume of Ringers' solution (previously heated for 10 minutes at 60º C). This mixture was then added into the tubes which contained the adult EPNs. The succeeding step involved the relaxation of the EPNs by cooling the specimens in the refrigerator at 4º C for 15 minutes. Simultaneously, 7 ml

of the TAF fixative was heated for 15 minutes in a water bath which was set to 65º C. The TAF solution was then added into each sample tube and incubated for 48 hours at 25º C. The specimens were thereafter mounted onto microscope slides for microscopic analysis. The specimens visualized and imaged on the Olympus BX63/OFM microscope fitted with a Nikon camera produced in Germany using different observation methods.

2.3   Molecular characterization of the EPNS

2.3.1   Genomic DNA extraction

The IJs were allowed to sediment by gravity in 50 ml Falcon tubes. The excess water was removed using sterile Pasteur pipettes and thereafter discarded. The IJs were surface sterilized by adding 5 ml of 0.1 % (w/v) sodium hypochlorite solution (see appendix I). The surface sterilization process was performed for 3 hours at 25º C. Subsequently, the IJs formed sediments from the sodium hypochlorite solution. The sodium hypochlorite solution was decanted. The surface sterilized IJs were rinsed thrice with 5 ml of sterile distilled water. The succeeding steps encompassed the isolation of genomic deoxyribonucleic acids (DNA). This method was conducted according to the protocol which was obtained from the Puregene® DNA Purification Kit Gentra systems 2003 (catalogue #D5004) (see appendix for protocol). The genomic DNA was quantified using the NanoDrop®-1000 Spectrophotometer (BioRad) and thereafter stored at 4º C. The concentration of the genomic DNA was measured in the ng/µl units and was assessed together with the 260/280 and 260/230 ratios for the subsequent polymerase chain reactions (PCR).

2.3.2   Polymerase chain reactions (18S rDNA gene amplification)

The PCR reaction was performed by Inqaba Biotechnological Industries (PTY) Ltd. South Africa. The PCR technique was used to amplify the small eukaryotic ribosomal sub-unit deoxyribonucleic acid (18S rDNA) gene for the subsequent evolutionary inference. The gene was targeted by the TW81 forward and AB28 reverse universal primers. The EconoTaq® PLUS GREEN 2× Master Mix was used for the experiment. The PCR was performed in the Thermocycler (BioRad) at 45 amplification cycles. The tables below describe the sequences of

the oligonucleotide primers, the PCR reagents in their respective concentrations and reaction volumes and lastly, the PCR amplification profile.

Table 2.1: The sequences of the forward and reverse oligonucleotide primers.

Oligonucleotide primers	Nucleotide sequence	Tm (° C)
TW81 Forward	5'-GCGGATCCGTTTCCGTAGGTGAACCTGC -3'	71.94
AB28 Reverse	5'-GCGGATCCATATGCTTAAGTTCAGCGGGT -3'	68.87

Table 2.2: The PCR reaction volumes and reagents in required concentrations.

Reagent	Reaction volume Experiment (25 μl)	Reaction volume control (25 μl)
2× Econo®TaqMM	12.5	12.5
TW81 FP [10μM]	1	1
AB28 RP [10μM]	1	1
Template DNA	1	0
Nuclease Free water	9.5	8.5

Table 2.3: The PCR profile (18S ribosomal DNA gene amplification).

PCR step	Temperature (º C)	Time (minutes)
Activation denaturation	95º C	5
Denaturation	95º C	0.5
Annealing	50º C	0.5
Extension	72º C	2
Final extension	72º C	10

2.3.3   Confirmation of PCR product and Gene sequencing

The 18S rDNA gene amplicons were cleaned using the Exonuclease Shrimp Alkaline Phosphotase (ExoSAP) protocol. The Exo/SAP master mix was prepared by adding 10 µl of the PCR mixture into 2.5 µl of the Exo/SAP master mix (comprising 50 µl of the Exonuclease I NEB M0293 and 200 µl of Shrimp Alkaline Phosphatase NEB M0371) into a 0.6 ml

microcentrifuge tube. The reaction was incubated for 30 minutes at room temperature. Subsequently, the reaction was stopped by heating the mixture at 95° C for 5 minutes. The purified 18S rDNA amplicons were then sequenced according to the manufacturer's instructions in the ABI v3.1 Big dye kit. This process was performed by Inqaba Biotechnological Industries PTY (Ltd) South Africa. The nucleotide sequences of the gene were retrieved in the form of a chromatogram. Each nucleotide base in the sequence was screened for errors and edited accordingly using the FinchTV.1.4.0 and the Molecular Evolutionary Genetic Analysis Program 6 (MEGA6). The edited sequences were thereafter used for the Basic Local Alignment Search Tool (BLAST) for building the phylogenetic tree.

2.4   Phylogenetic tree construction

The 18S rDNA gene sequence was deposited into the GenBank database to search for the closely related sequences using the BLAST algorithm on the National Center for Biotechnology Information (NCBI). The output which obtained the highest similarity score, percentage identity and query cover was considered as the isolate/organism which shared the highest sequence similarity with the input sequence which was the isolated species in this investigation. A set of 18S rDNA sequences was selected from the output obtained from the GenBank database and was imported into the MEGA6 algorithm. Some of these sequences shared high sequence similarities whereas other sequences shared low sequence similarity. The 18S rDNA gene sequence which was under investigation was aligned to the existing 18S rDNA sequences. The alignment also incorporated the out group species which was *Caenorhabditis elegans*. The sequences were aligned using the Multiple Sequence Comparison by Log-Expectation (MUSCLE) tool. The consensus region of the multiple sequence alignment was thereafter obtained and used to build the phylogenetic tree. The phylogenetic tree was constructed using the maximum likelihood model and bootstrapped at 1000 replicates. The succeeding experiments involved the isolation and characterization of the bacterial species.

2.5   Characterization of the symbiotic bacteria

2.5.1   The Haemolymph extraction technique

This method involved the isolation of phase I phenotypic variant cells of *Xenorhabdus* species from the haemolymph of infected insect larvae. This protocol was adapted from Akhurst (1980). The infected larvae were prepared by the methods which were described for Koch's postulate (see section 2.2.4). After a period of 48 hours, the infected larvae were removed and prepared for the isolation technique. The first process involved the surface sterilization of the infected larvae which entailed submerging the individual larva in 15 ml of 95 % (v/v) ethanol in a Petri dish. Each individual larva was gently held with sterile pair of forceps and passed swiftly through a Bunsen burner flame. The sterilized larva was dipped in 15 ml of sterile distilled water in a sterile Petri dish for cooling purposes. The cadaver was opened with a sterile hypodermic needle and syringe and an aliquot of haemolymph was extracted. The haemolymph was then mixed with 500 µl of sterile distilled water in a 1.5 ml Eppendorf tube. This mixture formed the inoculum for the streak plate method. The Nutrient Agar Bromothymol Blue Triphenyltetrazolium Chloride (NBTA) plates were prepared (see appendix). A drop of the inoculum was transferred onto the plates and the streak plate technique was performed to obtain individual bacterial colonies. The plates were then sealed with strips of Parafilm, inverted and incubated at 25º C for 48 hours. The colonies were then screened for phase I phenotypic variant bacterial cells.

2.5.2   Pure sub-culture of bacteria

One presumptive phase I phenotypic variant bacterial colony was used to prepare pure bacterial sub-cultures on freshly prepared NBTA plates. After the culture was established, 1 ml of nutrient broth (see appendix) was added into in a sterile 1.5 ml Eppendorf tube and inoculated with 3-4 bacterial colonies obtained from the pure culture. The inoculum was then incubated overnight at 25º C. The bacterial inoculum was used for the subsequent *in vitro* lipid agar investigations.

2.5.3   *In vitro* solid culture

This study served as a confirmation study. The spread plate technique was performed by spreading 0.1 ml of the bacterial inoculum onto freshly prepared lipid agar media (see appendix). The spread plates were incubated overnight at 25º C and bacterial lawns were established. Thereafter 500-1000 IJs were enumerated and transferred onto the bacterial lawn. The IJs were incubated for 5 days at 25º C. The development of IJs into first generation adults was then

monitored. This technique was performed to verify that the species which was isolated was the symbiotic partner of the EPN and not contaminants of the insect gut.

2.5.4   Genomic bacterial DNA isolation

The protocol was conducted in the laminar flow bench. Freshly streaked 24 hour bacterial colonies were transferred aseptically using a sterile inoculating loop into a 1.5 ml Eppendorf tube which contained 100 µl of sterile distilled water. The subsequent steps for the isolation of genomic DNA was performed according to the protocol which was obtained from the ZR Genomic DNA™- Tissue MiniPrep Kit (Zymo Research, catalogue #3050) (see appendix). The genomic DNA was quantified using the NanoDrop®-1000 Spectrophotometer (BioRad) and stored in the refrigerator at 4° C.

2.5.5   PCR (16S ribosomal DNA gene amplification)

The PCR was performed by the Inqaba Biotechnological industries (PTY) Ltd. South Africa. The PCR technique was used to amplify the small prokaryotic ribosomal sub-unit deoxyribonucleic acid (16S rDNA) gene region using the EUB968 and UNIV1382 forward and reverse universal primers. The EconoTaq® PLUS GREEN 2 × Master Mix was used for the experiment. The PCR was performed in the Thermocycler (BioRad) at 45 amplification cycles. The tables below describe the sequences of the oligonucleotide primers, the PCR reagents in their respective concentrations and reaction volumes and lastly, the PCR amplification profile.

Table 2.4: The sequences of the forward and reverse oligonucleotide primers.

Oligonucleotide primers	Nucleotide sequence	Tm (° C)
EUB968 (Forward)	5'-ACGGGCGGTGTGTRC-3'	62
UNIV1382 (Reverse)	5'-AACGCGAAGAACCTTAC-3'	66

Table 2.5: The PCR reaction volumes and reagents in required concentrations.

Reagent	Reaction volume Experiment (25 µl)	Reaction volume control (25 µl)
2× Econo®Taq MM	12.5	12.5

EUB968 FP [10µM]	1	1
UNIV1382 RP [10µM]	1	1
Template DNA	1	0
Nuclease Free water	9.5	8.5

Table 2.6: The PCR profile (16S rDNA gene amplification)

PCR step	Temperature (º C)	Time (minutes)
Activation denaturation	95º C	5
Denaturation	95º C	0.5
Annealing	50º C	0.5
Extension	72º C	2
Final extension	72º C	10

2.5.6   Confirmation of gene product and Gene sequencing

The 16S rDNA gene amplicons were purified and sequenced by Inqaba Biotechnological Industries (PTY) Ltd. South Africa according to the methods described above in section (see 2.2.3). The nucleotide sequences were retrieved and edited by the methods described above (section 2.2.3). The sequences were then used for the subsequent BLAST analysis and phylogenetic tree construction.

2.6   Phylogenetic analysis

The analysis of the 16S rDNA gene, importation of related 16S rDNA sequences, and multiple sequence alignment was performed according to the methods described above (section 2.4). The out-group species used for the multiple sequence alignment was *Escherichia coli*. The phylogenetic tree construction of the consensus multiple sequence alignment of the 16S ribosomal genes were performed according to the methods described in the section above (2.4).

2.7   RESULTS

2.7.1   Infection and Extraction
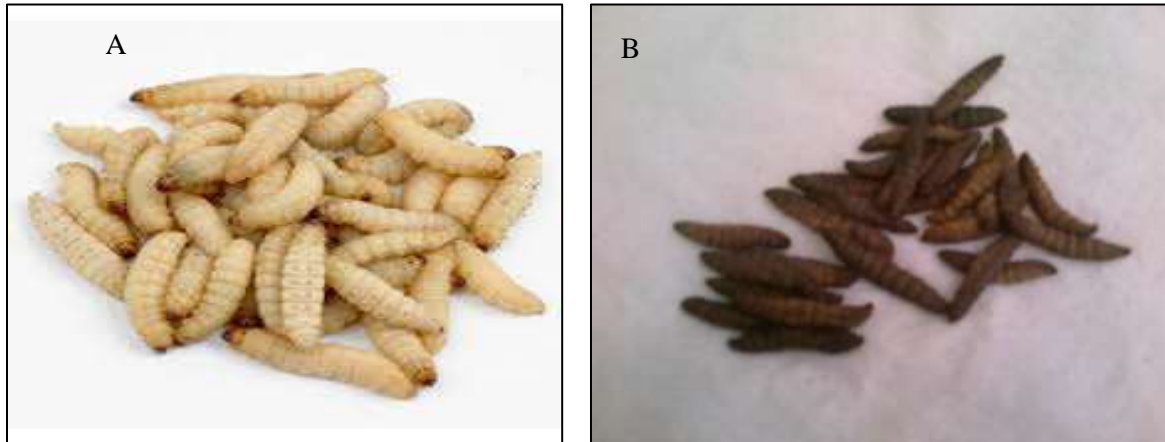
Figure 2.1: The healthy and infected last instar *G. mellonella*. The larvae transitioned in colour from light brown (A) to dark brown (B) after 72 hours of infection by one individual EPN species.

2.7.2   EPN isolation and re-infection
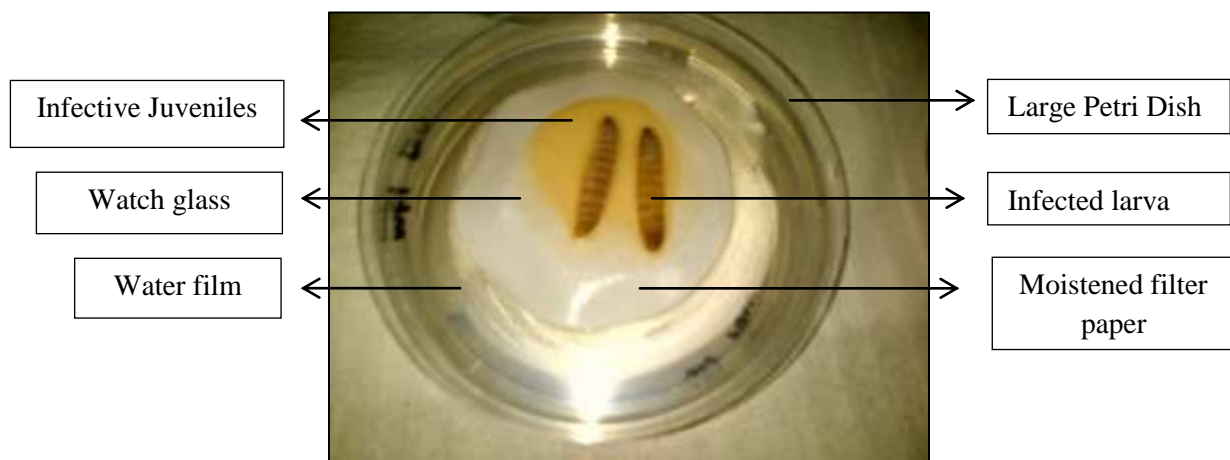
Figure 2.2: The White trap method (Kaya and Stock, 1997). The population of third staged infective juveniles emerged from the infected cadavers and migrated into the water film after 48 hours.
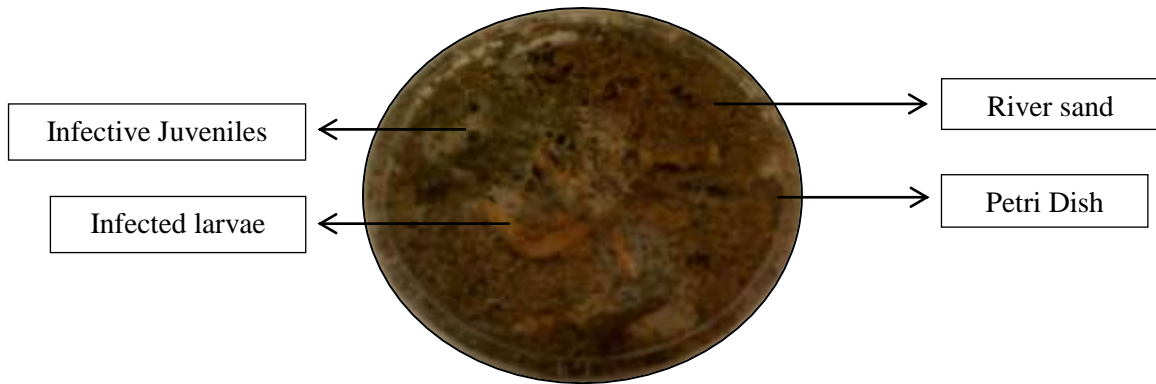
Figure 2.3: The pathogenicity confirmation sand-based assay which demonstrated the phenomenon of Koch's postulates. The last instar *G. mellonella* larvae were killed after 72 hours of re-infection. The infected larvae transitioned in colour from light to dark brown. The symptoms of larval infection were coherent with the initial symptoms of infection.

2.7.3   Morphological characterization

2.7.3.1   Maternal adult EPN (Anterior portion)
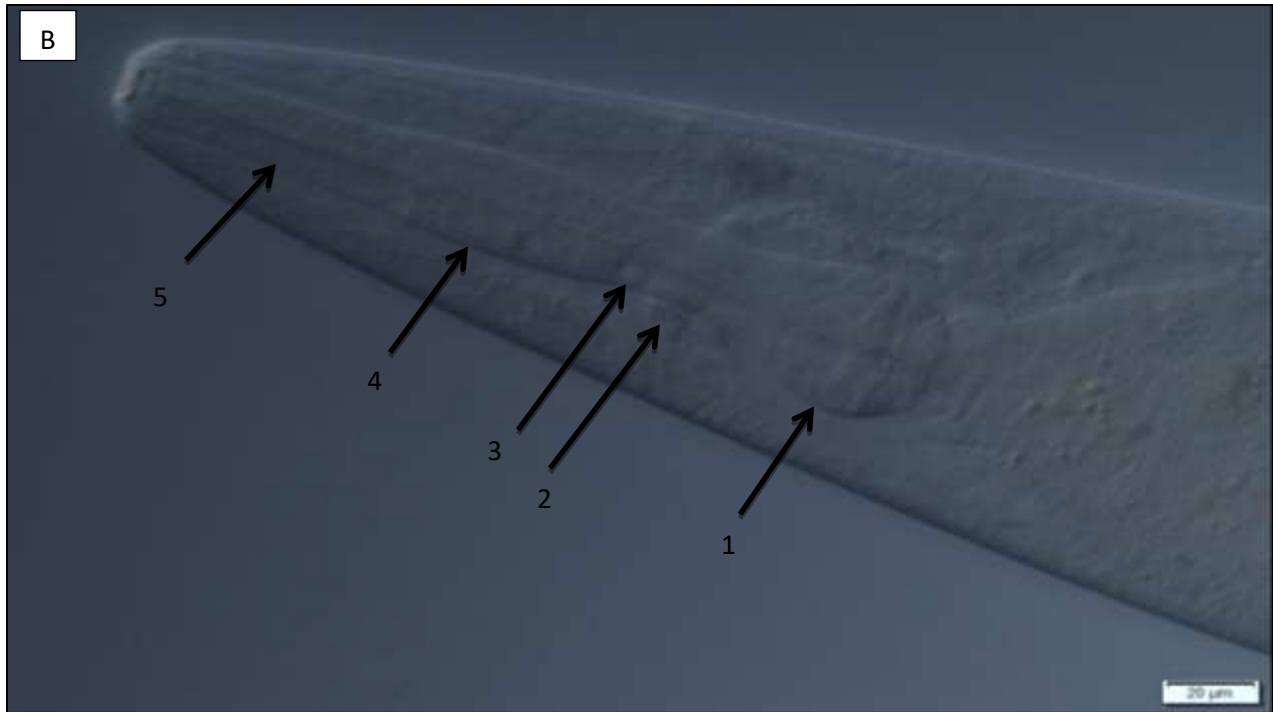
Figure 2.4 (A), (B): **The light microscope images of the anterior structures of the first generation female adult.** Image (A) showed the anterior parts of the female body imaged on the diffraction interference contrast (DIC) observation method with a magnifcation of 10 (×). The numbered arrows represented the follwowing structures: 1 = the vulva structure which was seen by a transerve non-pretuberant slit. 2 = represents the stoma region of the first generation female. Image (B) represented the anterior parts of the first generation female adult at higher magnifaction of 40 (×) on the DIC observation method. The numbers represented the following: 1 = the basal bulb, 2 = the nerve ring situated anterior to the posterior basal bulbsurrounding the isthmus, 3 = the isthmus structure, 4 = metacorpus, 5 = procorpus structure.

2.7.3.2   Maternal Adult (Posterior portion)

Figure 2.5: The light micrograph image of the posterior region of the first generation female adult. The image was taken using the DIC observation method at a magnification of 40 (×). The numberedarrows represented the following structures: 1 = Post-anal swelling, 2 = short tail tapering to blunt ends.

2.7.3.3   Second generation female
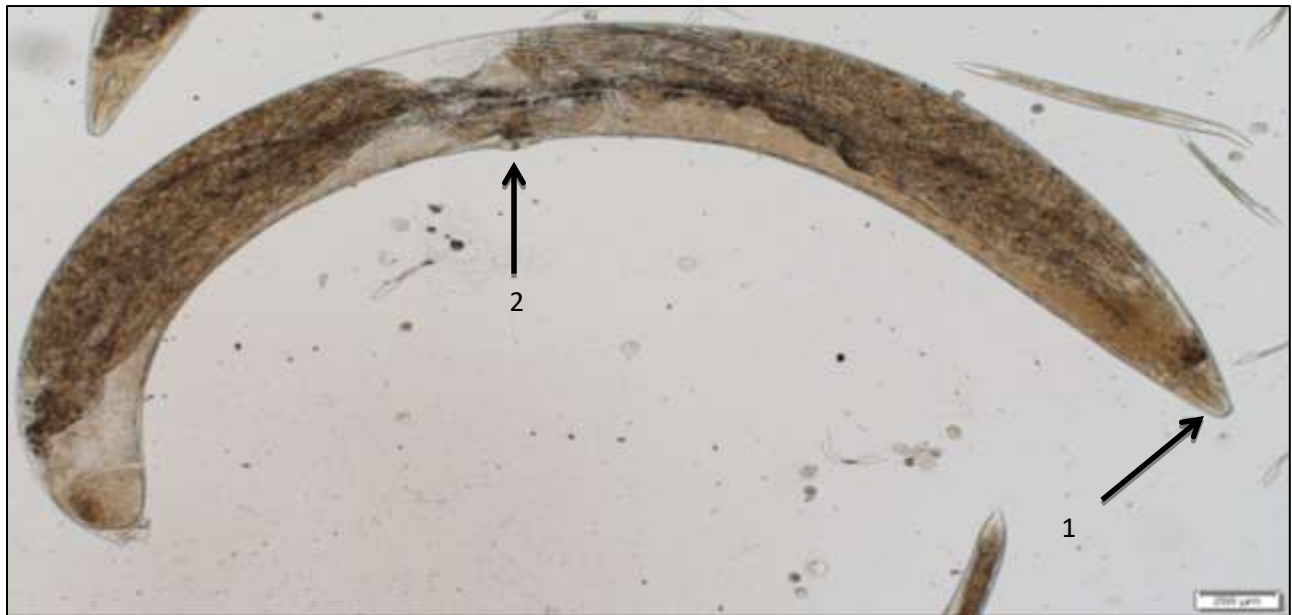
Figure 2.6: **The light micrograph image representing the entire body length of the second generation female adult.** The image was taken using the bright field (BF) observation method at a magnification at 10 (×). The numbered arrows represented the following structures: 1 = Stoma region, 2 = distinct protuberant vulva at the mid-body.

2.7.3.4   Anterior portion of the adult female (second generation)
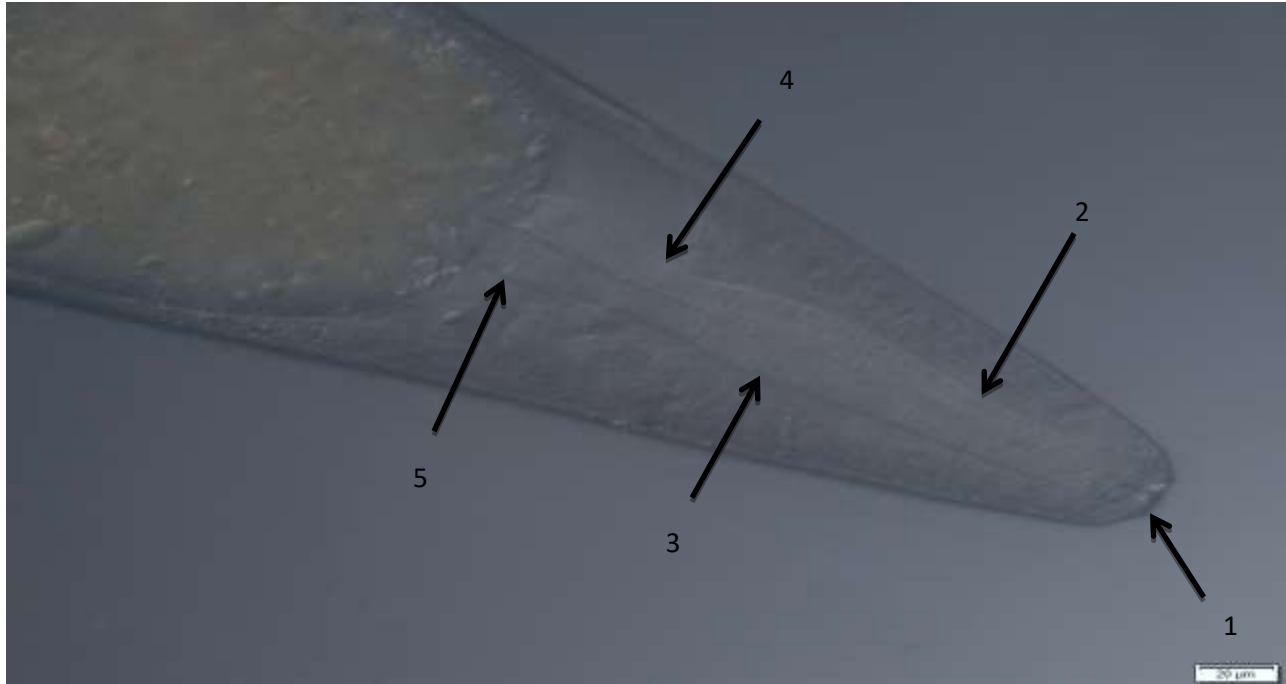
Figure 2.7: The anterior region of the second generation female adult. The image was taken using the BF observation method at a magnification of 40 (×). The numbered arrows represented the following structures: 1 = stoma region, 2 = procorpus, 3 = slightly swollen metacorpus, 4 = the isthmus structure and 5 = posterior basal bulb.

2.7.3.5   Posterior region of the second generation female
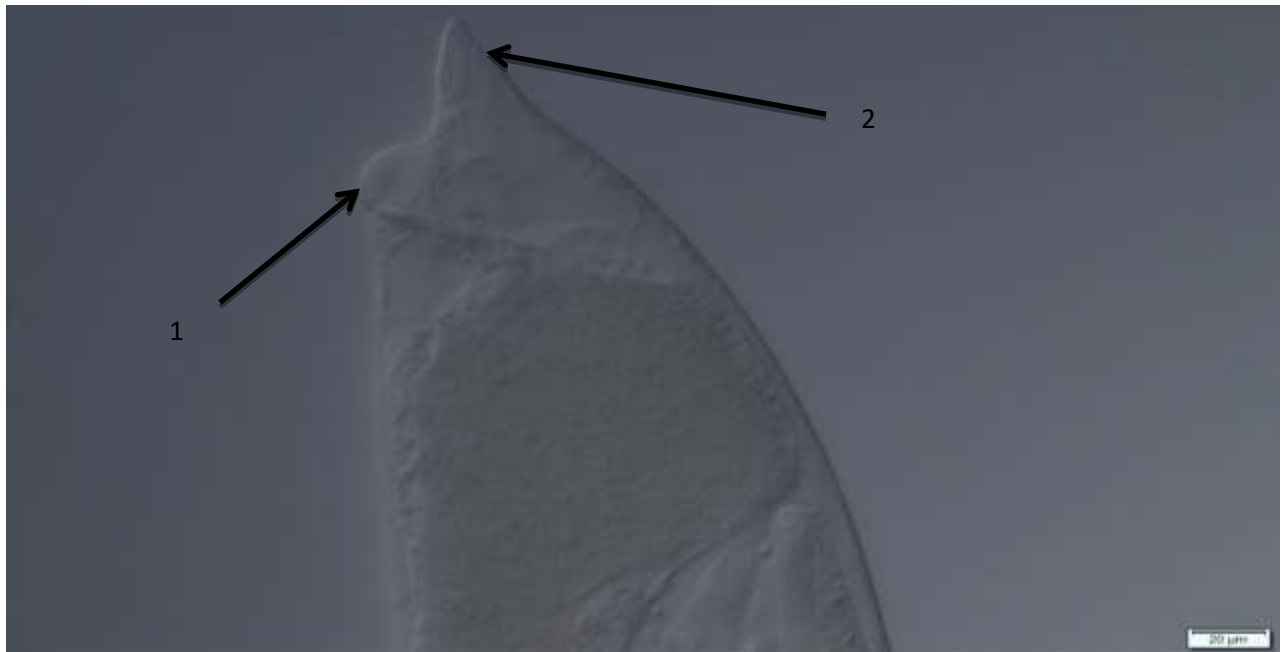
Figure 2.8: The posterior region of the second generation female adult. The image was taken using the DIC observation method at a magnification of 40 (×). The numbered arrows represented the following structures: 1 = the post-anal swelling and 2 = tail end tapering to a sharp point.

2.7.4   First generation male adult

2.7.4.1   Entire body length and anterior portion of the EPN
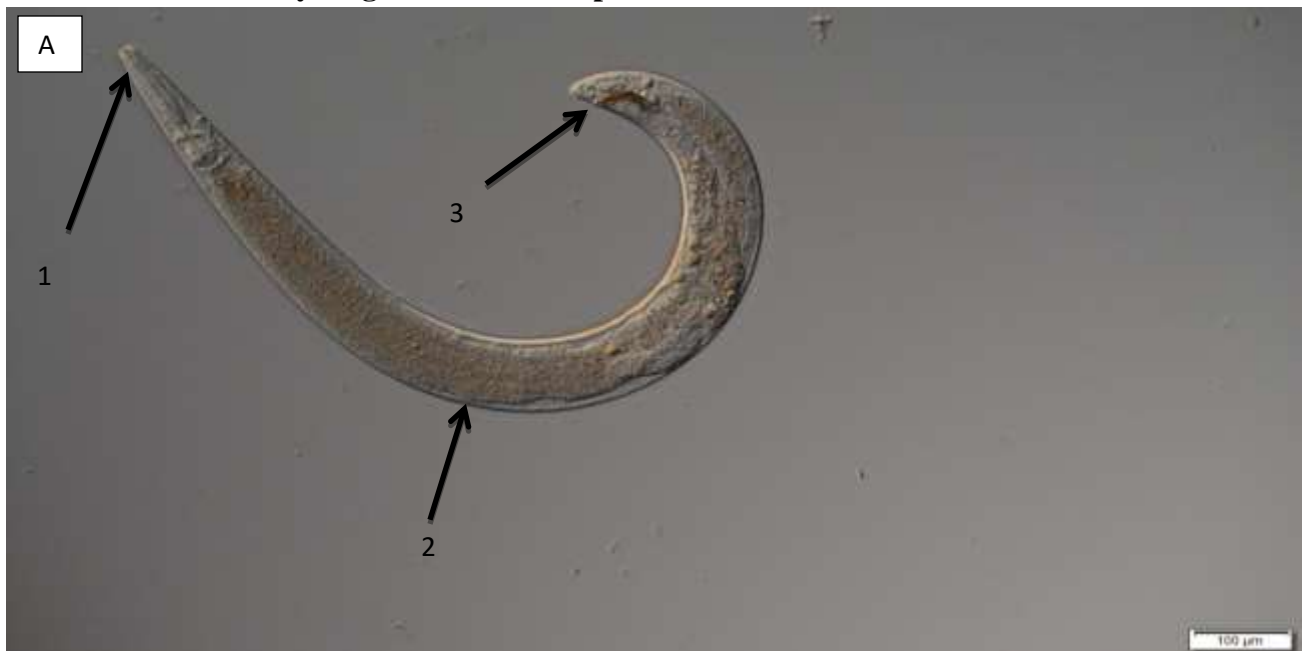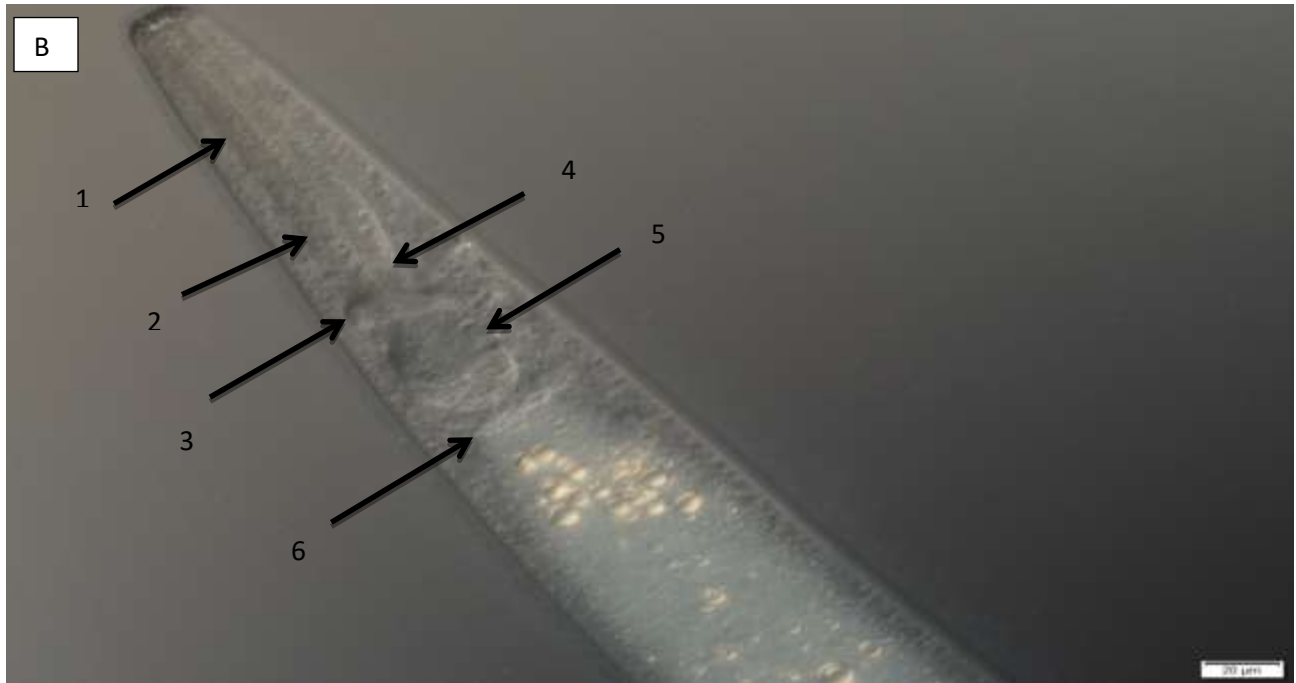
Figure 2.9: **The light micrograph images of the first generation adult male EPN**. Image (A) showed the overview of the structures along the entire length of the male EPN. The image was taken using the DIC observation method at a magnification of 10 (×). The numbered arrows represented the following structures: 1 = the anterior region of the EPNs represented by cephalic and pharynx region, 2 = the mid-section of the adult, 3 = the poster region of the EPN indicating the tail and male copulation structures. Image (B) indicated the anterior region of the male adult at a higher magnification of 40 (×) using the DIC observation method. The numbers represented the following structures, 1 = the procorpus, 2 = metacorpus, 3 = nerve ring which surrounded the isthmus, 4 = the isthmus structure, 5 = posterior basal bulb, 6 = prominent pharyngeal intestinal valve.

2.7.4.2   Posterior portion of male adult (first generation)

Figure 2.10: **The posterior tail-end of the first generation male adult EPN.** The observation method used for the light micrograph image was DIC at a magnification of $40 \, (\times)$. The numbered arrows represented the following structures: 1 = the pair of brown spicules, the head of the spicules (manubrim), 2 = the thick lamina which tapered to blunt ends, 3 = the velum structure and 4 = gubernaculum which was boat shaped.

2.7.5   Second generation adult male
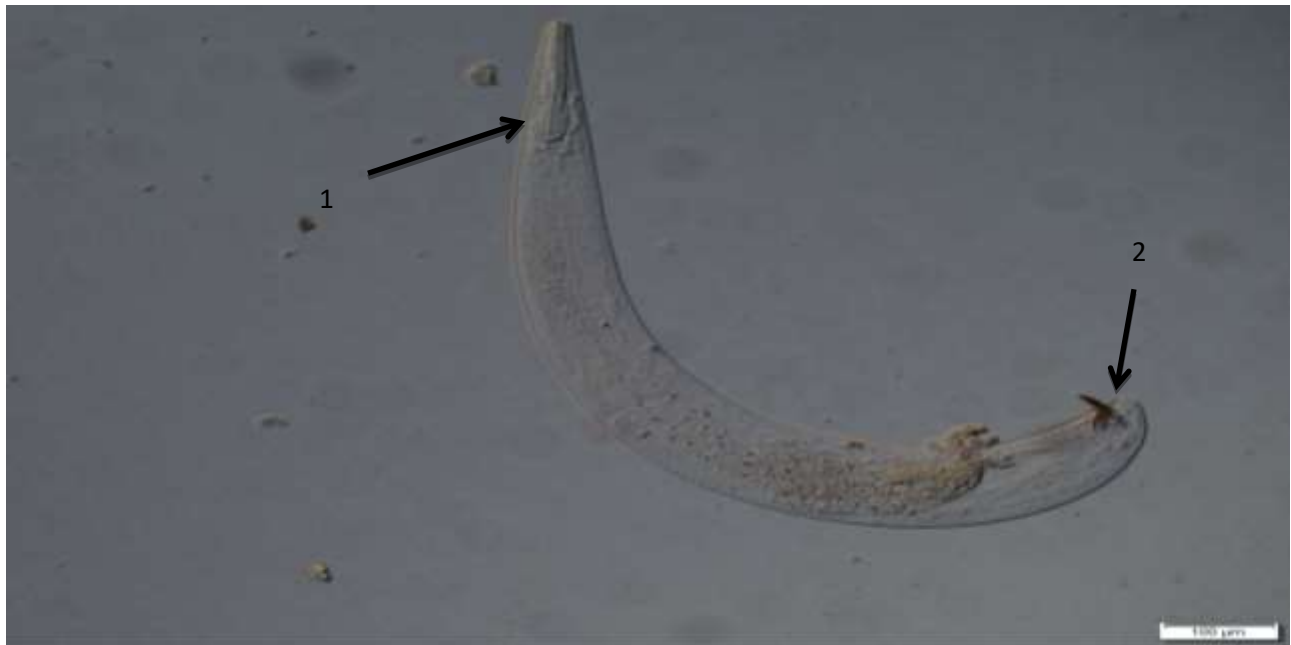
Figure 2.11: The light micrograph representing the entire body length of second generation adult male EPN. The observation method used for the light micrograph was the DIC at a magnification of 10 (×). The numbered arrows represented the following structures: 1 = the cephalic and pharynx region, 2 = the posterior tail end and male copulation structures.

2.7.5.1   Posterior structures of the adult male (G2)

Figure 2.12: The posterior tail-end region of the second generation adult male EPN. The posterior region consisted of the tail ends and male copulation structures. The light micrograph was imaged using the DIC observation method at a magnification of 40 (×). The numbered arrows represented the following structures: 1 = manubrim (spicule head), 2 = velum, 3 = lamina, 4 = protruding paired spicules, 5 = gubernaculum, 6 = posterior tail region.

2.7.6 The molecular characterization of the EPN species
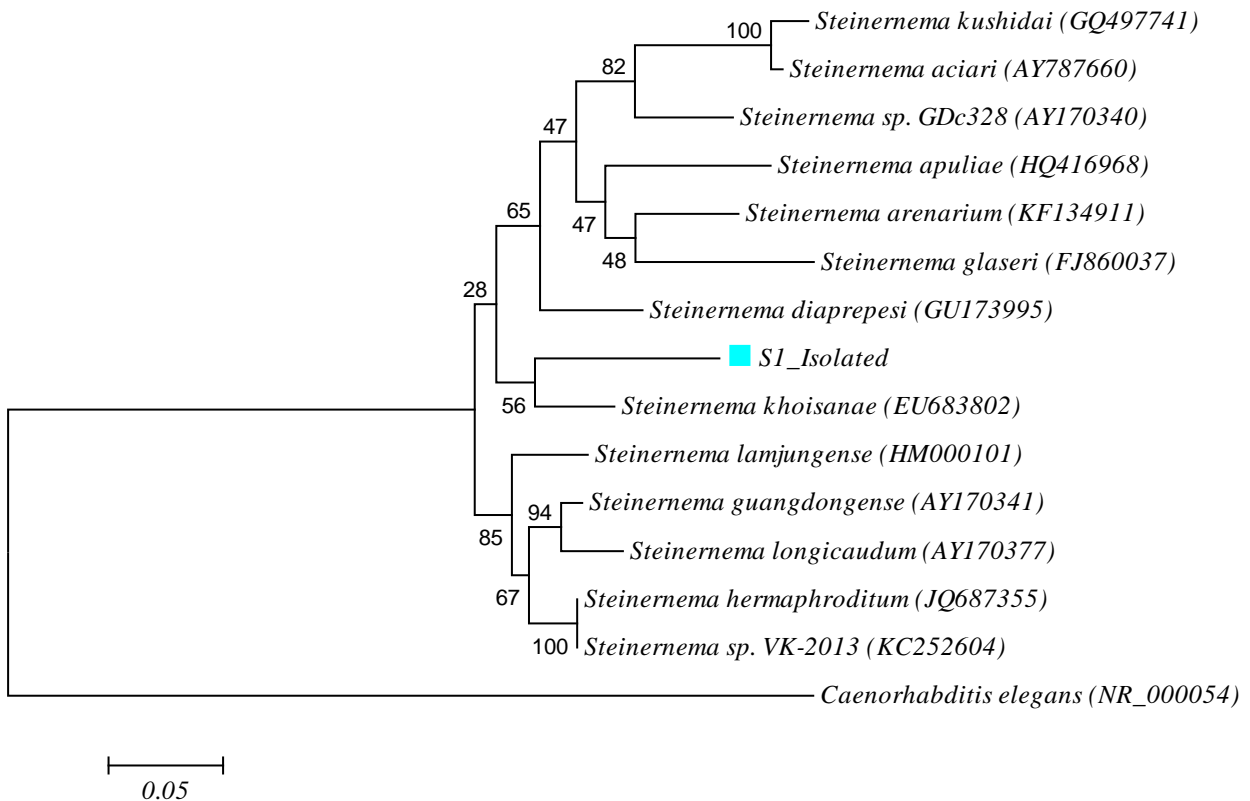
Figure 2.13: **The molecular phylogenetic tree constructed using the Maximum Likelihood method based on the Tamura-Nei model.** The phylogenetic tree was constructed from the consensus region of the multiple sequence alignment of the existing 18S rDNA EPN sequences together with the marked unidentified sequence under investigation (blue). The tree was rooted with the species *C. elegans*. The initial trees were obtained by applying two algorithms. These included the Neighbor Join and BioNJ algorithms to a matrix of pairwise distances which was estimated by the Maximum Composite Likelihood method. The percentage of trees in which the closely related taxa clustered was represented by the values adjacent to the nodes (bootstrap value of 1000 replicates).The tree was drawn to scale with the branch lengths measuring the

number of substitutions per site. The numbers following species represented the associated GenBank accession numbers of the sequences.

2.7.6.1   Result (NCBI and Phylogenetic tree)

The parameters on the NCBI BLAST algorithm revealed an 85 % maximum identity to the species *Steinernema khoisanae*. This subject sequence obtained the highest maximum score of 784 and percentage query cover of 97 %. Furthermore, this hit obtained an E-value of 0. The alignment between the subject and query showed 6 % gaps along the entire 18S ribosomal gene region. The phylogenetic tree revealed that a common ancestor was shared between the species under investigation and the South African isolate, *S. khoisanae* (EU683802). However, longer branch lengths were obtained for the species under investigation. There was a 56 % bootstrap value between the species under investigation and *S. khoisanae*.

2.7.7   Bacterial Isolation

2.7.7.1   Haemolymph extraction technique

Figure 2.14: **The 24-hour phase variant I bacterial colonies on Nutrient Bromothymol Blue Triphenyltetrazolium chloride Agar (NBTA).** The bacterial colonies grew convex and circular in shape. The colonies appeared dark green with sticky consistencies. The colonies were boarded by zones of clearance. The sizes of the bacterial colonies ranged from small to large.

2.7.7.2   *In vitro* lipid agar (confirmation studies)

Figure 2.15: The adult female EPNs obtained from the *in vitro* solid lipid agar plates. Image (A) represents the uterus structure of the adult female filled with ovoid eggs imaged by the DIC observation method at a magnification of 10 (×). The scale bar indicated a value of 100 µm. Image (B) represented the phenomenon of *endotokia matricida*. The image showed active and hatched infective juveniles within the uterus structure of the maternal body cavity by the DIC observation method at a magnification of 10 (×). The scale bar was represented by a value of 100 µm. The IJs were transferred onto a bacterial lawn on the lipid agar (spread plate of 0.1 ml bacterial inoculum). The IJs developed into adults and mated to produce the subsequent progenies.

2.7.8   Molecular characterization of the bacteria
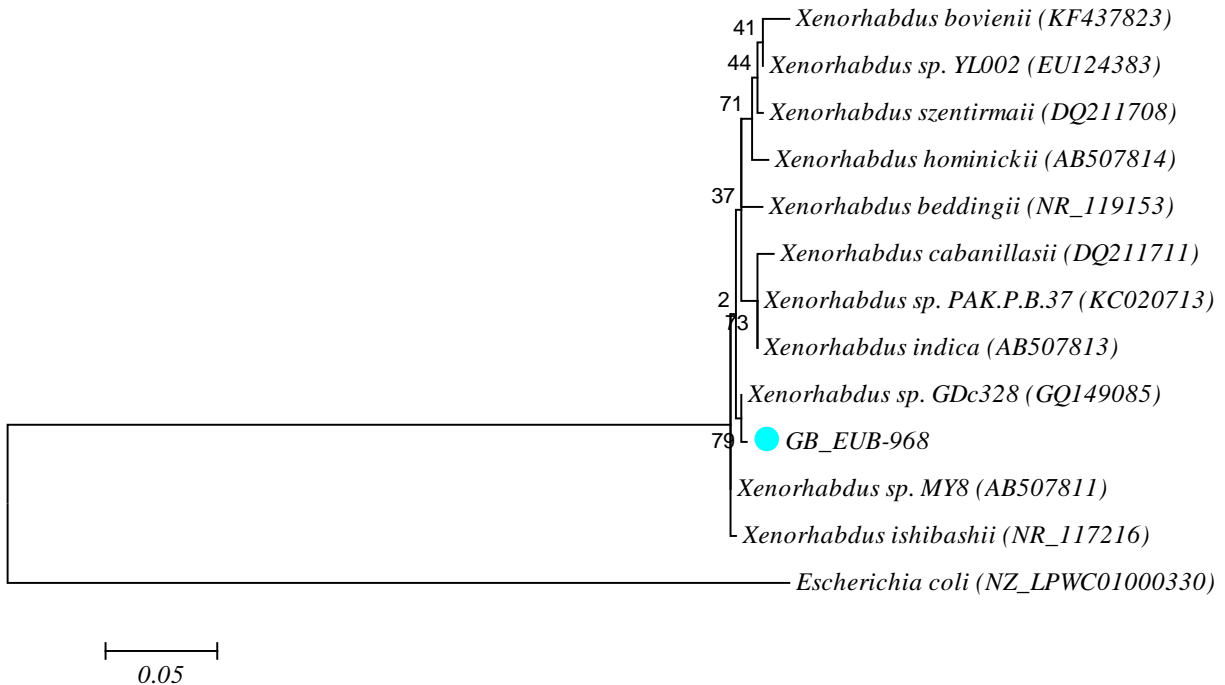
Figure 2.16: The molecular phylogenetic tree constructed using the Maximum Likelihood method based on the Tamura-Nei model. The phylogenetic tree was constructed from the consensus region of the multiple sequence alignment of existing 16S rDNA EPN sequences with the marked unidentified sequence under investigation (blue). The tree was rooted with the species *E. coli*. The initial trees were obtained by applying two algorithms. These included the Neighbor Join and BioNJ algorithms to a matrix of pairwise distances which was estimated by the Maximum Composite Likelihood method. The percentage of trees in which the closely related taxa clustered was represented by the values adjacent to the nodes (bootstrap value of 1000 replicates). The tree was drawn to scale with the branch lengths measuring the number of substitutions per site. The numbers following species represented the associated GenBank accession numbers of the sequences.

2.7.8.1   Result (NCBI parameters and phylogenetic tree)

The parameters on the NCBI BLAST algorithm revealed a 99 % maximum identity to the species *Xenorhabdus* sp. strain GDc328. This subject sequence obtained the maximum score of 894 and query cover of 98 %. Furthermore, this hit obtained an Expectation value (E-value) of 0. The alignment between the subject and query showed 0 % gaps along the entire 16S ribosomal

gene region. The phylogenetic tree revealed that a common ancestor was shared between the species under investigation and the isolate, *Xenorhabdus* sp. strain GDc328 (GQ149085). The two species taxonomically clustered by 79 % with negligible differences in the branch lengths.

2.8   DISCUSSION

2.8.1   Confirmation of EPN infection and isolation

The initial host infections obtained from the original soil samples may have been attributed to different pathogenic agents. It was imperative to identify the presence of the EPNs and establish their role the causative agents of larval mortality. One key indicator of EPN infection involved the transition in the color of the infected last instar *G. mellonella*. According to Koppenhöffer (2000), different strains and species of EPNs infect target hosts with characteristic colours. Also, the cuticles of the infected larvae remained intact with no putrid odor production. These symptoms served to confirm the possibility of EPN infection. The cadavers were set onto White traps (see figure 2.2) and the third stage progeny of infective juveniles (IJs) emerged from the infected cadavers after 48 hours. The emergence of the IJs from the cadavers indicated that the nutrients within the host became depleted. The pathogenic status of the EPNs against the last instar *G. mellonella* was established by the completion of Koch's postulates. The completion of Koch's postulates was achieved by the isolation of the EPNs from the infected larvae, the propagation of the individual EPN species, the re-infection of healthy larvae (see figure 2.3) and the re-isolation of the IJs (Kaya and Vega, 2012). The brown coloured insect cadavers (seen in figure 2.1) indicated that infection occurred by an individual EPN species which comprised the *Steinernema* genus (Koppenhöffer, 2000).

2.8.2   Morphological characterization

One distinctive feature of the EPNs which comprise the genus, *Steinernema* involves the presence of amphimictic (sexually distinct) males and females. The light micrograph images of both first and second generation males and females indicated distinct sexes. This finding confirmed that the individual population of EPNs under investigation belonged to the *Steinernema* genus. Comparisons were made to existing morphometric and phenotypic classifications of the different species which comprised the genus *Steinernema* (Nguyen et al.,

2007). It was observed the species under investigation was highly similar in structure to the indigenous South African isolate, *S. khoisanae*. However, differences were obtained in the morphology of the second generation adult males. This difference was observed in figure 2.7.5.1. The spicule structures protruded from the posterior end of the male adult. This was not characteristic of the second generation males which belonged to the species *S. khoisanae*. Molecular characterization was required to resolve the identity of the species.

2.8.3   Molecular characterization of the EPN

2.8.3.1   NCBI BLAST parameters
The NCBI BLAST parameters showed that subject sequence with the highest similarity to the species under investigation was *S. khoisanae* (highest hit). The maximum similarity score obtained for the hit was 784 which implied that a proportion of similarity was obtained between the query (species under investigation) and the subject sequence (*S. khoisanae*). The query coverage value implied that 97 % of the query length was incorporated in the aligned segments. The E-value described the number of hits with similar scores which were expected by chance when searching the database of particular size. Low E-values implied significance regarding the score and alignment for the hit. For the subject sequence, an E-value of 0 implied that the hit was significant. The maximum identity parameter described the extent to which the subject and query sequences were related over the length of coverage (Newell et al., 2013). The hit obtained a value of 85 % similarity. This value implied that the nucleotide sequences between the query and subject differed by 15 %. The phylogenetic analysis was performed to deduce the taxonomic affinities of the EPN species.

2.8.3.2   Phylogenetic analysis
The phylogenetic tree (figure 2.13) was used to infer the evolutionary relationship of the species under investigation. The tree showed that the species under investigation (marked in blue in figure 13) taxonomically grouped to the South African isolate *S. khoisanae* (GQ149085). The grouping obtained a percentage of 56 % which provided evidence that the two sequences clustered more strongly together than with other sequences in the alignment. However, this percentage value was low which implied that even though the species shared ancestry, both

could be regarded as distinct species. This finding was further substantiated by the length of branches obtained in figure 13. The species under investigation showed an extended branch length relative to the species *S. khoisanae*. The extended branch length implied that a higher degree of genetic variation over time was obtained. These findings contributed to the inference that the species under investigation was a novel South African isolate which belonged to the genus *Steinernema*.

2.8.4   The isolation of the symbiotic bacteria

The colonies which were isolated on differential NBTA media showed an initial red colour. This was attributed to the reduction of the Triphenyltetrazolium Chloride compound which was one of the constituents of the agar. Only phase I phenotypic variants were capable of reducing the compound. Also, the dark green coloured bacterial colonies in figure 2.14 were attributed to the adsorption of the Bromothymol Blue dye. This was also characteristic of phase I phenotypic variants. The colonies isolated were implicated as phase I variants since phase II variants were incapable of dye adsorption and compound reduction (see section 1.6.1) of the literature review for colonial characteristics of both phenotypic variants) (Kaya and Stock, 1997). These putative *Xenorhabdus* bacterial colonies were used for the subsequent lipid agar study.

2.8.4.1   Solid *in vitro* lipid culture: bacterial isolate confirmation

The purpose of the lipid agar study was to confirm that the bacteria which were previously isolated were the native endosymbiotic partners of the EPNs since numerous other bacterial species which inhabited the insect gut could possibly be isolated. Also, since enteric bacteria may exhibit similar properties on differential media, confirmation studies were required. The IJs ingested the bacteria and developed into adult males and females. The adult males and females mated and reproduced the subsequent IJ progenies. The uterus structure of the adult female contained ovoid eggs (figure 2.15A). The process of *endotokia matricida* was then demonstrated in figure 2.15B. During the process, the IJs fed of the internal tissue and developed within the maternal parent. Ultimately, the third staged IJs emerged through the body cavity of the maternal parent which consequently resulted in the death of adult female. According to Kaya and Stock (1997), the process of infective juvenile-adult development may not occur without the cognate symbiotic bacteria. However, development was seen in Figure 2.15 (A) and (B). This study

confirmed that the colonies which were isolated belonged to genus *Xenorhabdus*. However, molecular identification was required to taxonomically classify bacterial isolate to the species level.

2.8.5 Molecular characterization of the bacteria

2.8.5.1 NCBI BLAST parameters

The NCBI BLAST parameters showed that the output sequence (subject sequence) with the highest sequence similarity to the species under investigation (query sequence) was *Xenorhabdus* sp. strain GDc328 (GQ149085). This hit obtained the highest NCBI BLAST parameters which were defined in the discussion above (section 2.8.3.1). High similarity scores were obtained for the alignment between the subject and query sequences. This implied that the subject sequence shared high sequence similarity to the query sequence. Furthermore, the query coverage value implied that 98 % of the query was incorporated into the alignment. The E-value obtained for the hit was 0 which implied that the hit was significant. The maximum identity value obtained for the hit was 99 %. This implied a strong sequence relation between the subject and query over the length of coverage (Newell et al., 2013).

2.8.5.2 Bacterial phylogeny

The phylogenetic tree in figure 2.16 showed that the species under investigation (indicated by a blue circle) taxonomically grouped to the species *Xenorhabdus* sp. strain GDc328 (GQ149085). The taxonomic grouping obtained a value of 79 % which implied that the two species clustered together strongly. This percentage value was high. The species under investigation shared common ancestry with the species *Xenorhabdus* sp. strain GDc328. Also, the branch lengths of the two species were almost negligible. This finding implied that the species under investigation was highly related to *Xenorhabdus* sp. strain GDc328 and could possibly be regarded as taxonomically indistinguishable.

2.9 CONCLUSION

The species which was isolated in this investigation was a member of the *Steinernema* genus. The species was molecularly characterized by 18S rDNA gene amplification and phylogeny.

Two other genes may be used as diagnostic markers to further asses the molecular phylogenetic relationships of the South African isolate with other known EPN taxa. These genes include the large eukaryotic ribosomal sub-unit (28S rDNA) gene and the complete Internal Transcribed Spacer (ITS rDNA) gene. Also, more recently, the gene repertoire for the identification of *Steinernema* species expanded. These may include the cytochrome oxidase I (COI) and 12S mitochondrial genes. According to Stock (2015), these two genes provide important information regarding the taxonomic identification of species. The bacterial species isolated in this study comprised the genus *Xenorhabdus* and shared the highest sequence similarity to the species *Xenorhabdus* sp. strain GDc328.

CHAPTER 3: THE EFFECT OF TEMPERATURE ON EPN INFECTION, IJ RECOVERY AND PROGENY IJ YIELD

3.1   INTRODUCTION

The entomopathogenic nematodes (EPNs) which comprise the genus *Steinernema* are endemic to temperate climatic regions (Stock, 2015). The study conducted by Grewal et al., (1994) stated that the species *S. feltiae* was regarded as a species in which reproduction was most efficient at low temperature regimens. Other species such as *S. riboravis* and *S. scapteriscae* were regarded as warm temperature reproducing species (Atwa, 2015). Since the different species of the EPNs may be regarded as warm, cool or moderately adapted temperature organisms, some species may be more efficacious than others at different temperature regimes. Atwa (2015) suggested that that temperature may have influenced the host specialization of the EPNs during seasonal changes. It becomes imperative to seek out warm and cold adapted strains of EPNs for seasonal application and assess their adaptive capacity to different temperature regimes and rapid temperature fluctuations. Grewal et al., (1996) stated that the limited research in this area has contributed significantly in unpredictable field results of different entomopathogenic nematode (EPN) species.

van Zyl and Malan (2014) expressed the need to identify endemic EPN species previously (see chapter 2 section 2.1). The concerns of the authors were not only expressed as a result of the stringent safety regulation requirements but also due to the adaptive capacities of endemic species to local climatic conditions. Hill et al., (2015) stated that while the EPNs have adapted to the harsh environmental conditions inside the host larvae, the infective juveniles (IJs) are sometimes vulnerable to the variability in environmental temperature during the processes of host seeking.

The climate trends for the past five decades and the near future (2015-2035) temperature projections for South Africa were reported by the Department of Environmental Affairs (Kruger and Sekele, 2013). The report showed that the annual mean temperature was expected to increase by $1.5 \times$ the global average temperature of 0.65º C. The upper seasonal temperature extremes were expected to increase and the lower seasonal temperature limits were expected to

decrease annually throughout the country. Also, fluctuations in the rate at which temperature changed were documented. The fluctuations of temperature were reported to large extents in the years from 1990 to the mid-2000s. The near future (2015-2035) climate projection trends have been calculated and an increase by 1º C on the coastal areas and 1-2º C for the interior parts of the country were expected. The fluctuations of environmental temperature and the estimated increases may affect the efficacies of the EPNs as biological control agents. Byers and Poinar (1981) stated that some species of EPNs aggregate in temperatures 0.3º C above ambient temperature. The activity of the different biological processes of the EPNs functions in well-defined thermal niche breadths (see literature review table 1.3). It becomes imperative to ascertain the basal thermal profiles for endemic EPN species and evaluate their responses to rapid temperature changes.

The majority of temperature related investigative studies (see literature review 1.13.1) involved the acclimation of the EPNs to different temperatures for lengthy periods of time. However, given that environmental temperature may fluctuate rapidly, the efficacy of the EPNs for key biological processes should be evaluated by short term temperature acclimation. The key biological processes include host infection, infective juvenile (IJ) recovery and progeny IJ yield. The density of the EPN population may also contribute to successful insect host infections at different temperatures. The aim of this chapter was to evaluate the response of the endemic South African isolate, *Steinernema* spp. to two experimental temperature regimes by acute temperature acclimation for the biological processes of host infection, IJ recovery and progeny IJ yield.

3.2 METHODS AND MATERIALS

3.2.1 Temperatures study (Infection, recovery and yield)

The processes of EPN infection, recovery and yield were evaluated at the control and experimental temperatures. The control temperature was the standard 25º C and the experimental temperature values were 20º C and 30º C respectively. The process of infection was described by the events of EPN penetration, bacterial release and proliferation and lastly, larval mortality. The recovery process of the EPNs was described by the exit of the quiescent stage of IJs into active first generation male and female adults followed by the production and emergence of the new progeny of the infective juveniles. The yield of the EPNs was measured by the number of the IJs which were produced per infected larva.

3.2.2 EPN Infection

3.2.2.1 EPN Isolation and propagation

The virulence of the species was evaluated using the pathogenic sand-based assay at the control (25º C) and two experimental temperatures (20º C and 30º C). The virulence against freshly reared last instar *G. mellonella* was tested. The IJs that were used for the investigation was prepared by propagation in the last instar *G. mellonella* using the pathogenic sand-based assay. The EPNs were isolated using the White trap technique (see chapter 2 section 2.2.3). These two processes of propagation of the EPN species occurred for a period of 18 months at 25º C prior to the investigation.

3.2.2.2 The pathogenic sand based assay

The sand based assay was set up according to the methods described by Grewal et al., (1994). For this technique, 40 g of sterile river sand was measured and transferred into a sterile Petri dish (100×15 mm). The amount of water was then calculated to establish 16 % moisture content within the river sand. The IJs were collected and transferred from the White traps into 50 ml Falcon tubes. The IJs were allowed to sediment by gravity and volume of water was adjusted to 5 ml. The dilutions of 100 folds were thereafter performed with sterile distilled water (10 µl of sample was added into 990 µl of sterile distilled water). From this mixture, 10 µl of the diluted sample was then used to count the number of IJs using the dissecting light microscope. This

process was repeated 6 times and the average number of IJs from the 6 sets of 10 µl diluted samples was calculated. The number of IJs which were present in the original sample of 5 ml was then calculated by multiplying the number of IJs present in 10 µl by a factor of 100 and then dividing the IJs obtained by the volume used to count (10 µl). The units of the volume were then converted into milliliters and the estimated number of IJs present in 5 ml was calculated. Subsequently, the volume of the solution containing 500 IJs and 1000 IJs were then calculated and transferred into the river sand. The experiment was performed in triplicates with one second control for each selected temperature. The second controls were set up without the presence of the IJs. The Petri dishes were then transferred into the respective incubation chambers at 20º C, 25º C and 30º C for overnight temperature (acute) acclimation. Thereafter, 5 last instar *G. mellonella* larvae were transferred into each Petri dish at each temperature (experiment n = 15, control n = 5) labeled with the respective IJ population density (either 500 or 1000 IJs). Insect mortality was thereafter determined in 48 hour intervals.

3.2.3   Recovery

The infected cadavers were removed from the pathogenic sand based assay and sterilized by the removal of sand granules using a sterile pair of forceps and spraying the surfaces of the insect larvae with 70 % (v/v) ethanol. Each larva was set onto individual White traps (described in Chapter 2 section 2.2.3). The White traps (n = 15) were transferred into the respective temperature incubation chambers (20º C, 25º C and 30º C) for progeny infective juvenile emergence. The process of recovery was measured by the exit of the IJs from the infected cadavers and migration into the water film of the White traps.

3.2.4   Yield

The progenies of IJs which were recovered from the cadavers which were set onto White traps (n = 15) for each temperature regime (20º C, 25º C and 30º C) were collected into 50 ml Falcon tubes for the process of enumeration. The IJs were allowed to sediment by gravity and the volumes of the samples were adjusted to 5 ml. For the enumeration process, the samples were then diluted by 100 fold to a final volume of 1 ml (0.01 ml of sample into 0.99 ml of sterile distilled water). From this mixture, aliquots of 10 µl of the diluted samples were taken. This process was repeated 6 times for each recovered population of IJs at the different temperature

regimes and initial IJ population densities. In each 10 μl of diluted sample (× 6), the numbers of IJs were counted using the dissecting light microscope. The total number of IJs from the 6 samples was divided by a factor of 6 to obtain the average number of IJs in each 10 μl volume of the diluted sample. The number of IJs was then multiplied by the dilution factor of 100 fold to obtain the original count present in the 10 μl of the sample. Thereafter the original count within the standard 5 ml of sample was calculated and was regarded as the total number of IJs produced per larva. This process was performed for each selected temperature (n = 15) with their respective IJ population densities therefore a total of n = 90 samples were enumerated.

3.3 RESULTS

3.3.1 EPN Infectivity (1000 IJs)

3.3.1.1 Insect Mortality vs. time (20º C)
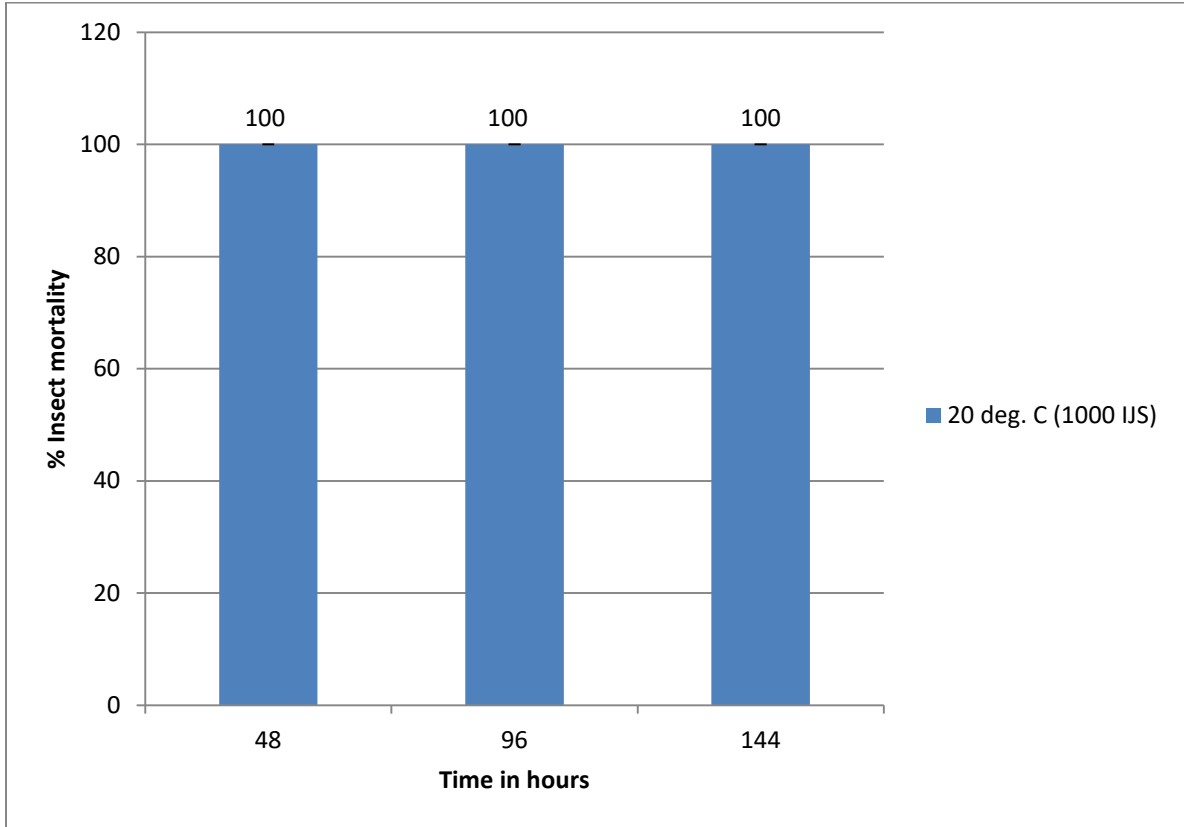
Figure 3.1 (a): The percentage of cumulative insect mortality (n = 15) measured at 48 hour intervals at 20º C for population density of 1000 IJs. The study was conducted at a moisture content of 16 %. At 20° C, 100 % insect mortality was achieved during the first 48 hours of infection. The standard error of the means (±) were represented by the standard error bars. The second controls yielded 0 % insect mortality.

3.3.1.2   Insect Mortaliy vs. time (25º C)
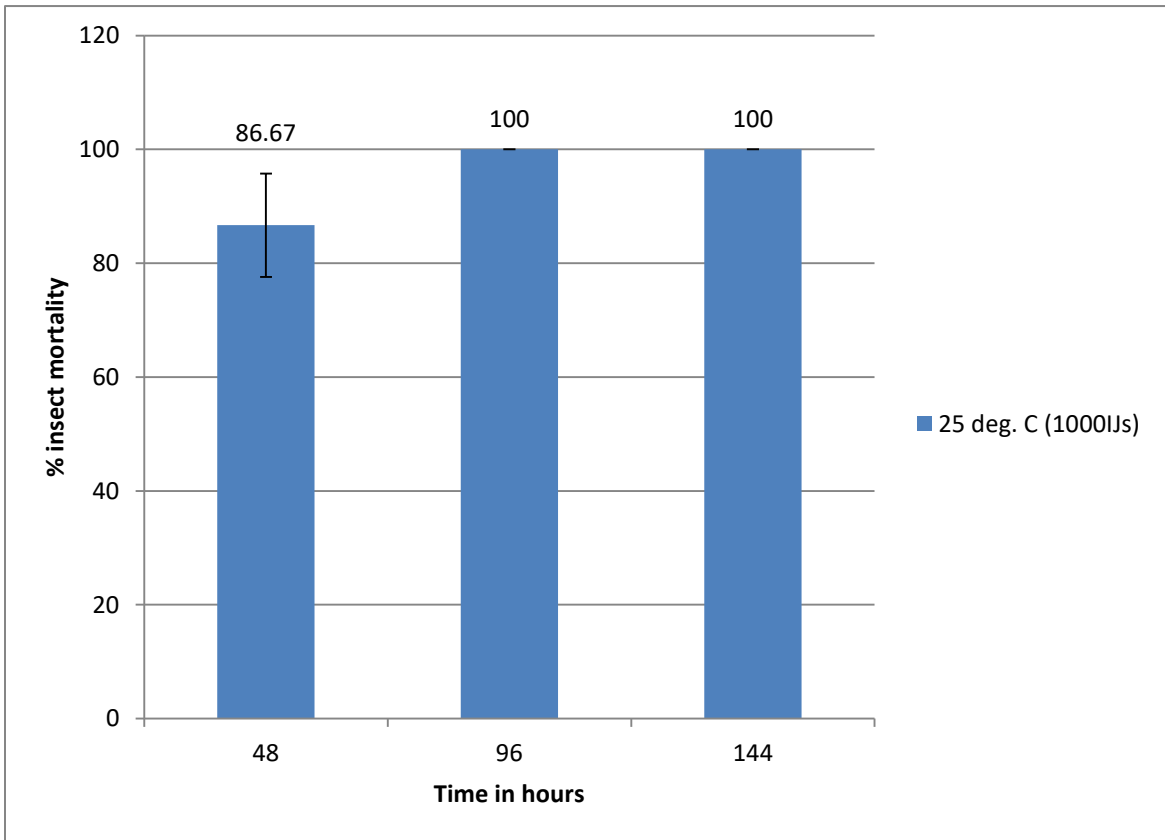
Figure 3.1 (b): The percentage of cumulative insect mortality (n = 15) measured at 48 hour intervals at 25º C for the population density of 1000 IJs. The study was performed at a moisture content of 16 %. High mortality was achieved during the first 48 hours of infection (86.67 %). At 96 hours, insect mortality increased by 13. 33 %. The standard error of the means (±) were represented by error bars. The second controls yielded 0 % of insect mortality. The percentage of host mortality was higher at 20° C for the first 48 hours of infection. However, 100 % insect mortality was achieved by 96 hours at the control temperature (25° C).

3.3.1.3　Insect Mortaliy vs. time (30º C)
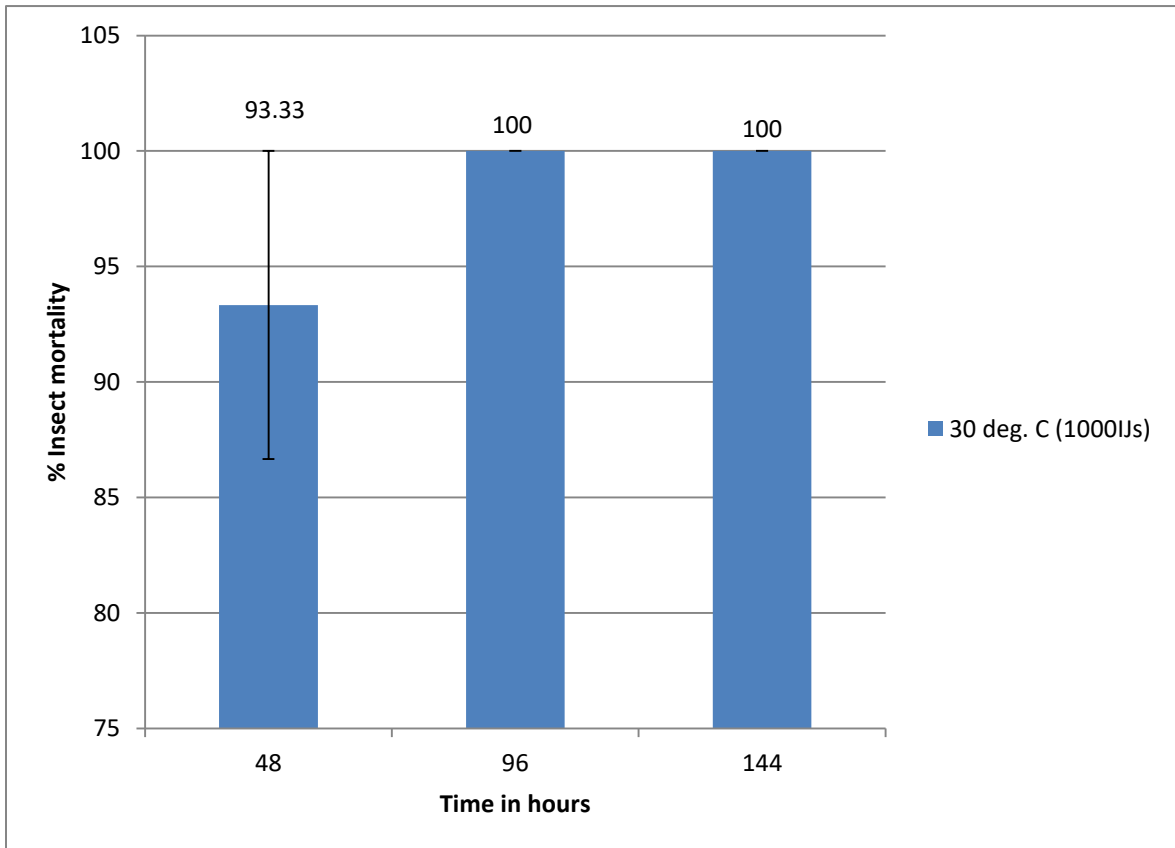
Figure 3.1 (c): The percentage of cumulative insect mortality (n=15) measured at 48 hour intervals at the experimental temperature of 30º C. The study was conducted at a population density of 1000 IJs and a moisture content of 16 %. The standard error of the means (±) were represented by standard error bars. The second controls yielded 0 % insect mortality. During the first 48 hours of infection, 93.33 % of insect mortality was achieved. This percentage was higher than the control temperature during 48 hours of infection. However, this percentage was lower when compared to the temperature regimen of 20° C. At 96 hours, 100 % host mortality was achieved.

3.3.1.4　Summary of figures 3.1(a-c)

The infectivity of the EPNs was characterized holistically by insect mortality. The processes which led to host death included infective juvenile penetration, bacterial release and the production of toxic virulence factors (Grewal et al., 1994; Yadav, 2012). According to the figures above, the species under investigation achieved high percentages of mortality at each

temperature regimen by 48 hours of infection. However, the highest percentage of mortality (100 %) was achieved at 20° C. The lowest percentage of mortality (86. 67 %) was achieved at 25° C. The species achieved 100 % insect mortality by 96 hours of infection at all temperature regimens. The Two-way Analysis of Variance (ANOVA) statistic test with replication was performed to evaluate the mean insect mortalities at different time intervals and temperature regimens.

3.3.1.5   Two-way ANOVA test

The Two-way ANOVA statistic test with replication was performed to determine the significance of the mean insect mortalities at each time interval and temperature regimen. The study was performed at a constant population density of 1000 infective juveniles.

Hypotheses

a) **Row factor (Time)**

H_o: There are no differences in the mean insect mortalities at different time intervals.

H_1: There is at least one mean mortality inequality present amongst the different time intervals.

$\alpha = 0.05$

b) **Column factor (Temperature)**

H_o: There are no differences in the mean mortalities at the control and experimental temperature regimens.

H_1: There is at least one mean mortality inequality present between the control and experimental temperature regimens.

$\alpha = 0.05$

c) **Interaction (Time and Temperature)**

H_o: There is no interaction between time and temperature.

H_1: There is interaction between time and temperature.

α = 0.05

Table 3.1: The summary statistics of the Two-way ANOVA test with replication. The mean mortalities at each time interval and temperature regimen were compared. The row factor represented temperature and the column factor represented temperature. The test included an interaction assessment between the two factors. The test was performed at a constant population density of 1000 IJs.

Source of variation	Sum of squares	Degrees of freedom	Mean square	F	P-value	F-critical
Rows	1333,33	2,00	666,67	3,15	0,05	3,07
Columns	444,44	2,00	222,22	1,05	0,35	3,07
Interaction	888,89	4,00	222,22	1,05	0,38	2,44
Within	26666,67	126,00	211,64			
Total	29333,33	134,00				

Decision

a) **Row factor (Time):** The P-value of 0.05 was equal to the significance level of 0.05. The test incorporated the F-statistic which employs the normal distibution under the null hypothesis. The F-value of 3.15 was higher than the F-critical value of 3.07 which implied that the data was categorized within the rejection region. The H_o was rejected and the H_1 hypothesis was accepted. At least one insect mortality mean was significantly different from the other two mortality means at the respective time intervals. This implied that the time taken to acheive insect mortality was significant.

b) **Column factor (Temperature)**: The P-value of 0.35 was higher than the significance level of 0.05. The test incorporated the F-statistic which employs the normal distribution under the null hypothesis. The F value of 1,05 was lower than the F-critical value of 3,07. This implied that the data was categorized in the acceptance region. The H_o was accepted and the H_1 was rejected. There was no statistical significance in the mean mortalities obtained at each temperature regimen.

c) **Interaction (Time and temperature):** The P-value of 0,38 was higher than the significance level of 0,05. The F-value of 1,05 was lower than the F-critical value of 2,44. The data was categorized within the acceptance region. The H_o was accepted and the H_1 was rejected. There was no interaction between time and temperature.

3.3.2   EPN Infectivity (500 IJs)
3.3.2.1   Insect Mortality vs. time (20⁰ C)
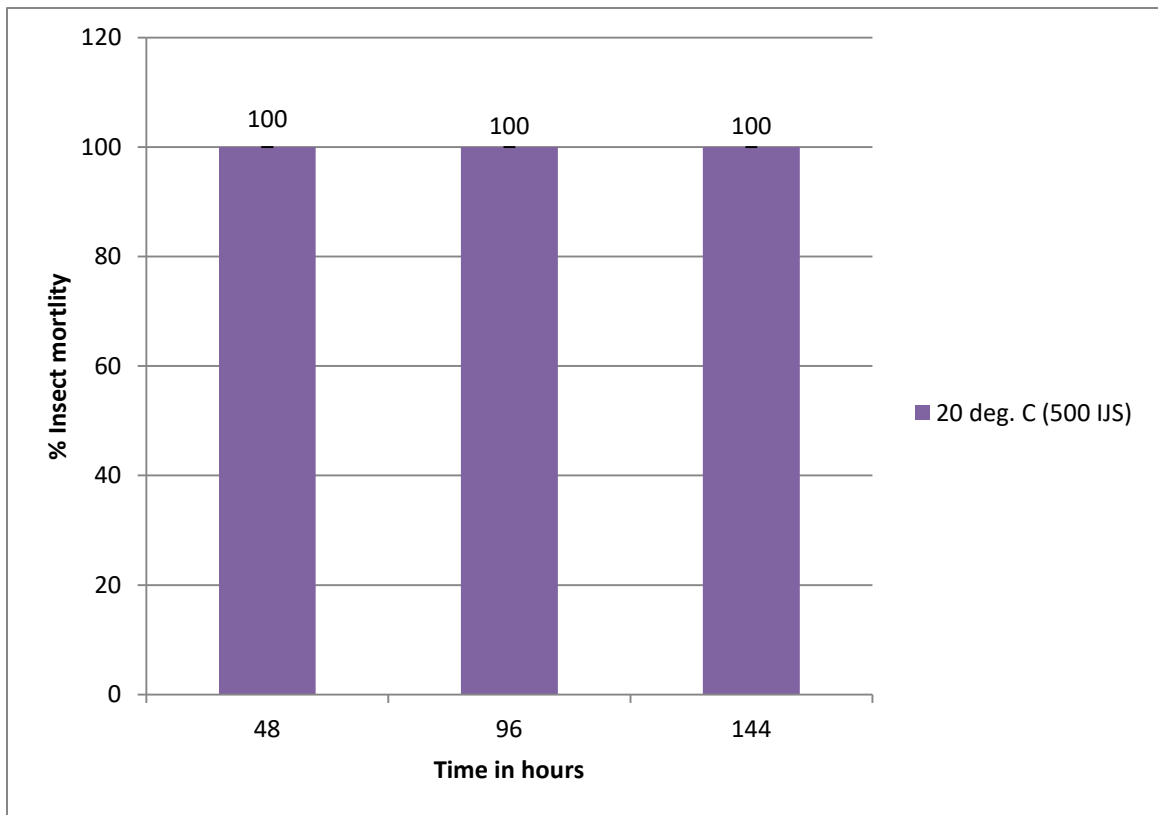
Figure 3.2 (a): The percentage of cumulative insect mortality (n = 15) measured at 48 hour intervals 20⁰ C. The study was performed at a population density of 500 infective juveniles. The standard error of the means (±) were represented by error bars. The study was conducted at a moisture content of 16 %. The second controls yielded 0 % of insect mortality. A high percentage of insect mortality (100 %) was achieved in the first 48 hours of infection.

3.3.2.2 % Insect Mortality vs. time (25º C)
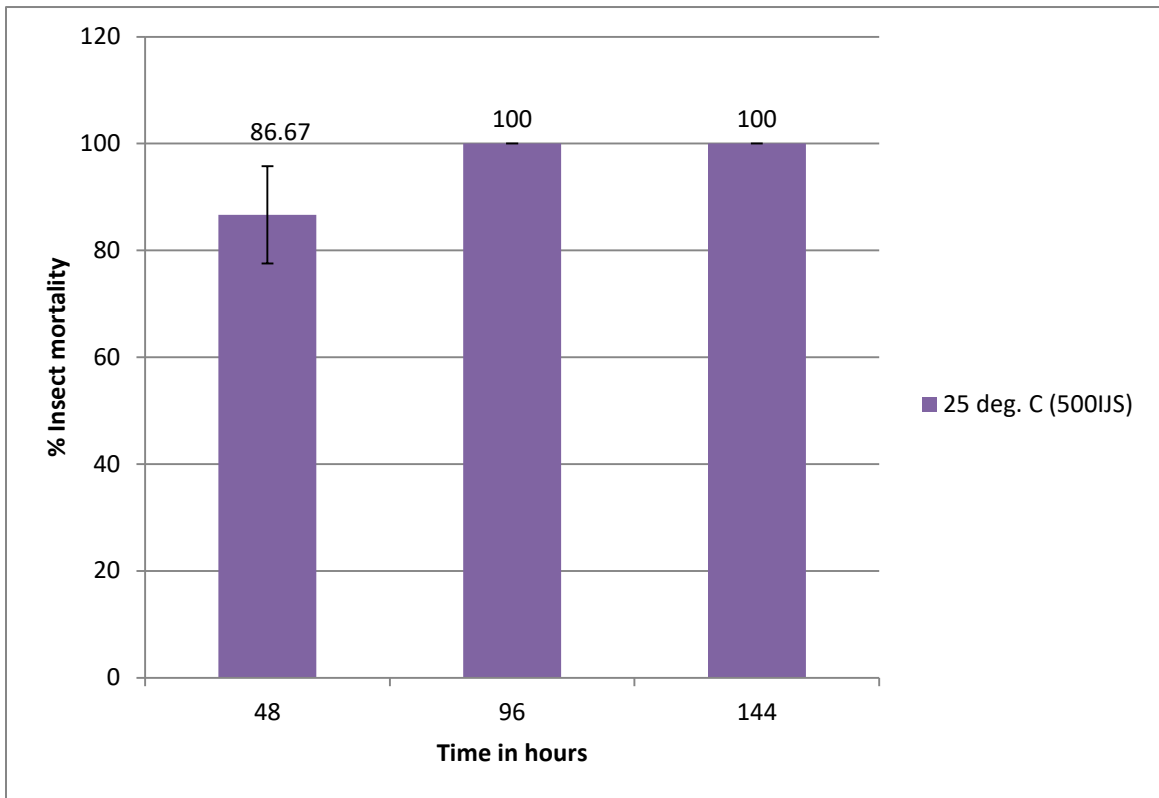
Figure 3.2 (b): The percentage of cumulative insect mortality (n = 15) measured at 48 hour intervals at 25º C. The study was performed at a population density of 500 infective juveniles. The standard error of the means (±) were represented by standard error bars**.** The study was conducted at a moisture content of 16 %. The second controls yielded 0 % of insect mortality. At 48 hours, 86.67 % insect mortality was achieved. A higher percentage of insect mortality was achieved by 48 hours of infection at 20° C. However, at 96 hours, 100 % insect mortality was achieved.

3.3.2.3   % Insect Mortality vs. time (30º C)
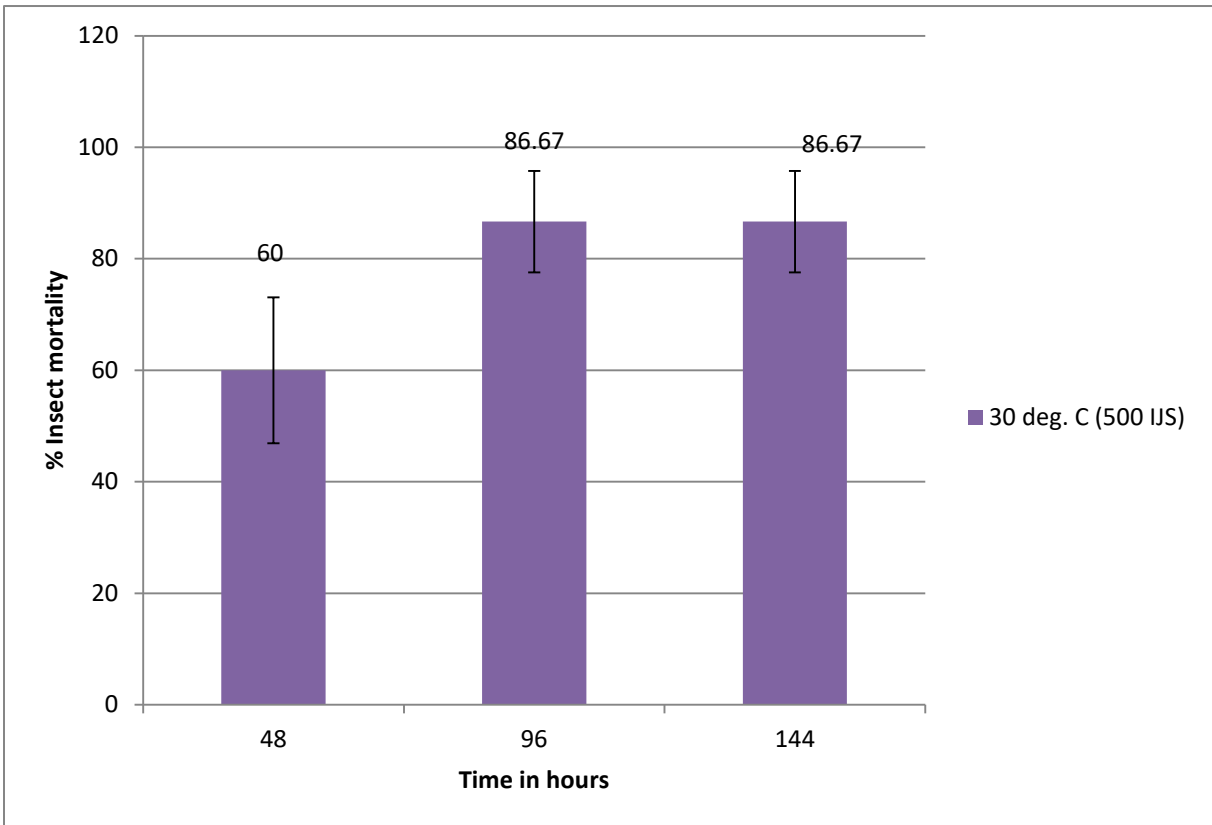
Figure 3.2 (c): The percentage of cumulative insect mortality (n = 15) measured at 48 hour intervals at 30º C. The study was performed at a population density of 500 infective juveniles. The standard error of the means ($\pm$) were represented by standard error bars. The study was conducted at a moisture content of 16 %. The second controls yielded 0 % of insect mortality. A low percentage of insect mortality (60 %) was obtained in the first 48 hours of infection. Higher percentages of insect mortalities were achieved at 20° C and 25° C. The percentage of insect mortality increased to 86.67 % by 96 hours. At this temperature regimen, 100 % insect mortality was not achieved.

3.3.2.4   Summary of figures 3.2 (a-c)

The infectivity of the EPNs were measured with a smaller population density of 500 infective juveniles. According to the figures above, insect mortality was achieved at all temperature regimens. However, different extents of mortality was obtained at the different time intervals and temperature regimens. At 20° C, rapid insect mortality was achieved by 48 hours of infection

(100 %). At this time interval, a high percentage of mortality was obtained at 25° C (86.67 %). The lowest percentage of insect mortality was obtained at 30° C (60 %). At 96 hours of infection, 100 % insect mortality was obtained at 25° C. At 30° C, the percentage mortality increased to 86.67 % by 96 hours of infection. The species was unable to acheive 100 % mortality at this temperature regimen. The Two-way ANOVA statistic test with replication was performed to evaluate the mean insect mortalities at different time intervals and temperature regimens.

3.3.2.5 Two-way ANOVA test

The Two-way ANOVA statistic test with replication was performed to determine whether significant differences were present in the mean larval mortalities obtained at each time interval and temperature regimen. The study was performed at a constant population density of 500 infective juveniles.

<u>Hypotheses</u>

a) **<u>Row factor (time)</u>**

H_o : There are no differences in the mean mortalities at different time intervals.

H_1: There is atleast one mean mortality inequality present at the different time intervals.

$\alpha = 0.05$

b) **<u>Column factor (temperature)</u>**

H_o: There are no differences in the mean mortalities at the control and experimental temperature regimens.

H_1: There is atleast one mean mortality inequality present between the control and experimental temperature regimens.

$\alpha = 0.05$

c) **<u>Interaction (Time and temperature)</u>**

H_o: There is no interaction between time and temperature

H_1: There is interaction between time and temperature.

$\alpha = 0.05$

Table 3.2: The summary statistics of the Two-way ANOVA test with replication. The mean mortalities at each time interval and temperature regimen were compared. The row factor represented time and the column factor represented temperature. The test included an interaction assessment between the two factors. The study was performed at a constant population density of 500 IJs.

Source of variation	Sum of squares	Degrees of freedom	Mean square	F	P-value	F-critical
Rows	5333,33	2,00	2666,67	3,82	0,02	3,07
Columns	12444,44	2,00	6222,22	8,91	0,00	3,07
Interaction	3555,56	4,00	888,89	1,27	0,28	2,44
Within	88000,00	126,00	698,41			
Total	109333,33	134,00				

Decision

a) **Row factor**: The P-value of 0.02 was lower than the significance level of 0.05. The test incorporated the F-statistic which employs the normal distribution under the null hypothesis. The F-value of 3.82 was greater than the F-critical value of 3,07. The data was categorized within the rejection region. The H_o was rejected and the H_1 was accepted. This implied that at least one mortality mean was significantly different from the other mortality means which were obtained from the respective time intervals. The time taken to achieve insect mortality was statistically significant.

b) **Column factor**: The the P-value of 0.00 was lower than the significance level of 0.05. The F-value of 8.91 was greater than the F-critical value of 3.07. The data was categorized into the rejection region. The H_o was rejected. The H_1 was accepted which implied that at least one mortality mean was significantly different from the other means which were obtained at the respective temperature regimens. The statistical findings of this test implied that insect mortality may be influenced by temperature.

c) **Interaction**: The P-value of 0.28 was lower than the significance level of 0.05. The F-value of 1.27 was lower than the F-critical value of 2.44. The data was catergorized into

the acceptance region. The H_o was accepted and the H_1 was rejected. The statistic values obtained from this test implied that there was no inetraction between time and temperature.

3.3.3 Population density and Temperature

3.3.3.1 Two-way ANOVA

The subsequent anlayses were performed to determine whether significant differences were present in the mean insect mortalities at each population density and temperature regimen. The two-way ANOVA test with replication was performed at a constant time interval of 48 hours.

Hypotheses

a) **Row factor (Temperature)**

H_o: There are no differences in the mean insect mortalities at each temperature regimen.

H_1: There is at least one mean mortality inequality present at the different temperature regimens.

$\alpha = 0.05$

b) **Column factor (Population density)**

H_o: There are no differences present in the mean insect mortalities at each population density of infective juveniles.

H_1: There is at least one mean mortality inequality present between the two population densities of infective juveniles.

$\alpha = 0.05$

c) **Interaction (Temperature and population density)**

H_o: There is no interaction between temperature and population density.

H_1: There is interaction between temperature and population density.

$\alpha = 0.05$.

Table 3.3 (a): The summary statistics of the Two-way ANOVA test with replication. The mean insect mortalities at each temperature regimen and population density were compared. The

row factor represented temperature and the column factor represented population density. The test included an interaction assessment between the two factors. The study was performed at 48 hours.

Source of variation	Sum of squares	Degrees of freedom	Mean square	F	P-value	F-critical
Row	8222,22	2,00	4111,11	4,32	0,02	3,11
Column	2777,78	1,00	2777,78	2,92	0,09	3,95
Interaction	5555,56	2,00	2777,78	2,92	0,06	3,11
Within	80000,00	84,00	952,38			
Total	96555,56	89,00				

Decision

a) **Row factor**: The P-value of 0.02 was lower than the significance level of 0.05. The F-value of 4.32 was higher than the F-critical value of 3.11. The data was categorized into the rejection region. The H_o was rejected and the H_1 was accepted. Statistical significance was obtained between the mean mortalities achieved at each temperature regimen. The statistical values obtained from the test implied that different temperature regimens may influence host mortality.

b) **Column factor**: The P-value of 0.09 was higher than the significance level of 0.05. The F-value of 2.92 was lower than the F-critical value of 3.95. The data was categorized into the acceptance region. The H_o was accepted. There was no statistical significance present between the mean insect mortalities at each population density. The statistical values obtained from the test implied that different population densities may not influence insect mortality.

c) **Interaction**: The P-value of 0.06 was higher than the significance level of 0.05. The F-value of 2.91 was lower than the F-critical value of 3.11. The data was categorized into the acceptance region. The H_o was accepted and the H_1 was rejected. There was no interaction present between temperature and population density.

3.3.3.2 Two-way ANOVA

The Two-way ANOVA test with replication was performed to determine whether significant differences were present in the mean insect mortalities achieved at each temperature regimen and population density. The study was performed at a time interval of 96 hours.

Hypotheses

a) **Row factor (Temperature)**

H_o: There are no differences in the mean insect mortalities at each temperature regimen.

H_1: There is at least one mean mortality inequality present at the different temperature regimens.

$\alpha = 0.05$

b) **Column factor (Population density)**

H_o: There are no differences in the mean insect mortalities at each population density of infective juveniles.

H_1: There is at least one mean mortality inequality present between the two population densities of infective juveniles.

$\alpha = 0.05$.

c) **Interaction (Temperature and population density)**

H_o: There is no interaction between temperature and population density.

H_1: There is interaction between temperature and population density.

$\alpha = 0.05$

Table 3.3 (b): The summary statistics of the Two-way ANOVA test with replication. The mean insect mortalities at each temperature regimen and population density were compared. The row factor represented temperature and the column factor represented population density. The test assessed the interaction between the two factors. The study was performed at 96 hours.

Source of variation	Sum of squares	Degrees of freedom	Mean square	F	P-value	F-critical
Row factor	888,89	2,00	444,44	2,15	0,12	3,11

Column factor	444,44	1,00	444,44	2,15	0,15	3,95
Interaction	888,89	2,00	444,44	2,15	0,12	3,11
Within	17333,33	84,00	206,35			
Total	19555,56	89,00				

Decision

a) **Row factor**: The P-value of 0.12 was higher than the significance level of 0.05. The F-value of 2.15 was lower than the F-critical value of 3.11. The data was categorised within the acceptance region. The H_o was accepted and the H_1 was rejected. This implied that the differences obtained in the mean mortalities of each temperature regimen were not statistically significant.

b) **Column factor**: The P-value of 0.15 was higher than the signifcance level of 0.05. The F-value of 2.15 was lower than the F-critical value of 3.95. The data was categorized in the acceptance region. The H_o was accepted and the H_1 was rejected. This finding implied that the differences obtained in the mean mortalities of each population density were not statistically significant.

c) **Interaction**: The P-value of 0.12 was higher than the significance level of 0.05. The F-value of 2,15 was lower than the F-critical value of 3.11. The data was categorized in the acceptance region. The H_o was accepted and the H_1 was rejected. This finding implied that no interaction was present between temperature and population density.

3.3.3.3   Two-way ANOVA

The Two-way ANOVA test with replication was performed to determine whether differences were present in the mean mortalities at each temperature regimen and population density of infective juveniles. The study was performed at a time interval of 144 hours.

Hypotheses

a) **Row factor (temperature)**

H_o: There are no differences in the mean mortalities at each temperature regimen.

H_1: There is at least one mean insect mortality inequality present at the different temperature regimens.

$\alpha = 0.05$

b) **Column factor (population density)**

H_o: There are no differences in the mean mortalities at each population density of infective juveniles.

H_1: There is at least one mean mortality inequality present between the two population densities of infective juveniles.

$\alpha = 0.05$.

c) **Interaction (temperature and population density)**

H_o: There is no interaction between temperature and population density.

H_1: There is interaction between temperature and population density.

$\alpha = 0.05$

Table 3.3 (c): The Two-way ANOVA test with replication. The mean insect mortalities at each temperature regimen and population density were compared. The row factor represented temperature and the column factor represented population density. The test assessed the interaction between the two factors. The study was performed at a time interval of 144 hours.

Source of variation	Sum of squares	Degrees of freedom	Mean square	F	P-value	F-critical
Row	888,89	2,00	444,44	2,15	0,12	3,11

Column	444,44	1,00	444,44	2,15	0,15	3,95
Interaction	888,89	2,00	444,44	2,15	0,12	3,11
Within	17333,33	84,00	206,35			
Total	19555,56	89,00				

Decision

a) **Row factor**: The P-value of 0.12 was higher than the significance value of 0.05. The F-value of 2.15 was lower than the F-critical value of 3.11. The data was categorized in the acceptance region. The H_o was accepted and the H_1 was rejected. This implied that the differences obtained in the mean mortalities of each temperature regimen were not statistically significant.

b) **Column factor**: The P-value 0f 0.15 was higher than the significance level of 0.05. The F-value of 2.15 was lower than the F-critical value of 3.95. The data was categorized within the acceptance region. The H_o was accepted and the H_1 was rejected. The statistical values obtained from the test implied that there was no statistical significance between the mortality means at each population density of infective juveniles.

c) **Interaction**: The P-value of 0.12 was higher than the significance level of 0.05. The F-value of 2, 15 were lower than the F-critical value of 3.11. The data was categorized within the acceptance region. The H_o was accepted and the H_1 was rejected. There was no interaction between temperature and population density.

3.3.4 Infective juvenile recovery
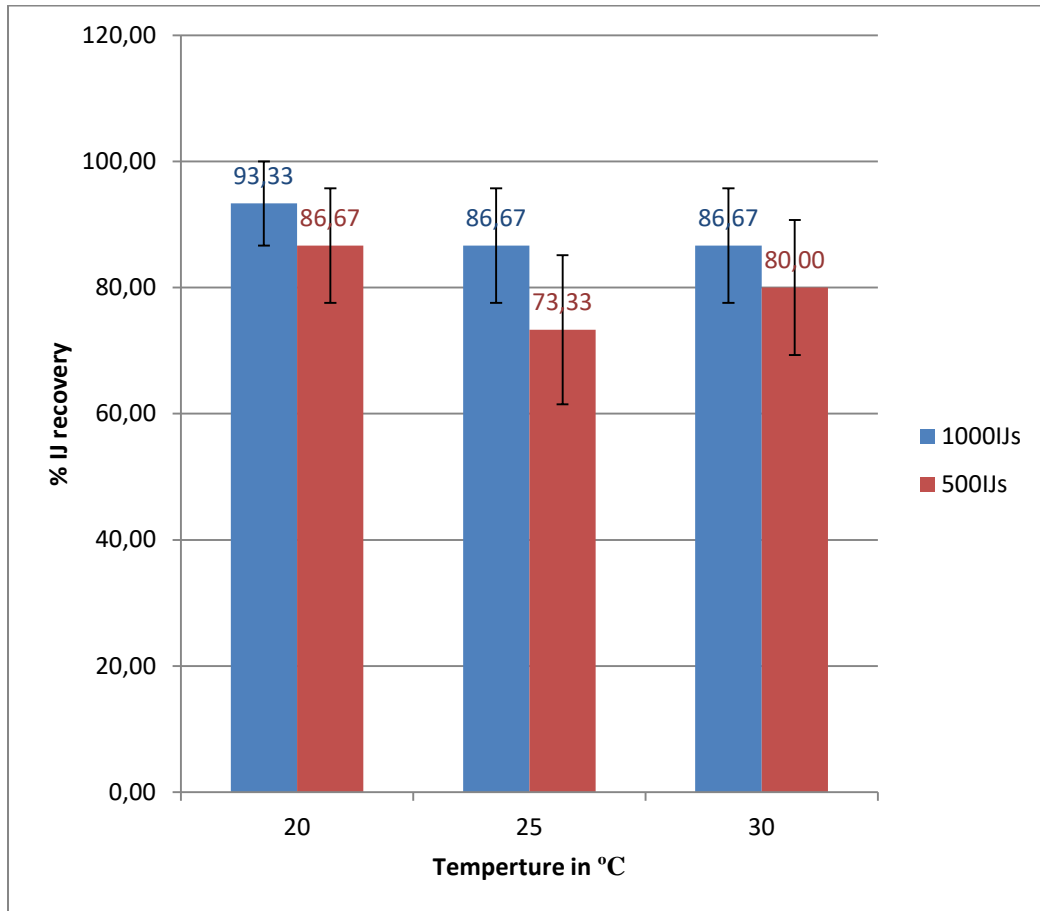
Figure 3.3: The percentage of recovered infective juveniles from two population densities at control and experimental temperature regimens. Recovery was expressed by percentage values and was recorded by the emergence of progeny infective juveniles. The standard error of the means (±) was represented by standard error bars. Each population density of infective juveniles yielded progeny which emerged at all temperature regimens. The population density of 1000 infective juveniles yielded the highest percentage of recovery at all temperature regimens. The population density of 500 infective juveniles yielded the lowest percentage of recovery at all temperature regimens. The temperature of 20° C yielded the highest percentage of recovery at the population density of 1000 IJs. The temperature regimens 25 and 30° C yielded the lowest percentages of recovery at the population density of 1000 infective juveniles. The temperature regimen 20° C also yielded the highest percentage of recovery at the population density of 500 infective juveniles. However, the percentage recovery achieved at 25° C was lower than the recovery achieved at 30° C for the population density of 500 infective juveniles.

3.3.4.1 Two-way ANOVA summary

The Two-way ANOVA test with replication was performed to determine whether significant differences were present in the mean infective juvenile recoveries at each temperature regimen and population density of infective juveniles.

Hypotheses

a) **Row factor (Temperature)**

H_o: There are no differences in the mean infective juvenile recoveries at each temperature regimen.

H_1: There is at least one mean recovery inequality is present amongst the different temperature regimens.

$\alpha = 0.05$

b) **Column factor (Population density)**

H_o: There are no differences in the mean recoveries at each population density of infective juveniles.

H_1: There is at least one mean recovery inequality present between the two population densities of infective juveniles.

$\alpha = 0.05$

c) **Interaction (Temperature and population density)**

H_o: There is no interaction between temperature and population density.

H_1: There is interaction between temperature and population density.

$\alpha = 0.05$

Table 3.4: The summary statistics of the Two-way ANOVA test with replication. The mean infective juvenile recoveries at each temperature regimen and population density were compared. The row factor represented temperature and the column factor represented population density. The test evaluated the interaction between the two factors.

Source of variation	Sum of squares	Degrees of freedom	Mean square	F	P-value	F-critical

Row	1555,56	2,00	777,78	0,57	0,57	3,11
Column	1777,78	1,00	1777,78	1,30	0,26	3,95
Interaction	222,22	2,00	111,11	0,08	0,92	3,11
Within	114666,67	84,00	1365,08			
Total	118222,22	89,00				

Decision

a) **Row factor**: The P-value of 0.57 was higher than the significance level of 0.05. The F-value of 0.57 was lower than the F-critical value of 3.11. The data was categorized in the acceptance region. The H_o was accepted and the H_1 was rejected. There was no statistical significance between the mean IJ recoveries at the different temperature regimens.

b) **Column factor**: The P-value of 0.26 was higher than the significance level of 0.05. The F-value of 1.30 was lower than the F-critical value of 3.95. The data was categorized into the acceptance region. The H_o was accepted and the H_1 was rejected. There was no statistical significance in the mean recoveries between the two population densities of infective juveniles.

c) **Interaction**: The P-value of 0.92 was higher than the significance level of 0.05. The F-value of 0.08 was lower than the F-critical value of 3.11. The data was categorized in the acceptance region. The H_o was accepted and the H_1 was rejected. There was no interaction between temperature and population density.

3.3.5 Progeny IJ yield
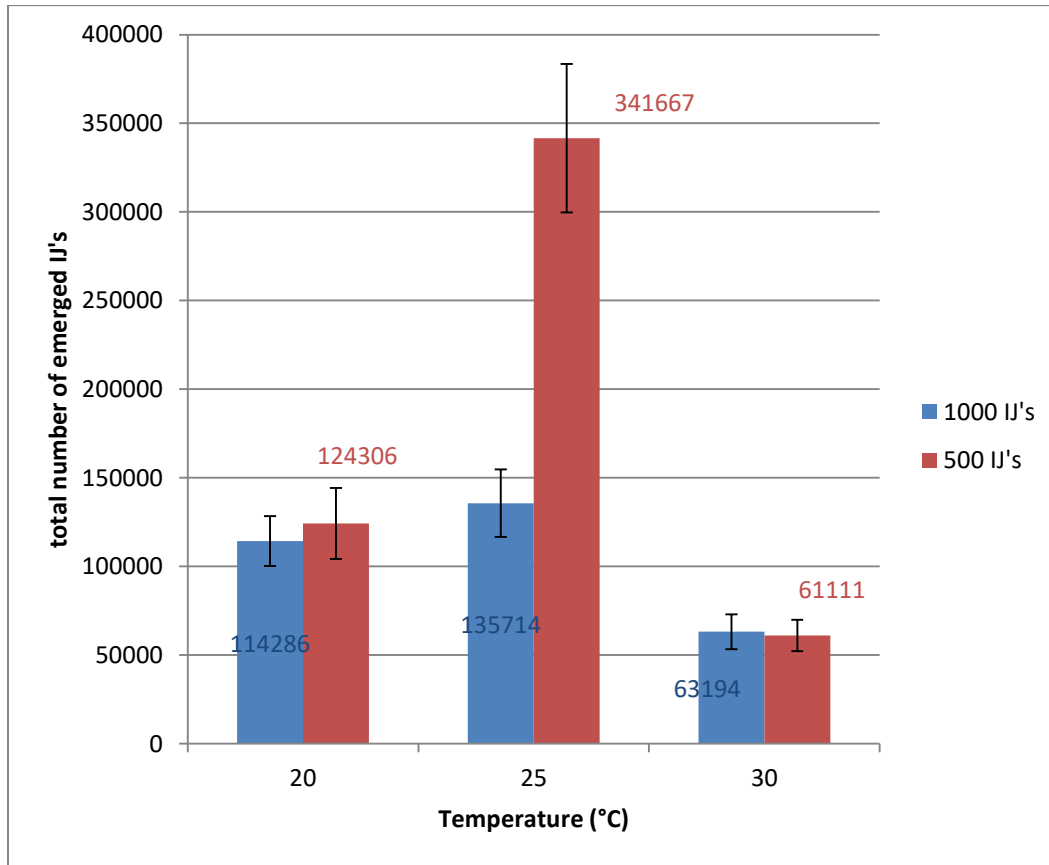
Figure 3.4: The yield of infective juveniles produced from two population densities at control and experimental temperature regimens. The yield was expressed as the number of infective juveniles produced per insect cadaver. The standard error of the means (±) was represented by standard error bars. Both population densities of infective juveniles produced yield at all temperature regimens. The population density of 500 infective juveniles produced higher yields at 20 and 25° C. However, lower yields were produced at 30° C. The population density of 1000 IJs produced lower yields than the population density of 500 IJs at 20 and 25° C. However, a higher yield was obtained at 30° C. Optimum infective juvenile yields were produced at the temperature regimen of 25° C. The yields of infective juveniles were also high at 20° C for both population densities. However, the yields of infective juveniles were lower at 30° C.

3.3.5.1 Two-way ANOVA

The Two-way ANOVA test with replication was performed to determine whether significant differences were present in the mean infective juvenile yields at each temperature regimen and population density.

Hypotheses

a) **Row factor (Temperature)**

H_o: There are no differences in the mean infective juvenile yields at each temperature regimen.

H_1: There is at least one mean infective juvenile yield inequality present between the different temperature regimens.

$\alpha = 0.05$.

b) **Column factor (Population density)**

H_o: There are no differences in the mean infective juvenile yields at each population density of infective juveniles.

H_1: There is at least one mean infective juvenile yield inequality present between the two population densities of infective juveniles.

$\alpha = 0.05$

c) **Interaction (Temperature and population density)**

H_o: There is no interaction between temperature and population density.

H_1: There is interaction between temperature and population density.

$\alpha = 0.05$

Table 3.5: The summary statistics of the Two-way ANOVA test with replication. The mean infective juvenile yields at each temperature regimen and population density were compared. The row factor represented temperature and the column factor represented population density. The interaction between temperature and population density was evaluated.

Source of	Sum of	Degrees of	Mean square	F	P-value	F-critical

variation	squares	freedom				
Row	1,38E+11	2,00	6,92E+10	7,16	0,00	3,11
Column	6,05E+10	1,00	6,05E+10	6,26	0,01	3,95
Interaction	1,43E+11	2,00	7,14E+10	7,39	0,00	3,11
Within	8,11E+11	84,00	9,66E+09			
Total	1,15E+12	89,00				

Decision

a) **Row factor:** The P-value of 0.00 was lower than the significance level of 0.05. The F-value of 7.16 was higher than the F-critical value of 3.10. The data was categorized within the rejection region. The H_o was rejected and the H_1 was accepted. This implied that at least one mean infective juvenile yield was significantly different to the other means at the respective temperature regimens. Temperature influenced infective juvenile yield.

b) **Column factor**: The P-value of 0.01 was lower than the significance level of 0.05. The F-value of 6.26 was higher than the F-critical value of 3.95. The data was categorized in the rejection region. The H_o was rejected and the H_1 was accepted. At least one mean infective juvenile yield was significantly different from the other mean at the respective population density. The statistical values obtained by the test showed that population density may influence the infective juvenile yield.

c) **Interaction:** The P-value of 0.01 was lower than the significance level of 0.05. The F-value of 7.39 was higher than the F-critical value of 3.11. The data was categorized in the rejection region. The H_o was rejected and the H_1 was accepted. The statistical values obtained from the test showed interaction between temperature and population density.

3.4   DISCUSSION

3.4.1   EPN infectivity

3.4.1.1   Time

The time taken to achieve host mortality is important when considering the biological control potential of the species under investigation. At a population density of 1000 infective juveniles, the Two-way ANOVA test generated a P-value of 0.05. The P-value implied statistical significance between the mean insect mortalities at different intervals of time. This led to the rejection of the null hypothesis. The rejection of the null hypothesis implied that at least one mean mortality inequality was present at a given time interval. These findings were consistent with the graphical representation of the cumulative mortality graphs (figures 3.1a-3.1c). According to the graphs, the species under investigation killed the larger proportion of insect hosts at the 48 hour time interval. According to Kalia et al., (2014), *Steinernema* spp. penetrates the insect host and releases the symbiotic bacteria into the haemocoel of the insect. The bacteria then proliferate and kill the insect host within 24-48 hours. This may account for the high percentage of mortality observed within the first 48 hours of infection. Since insect mortality was accumulated, there were almost negligible differences between 96 and 144 hours of infection. Although the percentages of mortality were high at the 48 hour time interval, 100 % mortality was not achieved at two temperature regimens. The possible explanations may be related to the delay in EPN penetration or release of bacterial cells which may be affected by minute effects of temperature. The interaction P-value of 0.38 led to the acceptance of the null hypothesis which stated no interaction was present between time and temperature. However, this did not imply that temperature had no influence on the time taken to achieve host mortality. The finding meant that the differences were not sufficient to be regarded as statistically significant. One possible explanation for this finding may related to the constant population density of infective juveniles at which the investigation was carried out. The high population density of 1000 infective juveniles may have overcome the effect of temperature to certain extents which contributed to the statistical findings regarding the interaction between time and temperature.

A second investigation was conducted to evaluate the time taken to achieve host mortality at a smaller population size of infective juveniles. The research findings obtained from the Two-way ANOVA analyses were similar to the results of the previous investigation. Significant

differences were present between the mean insect mortalities obtained at each time interval. The P-value of 0.002 led to the rejection of the null hypothesis. The rejection of the null hypothesis implied that at least one mean mortality inequality at a given time interval was present. According to the graphs (figures 3.2a-3.2c), this inequality may have been represented by the 48 hour time interval. This was attributed to the large proportion of insect mortality achieved during the first 48 hours of infection. This finding was unexpected. It was expected that a smaller population size of infective juveniles required an increase in the time taken to achieve host mortality. However, in this investigation, host mortality was still observed in the first 48 hours of infection with reduced population size of infective juveniles. This finding confirmed that the symbiotic bacteria of the species under investigation required at least 24-48 hours to achieve host mortality. The rapid infections obtained at 48 hours with a reduced population density of infective juveniles may have implied that the symbiotic bacteria were highly virulent. Although a proportion of insect hosts were killed at 48 hours, the percentages of insect mortality at 48 hours were lower than that achieved for the previous investigation. Also at 30° C, 100 % insect mortality was not achieved at the extended time intervals of 96 and 144 hours. This finding may have implied that temperature may have influenced the time taken to achieve 100 % host mortality at 30° C. The interaction P-value led to the acceptance of the null hypothesis which stated that no interaction was present between time and temperature. However, from the findings above, the presence of interaction was observed at 30° C. However, interaction was not sufficient to contribute to statistical significance.

3.4.1.2   Temperature

Host infection was obtained at the two experimental and control temperatures. At all temperature regimens, 100 % host mortality was achieved at a constant population density of 1000 infective juveniles. This finding was unexpected. The percentages of host mortalities achieved at the two experimental temperatures of 20° C and 30° C may be explained by the following outcomes. The first included the phenomenon of inadvertent temperature selection which may have occurred during the initial propagation of the species for 18 months at laboratory conditions (25° C). According to Jagdale and Gordon (1998) the propagation of the EPNs at laboratory temperatures may have caused changes in the genetic constitution of the EPNs which ultimately contributed to the performances of the EPNs at high and low temperature limits. This may serve as one

explanation for high percentages of host mortality at the two experimental temperatures. Alternatively, the performances of the EPNs may have resulted from acute acclimation. Hill et al., (2015) stated that the performances of some EPN species were enhanced by acute temperature acclimation whilst other species demonstrated decrements in performance. The results obtained from the Two-way ANOVA test showed no statistical significance (P = 0.35) between the mean mortalities at each temperature regimen. The statistical insignificance between the mean mortalities does not imply that temperature had no influence on host mortality. This finding implied that the differences between the mean mortalities were not large enough to be considered as statistically significant. The explanations above may explain the statistical insignificance between the mean mortalities at each temperature regimen. Also, the high percentages of infectivity at the two experimental temperatures may suggest that the temperatures represented a proportion of the infective thermal niche breadth of the species. One other possible explanation could involve the size of the EPN population. The large number of infective juveniles may have overcome the effects of temperature. To further validate the role of temperature on host mortality, further statistical analysis was performed.

The second investigation also tested host mortality at different temperature regimens. However, the study was performed at a reduced population density of 500 infective juveniles. Host infection was achieved at all temperature regimens. However, a reduction in host mortality was observed with an increase in temperature. At the temperature regimen of 30° C, 100 % host mortality was not obtained. The difference in host mortality at 30° C may have contributed to the statistical significance obtained between the mean insect mortalities at each temperature regimen (P = 0.00). The difference in the statistical significance between the two studies may be accounted for by the change in population density of infective juveniles. In the previous study the influence of temperature on the increments and decrements of host mortality may have been overcome by the large number of infective juveniles. In this study, a smaller population size was selected which may have elucidated the effect of the experimental temperatures on host mortality.

To evaluate the effect of the experimental temperatures on host mortality, further ANOVA analyses were performed. The test evaluated the effect of temperature and population density on

host mortality at a constant time interval. At 48 hours of infection, the mean mortalities at each temperature regimen were significantly different. The differences were most likely to be observed at this time interval since a large proportion of insect hosts were killed within 48 hours. This suggested that the two experimental temperature regimens influenced host mortality at different extents with decrements in the performance at upper thermal limits and improvements in performance at lower thermal limit. The high infectivity of the species at 20° C may be accounted for by the climatic origin of the species. The species *Steinernema* originated from temperate conditions. However, the extents of mortality at 30° C may have implied that the species demonstrated flexibility in adaptation either through inadvertent selection or acute acclimation.

3.4.1.3   Population density

The effect of population density on host mortality was evaluated. The studies were performed at constant time intervals. At 48 hours, host mortality was achieved at both population densities. However, there were large differences in the percentages of host mortalities between the two population densities at 30° C. The percentage of host mortality was lower at the reduced population density of 500 infective juveniles. This finding was expected. This assay was performed by numerous researchers which observed positive relationships between population density and host mortality (Kalia et al., 2014; Yadav, 2012). However, the Two-way ANOVA analysis showed that the mean mortalities at both population densities were not statistically significant. Although differences were present between the host mortality percentages at each population density, the differences were not sufficient to be regarded as statistically significant. One other explanation for the statistical insignificance could be related to the high percentages (100 %) of host mortality achieved at the population density of 500 infective juveniles at the two lower temperature regimens (25° C and 20° C). The high percentages of mortalities achieved at these lower temperature regimens may not be due to the effect of infective juvenile population size. Instead, these temperature regimens may be favourable for host infection. The previous study elucidated that the increments in EPN infectivity was observed at the lower temperature regimens. Also, the interaction P-value of 0.06 was not too far from the significance level of 0.05 which may have suggested that a small extent of interaction was present between population density and temperature although not sufficient to be regarded as statistically significant.

3.4.2 Recovery

3.4.2.1 Temperature

In this investigation, the emergence of progeny infective juveniles represented recovery at each temperature regimen. The study conducted by Rahoo et al., (2016) showed that the emergence of infective juveniles from the insect cadavers was a temperature dependant process. The recovery of infective juveniles was observed at all temperature regimens. This finding implied that the infective juveniles transitioned from developmental arrest into first generation adults. Furthermore, this result suggested that the process of reproduction occurred between subsequent adult generations at all temperature regimens (Moshoyav et al., 2013). For the population density of 1000 infective juveniles, as the temperature increased, the percentage recovery of infective juveniles decreased. This finding was similar to the study conducted by Rahoo et al., (2016). The authors observed that the emergence of progeny infective juveniles from insect cadavers was impeded by dry environmental conditions and encouraged by moisture. The authors then related this finding to temperature. The authors explained that the dryer conditions were experienced at the lower temperature regimen of 5° C and moist conditions were present at 10° C. For this investigation, the argument would be valid due to the temperature range which was chosen. The temperature regimens were low. However, in the present study, the inverse was observed. It was observed that the higher temperature regimen of 30° C was correlated to the rapid loss of water whereas the moisture was retained at the medium temperature of 20° C. However, no statistical significance was obtained between the mean infective juvenile recoveries at each temperature regimen. Although differences were present, there was not sufficient variation between the mean recoveries to be regarded as significant. One explanation for this finding may involve the high percentages of infective juvenile recoveries that were observed at each temperature regimen. These high percentages of recovery at each temperature regimen may have been attributed to the moisture produced from the initial volume of water in the White traps. The water from the White traps ensured that the filter paper remained moistened. The cadavers were placed on the filter paper.

At the population density of 500 infective juveniles, the lowest percentage of recovery was not observed at the higher temperature regimen but at 25° C. At this temperature regimen, a proportion of cadavers dried on the sand which may have contributed to the low percentage of

recovery. The loss of water contributed to the desiccation of the cadavers. The desiccation caused the cuticle to harden and shrink in volume which ultimately impeded the emergence of nematodes and affected their survival. Also, the texture of the cadavers was soft and tore during the White trap preparation. The observation of progeny infective juveniles at such an early stage was difficult because sufficient time was required for the processes of reproduction and subsequent infective emergence. In this instance, positive infective juvenile recovery could not be recorded which may have contributed to the lower percentage at 25° C when compared to 30° C. Also, no statistical significance was present between the mean infective juvenile recoveries at each temperature regimen. Although differences were present between the mean recoveries, it was not sufficient to be regarded as statistically significant. The similarity between the findings of both recovery investigations may suggest that the interaction between population density and temperature may not have been present.

3.4.2.2   Population density

The recoveries at both population densities of infective juveniles were high. The differences in infective juvenile recovery were not substantial. The emergence of infective juveniles indicated the depletion of host nutrients within the insect cadaver (San-Blas, Gowen, and Pembroke, 2008). One possible explanation for the higher percentage of recovery observed at the higher population density of infective juveniles may be related to the rapid depletion of host nutrients due to larger numbers of the initial infective juvenile dosage. The larger population density may have utilized the host nutrients in a shorter period of time when compared to the smaller population of infective juveniles. The recovery of infective juveniles from both investigations was recorded at a given interval of time. This meant that if the smaller population size took a longer period of time to emerge due to the longer period of time required depleting the host nutrients, a higher percentage of recovery would have been obtained. However, in this investigation, the time taken for infective juvenile recovery was not measured. The statistical insignificance obtained between the two recovery means may have implied that the recovery of infective juveniles were not entirely dependent on the chosen sizes of population densities. For future investigations, a different population density range may be required to justify these findings. According to the statistical analysis, no interaction was present between temperature and population density. A reduction in recovery was observed with an increase in temperature at

the population densities of 1000 infective juveniles. However, variation was observed by the increase in recovery at 30° C rather than a decrease for the population density of 500 infective juveniles which may have contributed to the statistical insignificance regarding the interaction between temperature and population density.

3.4.3 Yield

3.4.3.1 Temperature

Temperature is regarded as one of the most important parameters which affect the yield of infective juveniles (Hirao and Ehlers, 2009). Two investigations were performed to determine the effect of temperature on the yield of infective juveniles. These studies were conducted at two population sizes of infective juveniles. For both studies, good progeny yields were produced at all temperature regimens. According to Shapiro-Ilan, Han, and Dolinksi (2012), a single insect host may produce 30 000- 50 000 infective juveniles under laboratory conditions. The species under investigation produced progeny yields above 50 000 infective juveniles at all temperature regimens. However, the extents of juvenile production varied between the different temperature regimens. For both investigations, the progeny yields were higher at the temperature regimen of 20° C and 25° C and lower yields were produced at 30° C. This finding was coherent with the other temperature related investigations performed previously in this research project. According to the statistical analysis, the differences obtained between the mean yields at different temperature regimens were statistically significant. This finding was coherent with the study conducted by (Zervos, Johnson, and Webster, 1990). The authors observed that higher yields of infective juveniles were obtained at 20° C and 25° C. Temperature may have two types of effects. They are the direct and indirect effects.

The direct effect involves the number of generations passed through the insect host. Temperature may affect the growth rate of the symbiotic bacteria of the species under investigation. This type is regarded as an indirect effect. The two temperature regimens (20° C and 25° C) may have influenced an increase in the number of generations passed through the insect host. Alternatively, the temperature regimens (20° C and 25° C) may have been favourable for the growth of the symbiotic bacteria. The growth of the symbiotic bacteria may be correlated to the reproductive

fitness of the EPNs. This claim was supported by the study conducted by Ogier et al., (2014). The authors observed reduced reproductive fitness of two aposymbiotic EPN species. However, for this study, further investigations are required to validate this claim. The temperature regimen of 30° C may be unfavourable for the growth of the symbiotic bacteria and may have influenced the reduction in the number of progeny infective juveniles. According to the investigations performed previously in this research project, decrements in the performance of the EPNs were mostly observed at the temperature regimen of 30° C. The findings of this investigation were supported by the statistical difference observed between the mean yields at each temperature regimen. In this study, the actual progeny yields observed at 30° C may have been attributed to the high extent of interaction between temperature and population density.

3.4.3.2   Population density

The yield parameter is important when assessing the biological control potential of the EPN species. The yield of infective juveniles provides insight into the innate reproductive capacities of the species under investigation. The yield of infective juveniles may be strongly affected by the initial inoculum size of infective juveniles (Leite et al., 2016). The effect of two different population densities on the yield of progeny infective juveniles was assessed. In this investigation, good progeny yields were obtained at both population densities of infective juveniles. However, the unexpected finding was that higher progeny yields were obtained at the lower population densities of 500 infective juveniles. The study conducted by Yadav (2012) showed a positive relationship between juvenile dose and yield of infective juveniles for the species *Steinernema thermophilum*. However, the progeny soon declined with higher doses of infective juveniles. Contrastingly, the authors observed that species *S. glaseri* obtained low progeny yields with higher doses of infective juveniles. The authors then attributed the differences in progeny production at different doses of infective juveniles to species specificity.

In the present investigation, an inverse relationship was observed between population density and yield of progeny infective juveniles. This finding was coherent with the study conducted by Zervos, Johnson and Webster (1990). The authors observed higher progeny yields with lower doses of infective juveniles. The authors attributed this finding to a deleterious crowding effect. The sizes of Steinernematid females depend on nutrient availability. The number of progeny infective juveniles carried by the maternal adult depends on the size of the maternal adult. In this

investigation, the smaller number of infective juveniles may have utilized a lower proportion of nutrients. This implied that the nutrient availability in the cadavers were higher when infected with smaller population densities of infective juveniles. This possibly gave rise to large sized maternal adults. Large sizes implied a greater carrying capacity for progeny infective juveniles which may have contributed to the larger yields of infective juveniles observed at the low population densities. Conversely, due to the higher number of infective juveniles (1000 infective juveniles), more nutrients were utilized. The reduced nutrient availability may have contributed to smaller sized females which contributed to the lower progeny yields. However, at the temperature regimen of 30° C, the yields were slightly higher at 1000 infective juveniles. The differences between both population densities were not too substantial. However, this finding may be explained by the influence of temperature since strong interaction was present between temperature and population density.

3.5   CONCLUSION

The species *Steinernema* spp was investigated in the area of infectivity, recovery and yield at two experimental temperatures after acute temperature acclimation. The influence of time and population density on the different biological processes was also determined. The species achieved rapid infections within 48 hours at each temperature regimen. The effect of temperature on host mortality was elucidated at lower population densities of infective juveniles. The upper (30 °C) and lower temperature (20° C) regimens affected host infection to different extents. The differences obtained between the mortalities at the two experimental temperatures were explained by the climatic origins of the species and the performance of the species at higher temperatures was attributed to acute temperature acclimation. The species demonstrated efficacious abilities in host infection at both population densities of infective juveniles.

The percentages of recoveries of infective juveniles were also high at each temperature regimen. However 100 % recovery was not obtained. The effects of temperature on the recovery of the EPNs were not significant. However, the influence of temperature influenced the recovery process to certain extents. The infective juvenile recoveries at both population densities were high and the differences were not substantial. The effects of temperature on the progeny yield of infective juveniles were significant. Higher yields of infective juveniles were obtained at the

lower temperature regimen (20° C) whereas lower yields were obtained at the higher temperature regimen (30° C). The effect of population density also significantly affected the progeny yield of infective juveniles. The findings obtained from the investigations suggested that the species infectivity, recovery and progeny yield production were more favourable at the lower temperature regimens. However, the abilities of the species to perform these biological processes higher temperatures to certain extents demonstrated flexibility and adaptation. The endemic EPN isolate demonstrated good potential as a biological control agent of insect pests.

CHAPTER 4: THE DOSE RESPONSE ASSAY OF *XENORHABDUS* SP. STRAIN GDc328

4.1   INTRODUCTION

The symbiosis shared between microorganisms and animals have been documented in diverse ecological niches. The concept of mutualistic symbiosis involves mutual exploitation where each symbiotic partner contributes and receives net positive benefits. These benefits may be regarded as diverse and may include mutual influences on processes such as nutrition, development, reproduction and defence. As stated in the literature review, the species *Steinernema* shares an obligate mutualistic symbiotic relationship with *Xenorhabdus* species of bacteria. The symbiotic and pathogenic roles of the bacteria were previously mentioned (see section 1.6 of literature review). According to Ogier et al., (2014), no free living forms of the bacteria have been reported.

The contributions of the bacteria and helminthic partners during host infection may differ amongst the different entomopathogenic couples. The study conducted by Ogier et al., (2014) showed that two *Xenorhabdus* species (*Xenorhabdus nematophila* and *Xenorhabdus boveinii*) exhibited lethal attributes to insect hosts from bacterial dose of 100 colony forming units (CFU). The study performed by Han and Ehlers (2000) showed that two aposymbiotic species, *S. carpocapsae* and *S. feltiae* infected last instar *G. mellonella* with high efficiencies. The aposymbiotic EPNs lacked their associated *Xenorhabdus* species of bacteria. However, the authors observed reductions in the reproductive fitness of the entomopathogenic nematodes (EPNs). Under laboratory conditions, the pathogenic nature of both symbiotic partners may be demonstrated individually against different insect pests. This implies that the associations of some species may be regarded as facultative under laboratory conditions. However, some *Xenorhabdus* species such as *Xenorhabdus poinarii* demonstrated avirulent characteristics against insect hosts without their symbiotic nematode partner. The study conducted by Akhurst and Boemare (1990) showed that the LD_{50} (lethal dose required to kill 50 % of the insect population) without their entomopathogenic nematode (EPN) partners were as high as 10^5-10^6 cells. Ogier et al., (2014) stated that for some bacterial-nematode complexes, the co-operation of

both symbiotic partners may be required for the process of host infection. In some temperature-related investigations, the authors inferred that improved performances of the EPNs at experimental temperatures were attributed to the improvements in the growth rates of the bacteria (Grewal et al., 1996; Atwa, 2015).

The study conducted by Grewal et al., (1996) showed that the symbiotic bacteria isolated from a temperature acclimated EPN strain acquired quicker generation times during the log phase of growth when compared to the ancestral strain of bacterial isolate. The aims of this chapter involved investigating the infectious abilities of the species *Xenorhabdus* sp. strain GDc328 against last instar *G. mellonella* at different doses through intra-haemocoelic administration. The second aim of this chapter comprised the evaluating the infectious ability of the symbiotic bacteria without the contributions from their symbiotic EPN partners. The infectious abilities of the bacteria were further extended into temperature related investigations. This research aim included the evaluation of bacterial infection during rapid temperature changes without prior temperature acclimation. The infectious abilities of the bacteria were tested at the two experimental temperatures (20º C and 30º C) that were reported in the previous chapter.

4.2   METHODOLOGY

4.2.1   Bacterial stock solution

The inoculum was prepared using freshly streaked 24 hour bacterial colonies cultured at 25º C on nutrient bromothymol blue triphenyltetrazolium chloride agar (NBTA). The BBL™ Prompt™ Inoculation system (Catalogue #226306) was used for the direct standardization of the bacterial stock solution according to the manufacturer's manual. The apparatus comprised an inoculation wand. The properties of the wand were based on the 0.5 McFarland turbidity standards. Approximately 1.5×10^8 CFU were selected by touching 15 freshly streaked bacterial colonies with diameters of 2 mm with an inoculation wand. The colonies were then transferred into a tube containing 1 ml of sterile 0.85 % (w/v) saline solution. The bacterial cells in the saline solution were thoroughly mixed for 1 minute using the vortex. The wand was thereafter discarded. According to the manufacturer's protocol, the stock solution contained a bacterial cell count of 4.5×10^8 CFU/ml.

4.2.2 Serial dilutions

10 fold serial dilutions were prepared in the ranges of 10^{-1} to 10^{-7}. The cells were enumerated by preparing spread plates of the diluted samples on nutrient agar. 0.1 ml of sample was used for the spread plate technique. The nutrient agar plates (see appendix) were incubated at 25° C for 48 hours before the cell enumeration process. The bacterial stock and the diluted samples were used for the intra-haemocoelic administration (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, and 10^{-7}) into last instar *G. mellonella* (n = 10).
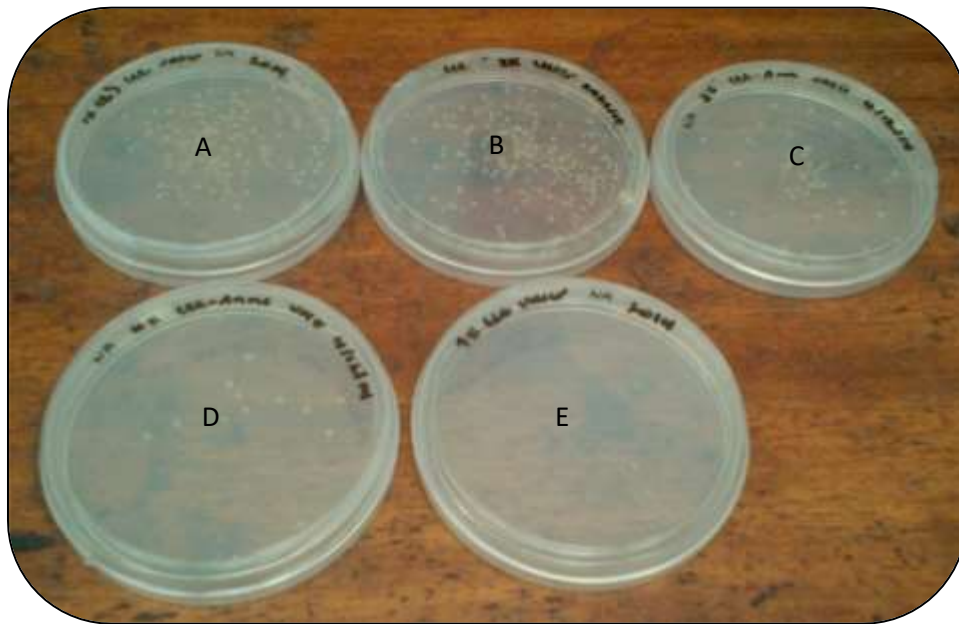
Figure 4.1: The spread plate technique on nutrient agar plates. The spread plate technique of each bacterial dilution (10^{-1} to 10^{-7}) was performed in triplicate. The spread plate technique was performed to approximate the bacterial dose which was expressed in CFU and administered to each larva at control and experimental temperature regimens. The alphabetical letters were represented by the following dilution factors: A = 10^{-1}, B = 10^{-2}, C = 10^{-3}, D = 10^{-4} and E = 10^{-7}. The bacterial colonies were incubated at 25° C for 48 hours. The bacterial colonies grew circular in morphology and white in colour. The image showed a reduction in the number of bacterial CFU with an increase in the dilution factor.

4.2.3 Cell enumeration

The bacterial colonies were counted and averaged. The average bacterial colony counts were obtained for each diluted sample. The averages were used to approximate the bacterial doses expressed in CFU for each diluted sample using the following formula:

$$\text{CFU/ml} = \frac{Cells\ (colony\ forming\ units) \times dilution\ factor}{0.1\ ml} \qquad (1)$$

The first two dilution sets (10^{-1} and 10^{-2}) were further diluted by an additional 100 fold to obtain countable bacterial colonies. The CFU/ml obtained for each sample was multiplied by the volume 0.05 ml. This was performed to obtain a close approximation of the bacterial CFU which were administered into each larva. The table below describes the approximate bacterial doses which were administered into the last instar *G. mellonella*.

Table 4.1: The approximate bacterial CFU represented by each sample which were administered into the larvae (n = 10) at 20º C, 25º C and 30º C.

The bacterial stock and Dilutions	Bacterial dose in Colony Forming Units (CFU)
Stock	2.30×10^8 CFU
10^{-1}	6.30×10^6 CFU
10^{-2}	1.70×10^6 CFU
10^{-3}	2.90×10^4 CFU
10^{-4}	1.38×10^3 CFU
10^{-7}	1.00×10^1 CFU

4.2.4 The intra-haemocoelic administration of *Xenorhabdus* sp. strain GDc328.

Last instar *G. mellonella* larvae (n = 10) were prepared for the investigation. The surfaces of the larvae were disinfected with 70 % ethanol (v/v) by moistening a dry piece of absorbent paper with ethanol and wiping the posterior right proleg of each larva. For each temperature regimen (20º C, 25º C and 30º C), n = 70 larvae were prepared (10 larvae were used for the stock, diluted samples and secondary controls). Sterile saline (0.85 % w/v) was used for the secondary control. Aliquots of 0.05 ml for the stock and diluted samples were drawn using an 18 G × 1.5 (1.20 ×

38 mm) hypodermic Terumo® Needle fitted onto a 1 ml Avacare syringe. The inoculum was injected into the right proleg of each larva. Aliquots of 0.05 ml of the sterile saline solution of 0.85 % (w/v) were also administered into 10 larvae which served as the secondary controls. The injected larvae were then placed into Petri dishes and incubated at the control (25º C) and experimental temperatures (20º C and 30º C). Larval mortality was measured in 24 hour intervals. The cumulative percentage of mortality graphs were drawn using Excel v. 2010 and the statistical analysis was also performed on this program. Two-way Analysis of Variance (ANOVA) tests were performed for the subsequent studies.

4.3 RESULTS

4.3.1 Bacterial infectivity

4.3.1.1 Insect mortality vs. bacterial dose (20º C)
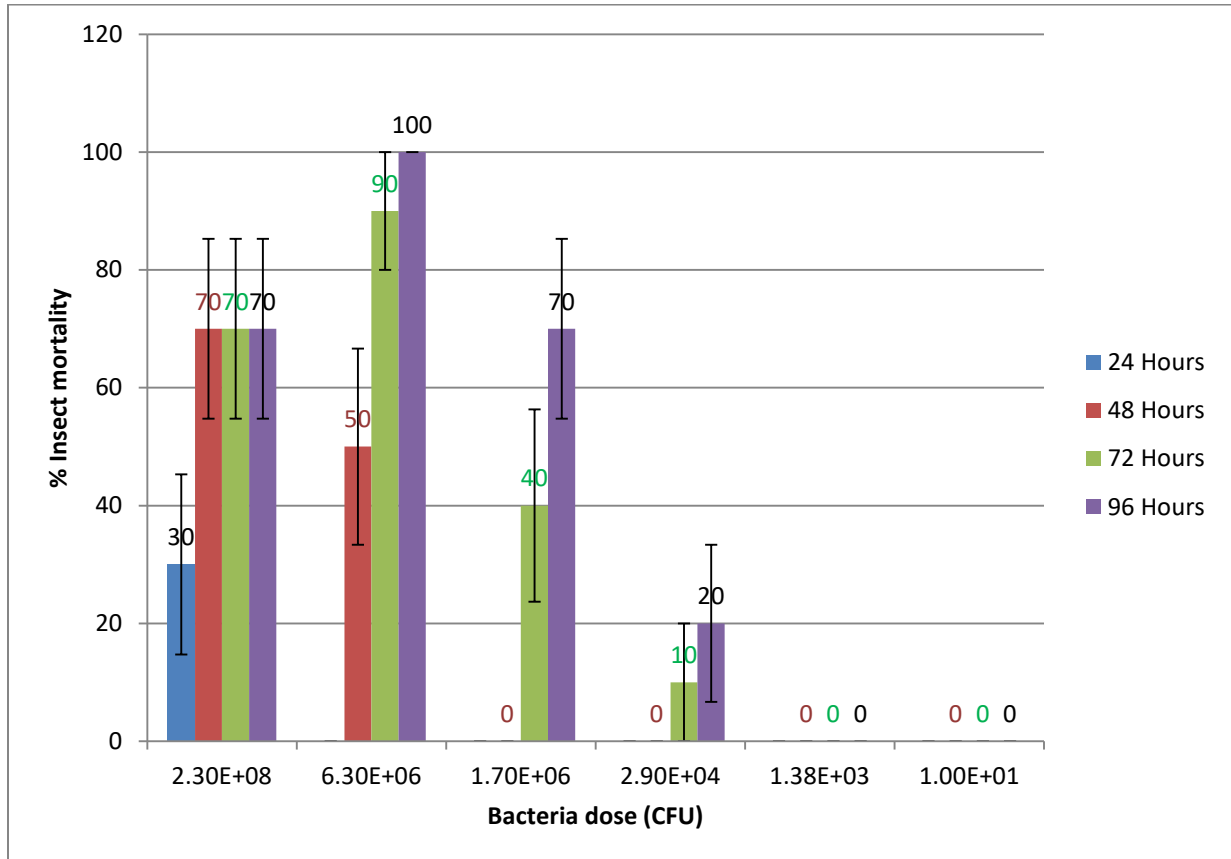
Figure 4.2 (a): The cumulative insect mortality measured at different bacterial doses and intervals of time at 20º C. The bacteria were administered into larvae (n = 10) at different bacterial doses through intra-haemocoelic injections. Insect mortality was thereafter assessed. The standard errors of the means (±) were represented by standard error bars. The standard error bars obtained for each bacterial dose and time interval were large. However, this excluded the error bars for the doses which obtained either 100 % or 0 % insect mortality. The secondary controls yielded 0 % larval mortality. Insect mortality was obtained to different extents from the highest dose (bacterial stock) until the third serial diluted dose. At 24 hours of infection, only the highest bacterial dose achieved a small extent of host mortality (30 %). At this time interval, no insect mortality was achieved at the lower doses of bacteria. By the 48 hour time interval, infection increased to 70 % for the bacterial stock dose and the first diluted dose obtained

mortality to 50 %. A large proportion of insect mortality was achieved by 72 hours of infection from the stock until the third diluted bacterial dose. However, at this time interval, the unexpected finding was the higher percentage of host mortality (90 %) obtained at the first diluted dose in comparison to the stock dose. By 96 hours, 100 % insect mortality was reached at the first diluted dose and an increase in the percentages of host mortality was observed at the subsequent doses. The lowest bacterial doses did not achieve host infection by 96 hours. The graphical representation above showed that longer times were required to achieve host mortality at different doses. Also, a positive relationship between host mortality and bacterial dose was observed.

4.3.1.2   The Two-way ANOVA summary

The Two-way ANOVA test with replication was performed to determine whether significant differences were present between the mean insect mortalities obtained at each bacterial dose and time interval. The study was performed at a temperature regimen of 20° C.

<u>Hypothesis</u>

a) **<u>Row factor (bacterial dose)</u>**

H_o: There are no differences between the mean insect mortalities obtained at each bacterial dose.

H_1: There is at least one mean mortality inequality present between the different bacterial doses.

$\alpha = 0.05$

b) **<u>Column factor (time)</u>**

H_o: There are no differences between the mean insect mortalities obtained at each time interval.

H_1: There is at least one mean mortality inequality present between the different time intervals.

$\alpha = 0.05$

c) **Interaction (bacterial dose and time)**

H$_o$: There is no interaction between bacterial dose and time.

H$_1$: There is interaction between bacterial dose and time.

α = 0.05

Table 4.2 (a): The summary statistics of the Two-way ANOVA test with replication. The mean insect mortalities at each bacterial dose and time interval were compared. The row factor represented bacterial dose and the column factor represented time. The test included an interaction assessment between the two factors. The study was performed at the temperature regimen of 20° C.

Source of variation	Sum of squares	Degrees of freedom	Mean square	F	P-value	F-critical
Rows	160333,33	5,00	32066,67	36,84	0,00	2,26
Columns	51500,00	3,00	17166,67	19,72	0,00	2,65
Interaction	60000,00	15,00	4000,00	4,60	0,00	1,71
Within	188000,00	216,00	870,37			
Total	459833,33	239,00				

Decision

a) **Row factor**: The P-value of 0.00 was lower than the significance level of 0.05. The F-value of 36.84 was higher than the F-critical value of 2.26. The data was categorized within the rejection region. The H$_o$ was rejected and the H$_1$ was accepted. This implied that at least one insect mortality mean was significantly different to the other means obtained at the respective bacterial doses.

b) **Column factor**: The P-value of 0.00 was lower than the significance level of 0.05. The F-value of 19.72 was higher than the F-critical value of 2.65. The data was categorized within the rejection region. The H$_o$ was rejected and the H$_1$ was accepted. This implied that at least one insect mortality mean was significantly different to the other means obtained at the respective time intervals.

c) **Interaction**: The P-value of 0.00 was lower than the significance level of 0.05. The F-value of 4.60 was higher than the F-critical value of 1.71. The data was categorized within the rejection region. The null hypothesis was rejected and the alternate hypothesis was accepted. The statistical values obtained from this test implied that the interaction present between the two independent factors were significant.

4.3.1.3   Insect mortality vs. bacterial dose at 25º C
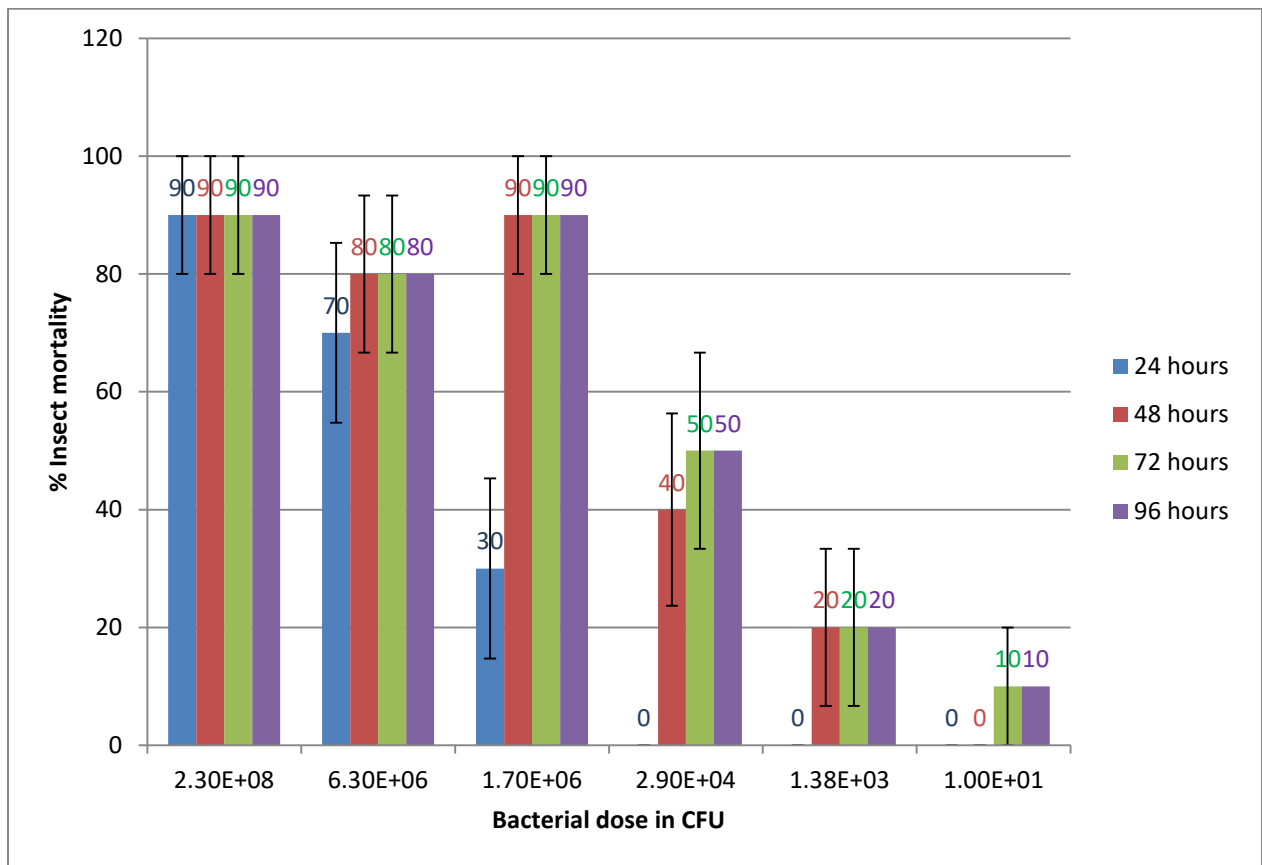
Figure 4.2 (b): The cumulative insect mortality measured at different bacterial doses and intervals of time at 25° C. The standard errors of the means (±) were represented by error bars. The sizes of the error bars were large. The secondary controls yielded 0 % insect mortality. Insect mortality was achieved at all bacterial doses. However, the extents of insect mortality differed at each bacterial dose. The percentages of insect mortality achieved at this temperature regimen (25° C) were higher than that achieved for the previous temperature regimen of 20° C. At the 24 hour time interval, insect mortality was achieved at the bacterial stock dose and the first two diluted doses. The stock bacterial dose achieved mortality as high as 90 %. A positive

relationship was observed between bacterial dose and insect mortality for the first two diluted bacterial doses. However, at 24 hours, no insect mortality was obtained at the lower bacterial doses. By the 48 hour time interval, the percentage of insect mortality increased at the first and second diluted doses. The bacterial doses of the third and fourth dilutions started to achieve host mortality at this time interval. However, an unexpected finding was observed. The percentage of mortality at the second diluted dose was higher (90 %) than that achieved for the first diluted dose (80 %) at the 48 hour time interval. At 72 hours of infection, increments in the percentages of host mortality were observed at the third and fourth diluted bacterial doses. At this time interval, the least diluted bacterial dose initiated host mortality. By 96 hours, host mortality accumulated to high percentages and there were no further increments observed between the different bacterial doses.

4.3.1.4   Two-way ANOVA test summary

The Two-way ANOVA test with replication was performed to determine whether significant differences were present between the insect mortality means obtained at each bacterial dose and time interval. The study was performed at a temperature regimen of 25° C.

Hypothesis

a) **Row factor (bacterial dose)**

   H_o: There are no differences between the mean insect mortalities obtained at each bacterial dose.

   H_1: There is at least one mean mortality inequality present between the bacterial doses.

   $\alpha = 0.05$.

b) **Column factor (time)**

   H_o: There are no differences in the mean insect mortalities at each time interval.

   H_1: There is at least one mean mortality inequality present between the time intervals.

   $\alpha = 0.05$.

c) **Interaction (bacterial dose and time)**

H$_o$: There is no interaction between bacterial dose and time.

H$_1$: There is interaction between bacterial dose and time.

α = 0.05.

Table 4.2 (b): The summary statistics of the Two-way ANOVA test with replication. The mean insect mortalities at each bacterial dose and time interval were compared. The row factor represented bacterial dose and the column factor represented time. The test included an interaction assessment between the two factors. The study was performed at the temperature regimen of 25° C.

Source of variation	Sum of squares	Degrees of freedom	Mean square	F	P-value	F-critical
Rows	258208,33	5,00	51641,67	38,07	0,00	2,26
Columns	26125,00	3,00	8708,33	6,42	0,00	2,65
Interaction	22625,00	15,00	1508,33	1,11	0,35	1,71
Within	293000,00	216,00	1356,48			
Total	599958,33	239,00				

<u>**Decision**</u>

a) **Row factor**: The P-value of 0.00 was lower than the significance level of 0.05. The F-value of 38.07 was higher than the F-critical value of 2.26. The data was categorized within the rejection region. The null hypothesis was rejected and the alternate hypothesis was accepted. This implied that significant differences were present between the mean mortalities obtained at each bacterial dose.

b) **Column factor**: The P-value of 0.00 was lower than the significance level of 0.05. The F-value of 6.42 was higher than the F-critical value of 2.65. The data was categorized within the rejection region. The null hypothesis was rejected and the alternate hypothesis was accepted. This implied that significant differences were present between the mean mortalities at each time interval.

c) **Interaction**: The P-value of 0.35 was higher than the significance level of 0.05. The F-value of 1.11 was lower than the F-critical value of 1.71. The data was categorized within the acceptance region. The null hypothesis was accepted and the alternate hypothesis was rejected. This implied that the interaction between the two independent factors were not sufficient to be regarded as statistically significant.

4.3.1.5   Insect mortality vs. bacterial dose (30º C)
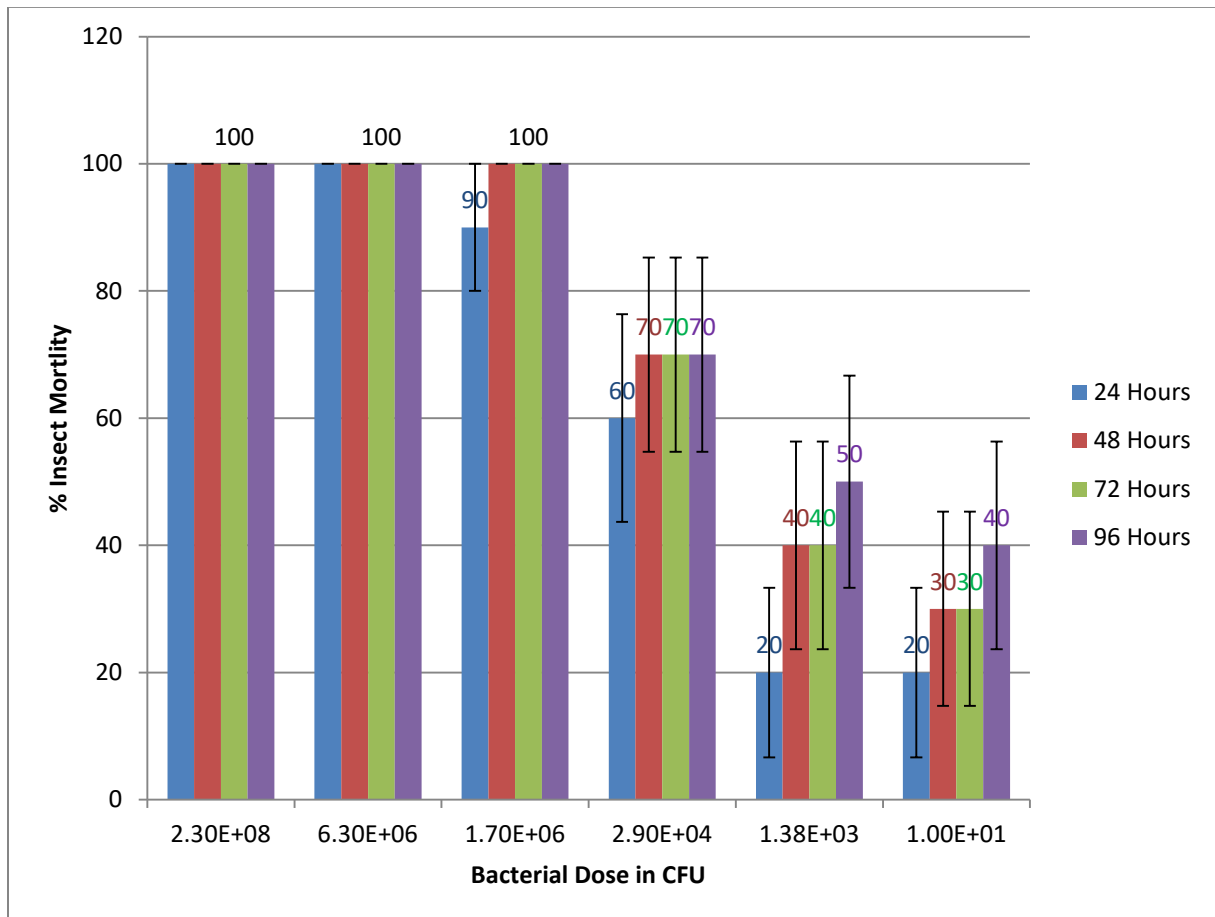
Figure 4.2 (c): The cumulative insect mortality measured at different bacterial doses over different intervals of time at 30º C. The standard errors of the means (±) were represented by standard error bars. The standard error bars obtained for each bacterial dose and time interval were large excluding the doses which obtained either 100 % or 0 % insect mortality. The secondary controls yielded 0 % larval mortality. Insect mortality was achieved at all bacterial doses. However, the extents of host mortality differed between each bacterial dose. The highest

percentages of insect mortality was achieved at the temperature regimen of 30° C. Rapid host infections were observed by the stock and first diluted bacterial doses. These doses were higher in the number of bacterial colony forming units. Also, at the 24 hour time interval, a high percentage of mortality (90 %) was observed by the second diluted bacterial dose. At the 24 hour time interval, host mortality was achieved to different extents at the lower bacterial doses. This finding was not observed from the first experimental temperature regimen of 20° C at 24 hours of infection. A positive relationship was observed between bacterial dose and host mortality at this time interval. At 48 hours of infection, increments were observed from the second to the seventh diluted bacterial dose. There were no increments in host mortality at the higher bacterial doses by the 48 hour time interval since 100 % insect mortality was achieved previously. There were no increments observed in the percentage of host mortality at 72 hour of infection by all bacterial doses. However, by the 96 hour time interval, increments in the percentages of host mortality were observed at the two lowest bacterial doses. However, there were no further mortality increments at bacterial doses which did not achieve 100 % host mortality.

4.3.1.6 Two-way ANOVA summary

The Two-way ANOVA test with replication was performed to determine whether significant differences were present between the insect mortality means obtained at each bacterial dose and time interval. The study was performed at a temperature regimen of 30° C.

Hypothesis

a) **Row factor (bacterial dose)**

H_o: There are no differences between the mean insect mortalities obtained at each bacterial dose.

H_1: There is at least one mean mortality inequality present between the different bacterial doses.

$\alpha = 0.05$.

b) **Column factor (time)**

H_o: There are no differences between the mean insect mortalities obtained at each time interval.

H_1: There is at least one mean mortality inequality present between the different time intervals.

$\alpha = 0.05$.

c) **Interaction (bacterial dose and time)**

   H_o: There is no interaction between bacterial dose and time.

   H_1: There is interaction between bacterial dose and time.

   $\alpha = 0.05$.

Table 4.2 (c): The summary statistics of the Two-way ANOVA test with replication. The mean insect mortalities at different bacterial doses and time intervals were compared. The row factor represented bacterial dose and the column factor represented time. The test included an interaction assessment between the two indepeendent factors. The study was performed at a temperature regimen of 30° C.

Source of variation	Sum of squares	Degrees of freedom	Mean square	F	P-value	F-critical
Rows	207708,33	5,00	41541,67	33,61	0,00	2,26
Columns	4458,33	3,00	1486,11	1,20	0,31	2,65
Interaction	3791,67	15,00	252,78	0,20	1,00	1,71
Within	267000,00	216,00	1236,11			
Total	482958,33	239,00				

Decision

a) **Row factor**: The P-value of 0.00 was lower than the significance level of 0.05. The F-value of 33.61 was higher than the F-critical value of 2.26. The data was categorized within the rejection region. The null hypothesis was rejected and the alternate hypothesis was accepted. This implied that significant differences were present between the mean insect mortalities obtained at different bacterial doses.

b) **Column factor**: The P-value of 0.31 was higher than the significance level of 0.05. The F-value of 1.20 was lower than the F-critical value of 2.65. The data was categorized within the acceptance region. The null hypothesis was accepted and the alternate hypothesis was rejected. This implied that there were no significant differences present between the mean insect mortalities at each time interval.

c) **Interaction**: The P-value 1.00 was higher than the significance level of 0.05. The F-value of 0.20 was lower than the F-critical value of 1.71. The data was categorized within the acceptance region. The null hypothesis was accepted and the alternate hypothesis was rejected. This implied that the interaction between the two independent factors were not sufficient to be regarded as statistically significant.

4.3.2   Infectivity (Bacterial dose and temperature)

4.3.2.1   Two-way ANOVA test summary

The Two-way ANOVA test with replication was performed to determine whether significant differences were present between the insect mortality means at different bacterial doses and temperature regimens. The study was performed at a time interval of 24 hours.

Hypothesis

a) **Row factor (bacterial dose)**

H_o: There are no differences between the insect mortality means obtained at each bacterial dose.

H_1: There is at least one mean mortality inequality present between the different bacterial doses.

$\alpha = 0.05$.

b) **Column factor (temperature)**

H_o: There are no differences between the insect mortality means obtained at each temperature regimen.

H_1: There is at least one mean mortality inequality present between the different temperature regimens.

α = 0.05

c) **Interaction (bacterial dose and temperature)**

H_o: There is no interaction between bacterial dose and temperature.

H_1: There is interaction between bacterial dose and temperature.

α = 0.05.

Table 4.3 (a): The summary statistics of the Two-way ANOVA test with replication. The mean insect mortalities at each bacterial dose and temperature regimen were compared. The row factor represented bacterial dose and the column factor represented temperature. The test included an interaction assessment between the two factors. The study was performed at a time interval of 24 hours.

Source of variation	Sum of squares	Degrees of freedom	Mean square	F	P-value	F-critical
Rows	113611,11	5,00	22722,22	26,87	0,00	2,27
Columns	108444,44	2,00	54222,22	64,12	0,00	3,05
Interaction	44222,22	10,00	4422,22	5,23	0,00	1,89
Within	137000,00	162,00	845,68			
Total	403277,78	179,00				

Decision

a) **Row factor:** The P-value of 0.00 was lower than the significance level of 0.05. The F-value of 26.87 was higher than the F-critical value of 3.05. The data was categorized in the rejection region. The null hypothesis was rejected and the alternate hypothesis was accepted. This implied that there were significant differences in the mean insect mortalities obtained at the different bacterial doses.

b) **Column factor**: The P-value of 0.00 was lower than the significance level of 0.05. The F-value of 64.12 was higher than the F-critical value of 3.05. The data was categorized within the rejection region. The null hypothesis was rejected and the alternate hypothesis

was accepted. This implied that there were significant differences present in the mean mortalities obtained at different temperature regimens.

c) **Interaction**: The P-value of 0.00 was lower than the significance level of 0.05. The F-value of 5.23 was higher than the F-critical value of 1.89. The data was categorized within the rejection region. The null hypothesis was rejected and the alternate hypothesis was accepted. This implied that interaction was present between the two independent factors. The interaction was statistically significant.

4.3.2.2   Two-way ANOVA test summary

The Two-way ANOVA test with replication was performed to determine whether significant differences were present in the mean insect mortalities at different bacterial doses and temperature regimens. The study was performed at a time interval of 48 hours.

Hypothesis

a) **Row factor (bacterial dose)**

H_o: There are no differences between the mean insect mortalities obtained at each bacterial dose.

H_1: There is at least one mean mortality inequality present between the different bacterial doses.

$\alpha = 0.05$.

b) **Column factor (temperature)**

H_o: There are no differences between the mean insect mortalities obtained at each temperature regimen.

H_1: There is at least one mean mortality inequality present between the temperature regimens.

$\alpha = 0.05$

c) **Interaction (bacterial dose and temperature)**

H$_o$: There is no interaction between bacterial dose and temperature.

H$_1$: There is interaction between bacterial dose and temperature.

α = 0.05

Table 4.3 (b): The summary statistics of the Two-way ANOVA test with replication. The mean insect mortalities at each bacterial dose and temperature regimen were compared. The row factor represented bacterial dose and the column factor represented temperature. The test included an interaction assessment between the two factors. The study was performed at a time interval of 48 hours.

Source of variation	Sum of squares	Degrees of freedom	Mean square	F	P-value	F-critical
Row	147111,11	5,00	29422,22	25,63	0,00	2,27
Column	87111,11	2,00	43555,56	37,94	0,00	3,05
Interaction	29555,56	10,00	2955,56	2,57	0,01	1,89
Within	186000,00	162,00	1148,15			
Total	449777,78	179,00				

Decision

a) **Row factor**: The P-value of 0.00 was lower than the significance level of 0.05. The F-value of 25.63 was higher than the F-critical value of 2.27. The data was categorized in the rejection region. The null hypothesis was rejected and the alternate hypothesis was accepted. This implied that there were significant differences in the mean insect mortalities obtained at different bacterial doses.

b) **Column factor:** The P-value of 0.00 was lower than the significance level of 0.05. The F-value of 37.94 was higher than the F-critical value of 3.05. The data was categorized within the rejection region. The null hypothesis was rejected and the alternate hypothesis was accepted. This implied that significant differences were present between the mean mortalities obtained at each temperature regimen.

c) **Interaction**: The P-value of 0.01 was lower than the significance level of 0.05. The F-value of 2.57 was higher than the F-critical value of 1.89. The data was categorized within the rejection region. The null hypothesis was rejected and the alternate hypothesis was accepted. This implied that interaction was present between the two independent factors. The interaction was statistically significant.

4.3.2.3 Two-way ANOVA test summary

The Two-way ANOVA test with replication was performed to determine whether significant differences were present between the mean insect mortalities at different bacterial doses and temperature regimens. The study was performed at a time interval of 72 hours.

Hypothesis

a) **Row factor (bacterial dose)**

H_o: There are no differences between the mean insect mortalities obtained at each bacterial dose.

H_1: There is at least one mean mortality inequality present between the different bacterial doses.

$\alpha = 0.05$.

b) **Column factor (temperature)**

H_o: There are no differences between the mean insect mortalities obtained at each temperature regimen.

H_1: There is at least one mean mortality inequality present between the temperature regimens.

$\alpha = 0.05$.

c) **Interaction (bacterial dose and temperature)**

H_o: There is no interaction between bacterial dose and temperature.

H_1: There is interaction between bacterial dose and temperature.

$\alpha = 0.05$.

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Table 4.3 (c): The summary statistics of the Two-way ANOVA test with replication. The mean insect mortalities at different bacterial doses and temperature regimens were compared. The row factor represented bacterial dose and the column factor represented temperature. The test included an interaction assessment between the two indpeendent factors. The study was performed at a time interval of 72 hours.

Source of variation	Sum of squares	Degrees of freedom	Mean square	F	P-value	F-critical
Rows	173833,33	5,00	34766,67	26,44	0,00	2,27
Columns	44333,33	2,00	22166,67	16,86	0,00	3,05
Interaction	14333,33	10,00	1433,33	1,09	0,37	1,89
Within	213000,00	162,00	1314,81			
Total	445500,00	179,00				

<u>Decision</u>

a) **Row factor**: The P-value of 0.00 was lower than the significance level of 0.05. The F-value of 26.44 was higher than the F-critical value of 2.27. The data was categorized in the rejection region. The null hypothesis was rejected and the alternate hypothesis was accepted. This implied that there were significant differences in the mean insect mortalities obtained at different bacterial doses.

b) **Column factor**: The P-value of 0.00 was lower than the significance level of 0.05. The F-value of 16.86 was higher than the F-critical value of 3.05. The data was categorized within the rejection region. The null hypothesis was rejected and the alternate hypothesis was accepted. This implied that significant differences were present between the mean mortalities obtained at each temperature regimen.

c) **Interaction**: The P-value of 0.37 was higher than the significance level of 0.05. The F-value of 1.09 was lower than the F-critical value of 1.89. The data was categorized within the acceptance region. The null hypothesis was accepted and the alternate hypothesis was

rejected. This implied that the interaction between the two independent factors was not regarded as statistically significant.

4.3.2.4   Two-way ANOVA summary test

The Two-way ANOVA test with replication was performed to determine whether significant differences were obtained between the mean insect mortalities at different at bacterial doses and temperature regimens. The study was performed at a time interval of 96 hours.

Hypothesis

a) **Row factor (bacterial dose)**

H_o: There are no differences between the mean insect mortalities obtained at each bacterial dose.

H_1: There is at least one mean mortality inequality present between the bacterial doses.

$\alpha = 0.05$.

b) **Column factor (temperature)**

H_o: There are no differences between the mean insect mortalities obtained at each temperature regimen.

H_1: There is at least one mean mortality inequality present between the temperature regimens.

$\alpha = 0.05$.

c) **Interaction (bacterial dose and temperature)**

H_o: There is no interaction between bacterial dose and temperature.

H_1: There is interaction present between bacterial dose and temperature.

$\alpha = 0.05$.

Table 4.3 (d): The summary statistics of the Two-way ANOVA test with replication. The mean insect mortalities at different bacterial doses and temperature regimens were compared. The row factor represented bacterial dose and the column factor represented temperature. The

test included an interaction assessment between the two indpeendent factors. The study was performed at a time interval of 96 hours.

Source of variation	Sum of squares	Degrees of freedom	Mean square	F	P-value	F-critical
Rows	177777,78	5,00	35555,56	27,17	0,00	2,27
Columns	33777,78	2,00	16888,89	12,91	0,00	3,05
Interaction	12222,22	10,00	1222,22	0,93	0,50	1,89
Within	212000,00	162,00	1308,64			
Total	435777,78	179,00				

Decision

a) **Row factor**: The P-value of 0.00 was lower than the significance level of 0.05. The F-value of 27.17 was higher than the F-critical value of 2.27. The data was categorized within the rejection region. The null hypothesis was rejected and the alternate hypothesis was accepted. This implied that significant differences were present between the insect mortality means obtained at each bacterial dose.

b) **Column factor:** The P-value of 0.00 was lower than the significance level of 0.05. The F-value of 12.91 was higher than the F-critical value of 3.05. The data was categorized within the rejection region. The null hypothesis was rejected and the alternate hypothesis was accepted. This implied that significant differences were present between the mean mortalities obtained at each temperature regimen.

c) **Interaction**: The P-value of 0.50 was higher than the significance level of 0.05. The F-value of 0.93 was lower than the F-critical value of 1.89. The data was categorized in the acceptance region. The null hypothesis was accepted and the alternate hypothesis was rejected. This implied that the interaction between the two independent factors was not regarded as statistically significant.

4.4 DISCUSSION

4.4.1 Bacterial infectivity

4.4.1.1 Bacterial dose

It was observed that host infection was achieved without the contributions from the EPN partners. According to the study conducted by Mahar et al., (2005), the sole administration of bacterial cells was sufficient to kill insect hosts. The authors further stated that researchers were taking much interest in the area of bacterial cell administration against insect pests. This investigation measured host infectivity in relation to different bacterial doses. A directly proportional relationship was obtained between bacterial dose and host mortality. The statistical findings confirmed that strong significant differences were present between the mean mortalities at each bacterial dose. These findings were coherent with the study conducted by Sicard et al., (2004). The study conducted by Sicard et al., (2004) investigated the virulence of the fluorescently tagged species *X. nematophila* by intra-haemocoelic injection at different bacterial doses. The authors observed a directly proportional relationship between bacterial dose and host mortality. A positive relationship was observed between high bacterial doses and bacterial growth within the hemocoel of the insect. The rapid growth of the bacteria contributed to lethal septicaemia of the insect host (Thaler et al., 1998) and ultimately death (Ramarao, Nielsen-Leroux, and Lereclus, 2012). Furthermore, at high bacterial doses, the authors observed reductions in the nodulation process which was a cellular response exhibited the insect host. In this investigation, the lower bacterial doses did not exhibit high extents of host infection.

The reduction in host mortality at the lower bacterial doses may be explained by partial clearance of the bacterial population. According to the study conducted by Sicard et al., (2004), this phenomenon was observed when the bacterial cells were present in low doses. However, this was not observed for the high bacterial doses administered into the insect larvae. Also, bacterial cells may re-emerge back into the haemolymph of the insect host. The study conducted by Sicard et al., (2004) showed that re-emergence of bacterial cells may occur by proliferation within the nodule structures which may have contributed to the small extents of infection at the lower bacterial doses. This implied that longer periods of time may have been required to achieve higher percentages of host infection at the lower bacterial doses. At the lower bacterial doses, host infection may be regarded as a time-dependent process. The absence of host mortality from

the two lowest bacterial doses at 20° C may have suggested the presence of interaction between bacterial dose and temperature.

The graphical representations of two investigations (20° C and 25° C) showed that higher extents of host mortality were obtained at the less concentrated bacterial dose. This finding deviated from the directly proportional relationship observed between bacterial dose and host mortality. This deviation was attributed to the process of intra-haemocoelic administration. Minute volumes of bacterial inocula may have been ejected from the exposed wound on the base proleg of the insect host. This may have contributed to the lower extents of host mortality obtained at the higher bacterial dose since fewer cells were administered into the haemolymph of the host. Host mortality was investigated with two other factors in addition to bacterial dose. These factors were time and temperature.

4.4.1.2   Time

A large proportion of the bacteria administered in different bacterial doses were able to overcome host barriers within 96 hours. The study conducted by Nielsen-Leroux et al., (2012) showed that overcoming different barriers of the host immune system were required to achieve host infection. The authors stated that subsequent to intra-haemocoelic administration, the host released antimicrobial peptides which targeted the envelope structure of the bacteria. The bacterial species *X. nematophila* counteracted the humoral response of the host by inhibiting initial peptide synthesis. The authors noticed that administering the non-pathogenic species *Salmonella enterica* into insect larvae triggered the production of the cecropin antimicrobial peptide. However, the transcript for the cecropin antimicrobial peptide was not induced after the intra-haemocoelic injection of *X. nematophila* into the insect host. Also, *Xenorhabdus* species produces an array of cytolysins, hemolysins and toxins which function in the induction of host cell necrosis. One other barrier includes the competition between the host and the bacteria for ion sources. The sequestration of this component was essential for bacterial growth.

The effect of time on host mortality at each bacterial dose was measured at the control and experimental temperature regimens. At the temperature regimen of 20° C, a directly proportional relationship was observed between time and host mortality. However, this excluded the time intervals in which mortality remained constant. This finding was coherent the study conducted

by Mahar et al., (2005). The authors observed a significant increase in host mortality with time. In this investigation, the rapid infections observed at the 24 hour time interval may not have been solely attributed to the virulence of the species since interaction between bacterial dose and time were present at the temperature regimen of 20° C. This was supported by the high percentages of infection observed only at the higher bacterial doses. The large number of bacterial colony forming units may have overcome the time factor which may have contributed to the extents of infection observed at the 24 hour time interval. This was further substantiated by the strong interaction present (P = 1.35E – 07) between time and bacterial dose at the temperature regimen of 20° C.

Higher host mortalities were observed at the time intervals measured at temperature regimens of 25° C and 30° C. These increments were not related to the factor of time but rather to the interaction between bacterial dose and temperature. At the temperature regimen of 25° C, the time taken to achieve host mortality increased as the bacterial doses decreased. This suggested a level of interaction between bacterial dose and time. However, the interaction was not regarded as statistically significant. This may be explained by the different patterns of host infection obtained at the 48, 72 and 96 hour time interval. At these intervals, the second diluted bacterial dose achieved a higher percentage of mortality than the first diluted dose which may have contributed to the statistical insignificant interaction between bacterial dose and time. Also, at each bacterial dose, the increments in host mortality over time were not substantial. This may have further contributed to the statistical insignificant interaction between bacterial dose and time.

Lastly, at the temperature regimen of 30° C, there were no significant differences in the times taken to achieve host mortality. This may have been attributed to the optimum host mortality obtained within the first 24 hours of infection at higher bacterial doses. Since mortality was accumulated, the percentages were carried to the subsequent time intervals. Also, slight increments in mortality were observed over time. The percentage in host mortality increased at lower bacterial doses with time. This suggested a level of interaction between bacterial dose and time. However, since the increments were not substantial, the interaction was not sufficient to be

regarded as statistically significant. The last investigation measured host mortality in relation to one other independent factor which was temperature.

4.4.1.3 Temperature

The bacterial cells may be considered as effective biological control agents of noxious insects present in cryptic habitats (Mahar et al., 2005). Temperature is an important factor when considering the biological control potential of the bacterial species. The effect of temperature on host mortality at each bacterial dose was evaluated at four time intervals. At each time interval, the highest extents of host mortality was obtained at the temperature regimen of 30° C and the lowest at 20° C. This finding was unexpected. The differences in the host mortalities obtained at each temperature regimen were strongly statistically significant at all intervals of time. These findings were not coherent with the findings of the previous chapter. The EPN-bacterial complex achieved higher mortalities at the temperature regimen of 20° C and smaller extents at 30° C. The study conducted by Grewal et al., (1996) stated that the bacteria and nematode co-evolved together as a bi-partite complex. However, in this investigation, the bacteria were removed from the bi-partite complex with the nematode and propagated solely *in vitro* prior to intra-haemocoelic administration. It was a possibility that external factors may have re-shaped the bacteria to function as an independent infectious microorganism. This re-shaping may have contributed to differences observed in performances at the two experimental temperature regimens between the bacteria and the bacterial-EPN complex. The study conducted by Hinchcliffe et al., (2010) elucidated one of the toxin complexes produced by the symbiotic bacteria of the EPNs. This was the toxin complex (Tc). The authors also observed that the subunits of the gene were preferentially expressed according to temperature. This may have implied that the gene was preferentially expressed to greater extents at 30° C which contributed to the higher extents of host mortality. The decrements in performance observed at 20° C may have implied that the sub-units of the gene were not expressed. However, host mortality was still observed because infection was a collaborative effect from different complexes other than the Tc complex. At the temperature regimen of 25° C, the host mortality achieved was high. This was expected since the bacteria were initially propagated at this regimen.

As the bacterial dose decreased, higher temperature regimens were required to achieve host mortality. This suggested that interaction was present between bacterial dose and temperature.

However, there were differences regarding the statistical significance of the interaction between temperature and bacterial dose at certain time intervals. This was expected as the significant interactions would have been elucidated to higher extents at the 24-48 hour time intervals since the large proportion of insect hosts were killed to different extents between those time intervals. By the 72-96 hour time interval, host mortality was already accumulated from the previous intervals. This meant that the percentages of host mortality achieved at each bacterial dose were already high. The increments in host mortality at each temperature regimen were not substantial. This may have contributed to the non-significant statistical interaction at the higher time intervals of 72-96 hours.

4.5 CONCLUSION

The study conducted by Mahar et al., (2005) demonstrated the potential of utilizing bacterial cell suspensions for plant protection. In the present investigations, the bacteria killed effectively at higher dosages. Host infections were observed as early as 24 hours after administration. A directly proportional relationship was established between host mortality and the three factors of bacterial dose, time and temperature. The investigations showed that strong significant statistical differences were present in the host mortalities obtained between different bacterial doses, time intervals and temperature regimens. Also, interaction was present between bacterial dose and each independent factor of time and temperature. It is important to utilize present knowledge and tools to develop the symbiotic bacteria as an effective bio-pesticide. The bacteria demonstrated virulence at the two experimental temperatures (20° C and 30° C) without prior acclimatization. The South African bacterial isolate may be regarded as a good bio-pesticide candidate. Furthermore, the temperature-regulated genes responsible for host infectivity may be desirable for future prospects in plant genetic transformation.

CHAPTER 5: THE DRAFT WHOLE-GENOME SEQUENCE OF *XENORHABDUS* GDc328

1.1   INTRODUCTION

Xenorhabdus bacteria may be considered as interesting models for studying the evolution of molecular mechanisms which facilitate the symbiosis between an insect pathogenic bacterium and animal vector. *Xenorhabdus* bacteria are suitable pathogenic models because of their obligate mutualistic association with entomopathogenic nematode (EPN) vectors and also because they are pathogens of a wide range of insect hosts (Ogier et al., 2014). The combination of both attributes represented by one organism may provide insight into the beneficial and detrimental interactions which exist between the microbial species and their respective invertebrate hosts. The developments in the field of molecular biology established new possibilities to investigate the evolution of insect pathogenic bacteria, the symbiotic partnership shared with the entomopathogenic nematodes (EPNs) and their interactions with insect hosts (Vivas and Goodrich-Blair, 2001). These developments included the rapid high throughput sequencing technique referred to as whole genome sequencing or alternatively known as Next Generation Sequencing (NGS).

Next Generation Sequencing is regarded as a technique in which the genome sequences of prokaryotic species may be generated rapidly in-house using the different bench top sequencing machines. These sequence instruments includes the Roche 454 FLX Junior, Ion Torrent PGM and the Illumina MySeq (Edwards and Holt, 2013). The sequence data is made available on public domains which permit extensive comparative genome studies. In the year 2013, more than 6500 prokaryotic genomes were deposited into the GenBank database. Stock (2015) stated that more researchers have applied the whole genome sequencing technique to analyse the symbiotic bacteria of the different EPN species.

Bisch et al., (2016) explored two strains of one individual species through comparative genomics and observed differences in each individual strain regarding host exploitation. The authors stated the divergence in host exploitation was correlated to the sizes of each individual genome. One

other study by Ogier et al., (2014) involved the comparative genome analysis between virulent and avirulent species of *Xenorhabdus* bacteria. For the past years, extensive studies were performed on the species *X. nematophila* followed by *Xenorhabdus boveinii*. The genome of *X. nematophila* consisted of a megaplasmid of 155 Kb with a total genome size of 4.3 Mb. The genome of *X. boveinii* comprises a total genome size of 4.63 Mb and a mega plasmid of 177 Kb and a plasmid of 8 Kb. The comparative genome study conducted by Bisch et al., (2016) showed that a large proportion of genes from two *X. boveinii* strains encoded for insecticidal proteins and antimicrobial compounds. Furthermore, Stock (2015) stated that the majority of genes functioned in host colonization, evasion and nematode symbiosis.

The identification of important genes may expand the capacity of the EPN-bacterial partnership as biological control agents of insect pests by genetic improvements. Two important areas which may require enhancement includes the areas of virulence towards insect pests and the resistance to harsh environmental conditions such as lethal temperatures (Gaugler et al., 1997). The study conducted by Gaugler et al., (1997) identified a lethal toxin which killed the insect hosts within 48 hours. The authors stated that attempts have been made to clone the gene which encoded for the toxin. The authors aimed at producing a gene which encoded for the toxin with increased toxicity. Furthermore, the study showed that the insertion of a heat shock resistant protein into EPNs increased their tolerance to lethal temperatures by 18 fold. Selvan et al., (1996) stated that the efficiencies of the nematodes may be enhanced at warm and cold temperatures by enhancing their tolerances to environmental extremes.

The aim of this chapter entailed the acquisition of the draft whole genome sequence of the bacterial species *Xenorhabdus* sp. strain GDc328 by next generation sequencing methods. The second aim encompassed screening the genome to establish the genes which functioned in bacterial temperature tolerance, bacterial virulence and symbiosis.

1.2   METHODS AND MATERIALS

1.2.1   Genomic bacterial DNA extraction

The genomic deoxyribonucleic acid (DNA) extraction was performed in a laminar flow bench. The genomic DNA was isolated from freshly streaked 24 hour bacterial colonies cultured on nutrient bromothymol blue triphenyltetrazolium chloride agar (NBTA) at 25° C. An entire pure bacterial culture plate was used for the study. The bacterial colonies were transferred with a flamed inoculating loop into a sterile 1.5 ml Eppendorf tube containing 500 µl of sterile distilled water. The genomic bacterial DNA was then isolated according to the methods described in the protocol obtained from the ZR Genomic DNA™- tissue miniprep kit (Zymo Research, catalogue #3050) (see appendix). The genomic DNA was thereafter purified according to the manufacturer's manual obtained from the ZR Bacterial DNA Clean and Concentrator-5 Kit (catalogue #D4013). The purified genomic DNA of the bacteria was quantified using the NanoDrop-1000 spectrophotometer (Thermo-scientific). The genomic DNA concentration requirement stipulated by the Agricultural Research Council (ARC) Biotechnology Platform South Africa was to be in the range of at least 50 ng/µl of DNA. The purity of the extracted DNA was also assessed by using the 260/280 ratio. A second ratio was used as a secondary measure to assess the purity of the DNA. This was the 260/230 ratio. The ratios were required to be in the ranges of 1.8 and 2.0-2.2 respectively. The DNA was stored at 4° C.

5.2.2   Genomic sequencing

The paired-end genomic DNA libraries were prepared according to the manufacturer's instructions of the NextEra DNA sample preparation kit (Illumina). The genomic DNA libraries were also indexed using the NextEra index kit (Illumina) according to the protocols stipulated by the manufacturer. Paired-end *de novo* sequencing (2×300 bp) was performed on the MySeq™ Illumina Sequencing Platform using the MySeq reagent kit version 3 at the ARC Biotechnology Platform.

5.2.3   FastQC analysis- Untrimmed sequence data

The sequence reads were retrieved in the FastQ format. To assess the quality of the forward and reverse paired end sequence reads, quality reports were generated on the FastQC version 0.11.3 bioinformatics algorithm. The script for the algorithm was generated on Linux-based servers of

the Wits University. The reports which were generated guided the process of trimming the adapter content and poor quality reads.

5.2.3.1   Quality and Adapter trimming of sequence reads

The multithreaded bioinformatics algorithm Trimmomatic version 0.32 was used for the quality and adapter trimming of the Illumina sequence reads (FASTQ format). The paired end mode was used for the program and the following trimming tasks were executed through the Linux based command lines. The ILLUMINACLIP option was used to search and remove the NextEra adapters from the forward and reverse paired end reads using the sequence file NexteraPE.PE.fa. The second trim task included MAXINFO with the parameters of 50: 0.75. This task maximized the value of each read by balancing each read length with respective error rates. The last trim option which was applied to the sequence reads was MINLEN (50). This option discarded read lengths with sizes below 50 bp.

5.2.4   FastQC analysis (Trimmed data)

A second set of reports were generated for the trimmed forward and reverse paired-end sequence reads using the FastQC bioinformatics tool. This program was used to verify the quality of the trimmed forward and reverse paired-end reads. The script was generated on the Linux-based servers of the Wits University. The data was used to perform the subsequent assembly and annotation.

5.2.5   Genome Assembly and Annotation

The trimmed paired-end sequence reads of *Xenorhabdus* sp. strain GDc328 were assembled into the *de novo* draft genome sequence using the SPAdes version 3.5 Prokaryotic Genome Assembler. The algorithm was executed on the Linux-based servers of the Wits University using the careful mode pipeline which incorporated a miss match correction tool. This pipeline ensured that the number of mismatches and short indels were reduced. The following Kmer sizes were used for the assembly pipeline; K127, K21, K33, K55, K77 and K99. The assembly of the sequence reads occurred at two levels. The first included the contiguous fragments (the sequence reads were joined together into long continuous DNA fragments). The second involved the

scaffold assembly which involved the joining of contiguous DNA fragments. The output files (scaffold.fa and contigs.fa) were evaluated using the Quality Assessment Tool for Genome Assemblies (QUAST). The script for the program was generated and executed by a subset of command lines on the servers of the Wits University. The genome assembly was submitted onto the BioProject and BioSample databases on NCBI and received the identification numbers: PRJNNA291055 and SAMN03939244 respectively. The complete genome annotation was achieved using the National Center for Biotechnological Information / Prokaryotic Genome Annotation Pipeline (NCBI / PGAP). The whole genome shotgun project of *Xenorhabdus* sp. GDc328 was deposited into the Data Bank of Japan / European Molecular Biology Laboratory / GenBank (DDBJ/EMBL/GenBank) with the accession number LGYQ01000000.

5.2.6 Screening the genome annotation

The existing shotgun genome projects were screened for genes which functioned in symbiosis, virulence and temperature tolerances and compared to annotation of *Xenorhabdus* sp. strain GDc 328. Thereafter, selected protein sequences were deposited into the Universal Protein Knowledge Base (UniProtKB) database to search for related protein sequences using the UniProtKB Basic Local Alignment Search Tool (BLAST) algorithm.

5.3   RESULTS

5.3.1   FastQC Modules for untrimmed data

5.3.1.1   Basic statistics and Per Base Sequence Quality (Module 1 and 2)

The graphs for each module was included into the appendix section (see appendix section). The first module entailed the basic statistics report. This report revealed that 141 4200 sequence reads were generated in total with sequence lengths of 301 bp and a molecular G+C content of 44 %. The second module was the Per Base Sequence Quality module. The graphs showed that the quality scores were high for the forward and reverse paired-end reads at the 5' regions. The instructor's manual stated that high quality scores implied better base calls. However, the quality of the both reads deteriorated as the sequences progressed into the 3' regions (see appendix for graphs). According to the instructor's guide, quality scores of less than 20 implied poor base calls. This graph indicated that both the forward and reverse paired-end reads required trimming towards the 3' ends of the sequences to achieve improved sequence qualities.

5.3.1.2   Module (3) Per Sequence Quality Scores

This module analyzed the quality scores of a subset of sequence reads based on the PHRED score quality score. A large proportion of the forward and reverse paired-end reads showed mean scores from 27 and higher which implied that good qualities were achieved. The forward sequence reads obtained quality scores from 33 and above (see appendix for graphs).

5.3.1.3   Module (4) Per Base Sequence content

This module measured the proportion of the four nucleotide base positions in a sequence file of the library. According to instructor's manual, the proportions of each nucleotide base should be the same for a random library. The lines which were plotted on the graph (see appendix) were expected to be parallel to each other for the paired-end reads. The modules yielded errors for the forward and reverse paired-end reads. The instructor's guide attributed the error to the differences in the base composition which exceeded the value of 20 %. However, the author's manual stated that the difference obtained in the proportion of nucleotide bases had minute effects on the downstream processes.

5.3.1.4 Module (5) Per Sequence G+C content

This module measured the G+C content across the entire length of each sequence and compared the values to a theoretical normal distribution of G+C content observed by the blue line on the graph (see appendix). The red line represented the actual G+C content of the sequence reads. For both the forward and reverse paired-end reads, large proportions of the sequence reads were close to the average G+C content. A small proportion of reads had G+C contents higher or lower than the average. A match was obtained between the G+C content of the actual reads and the normal theoretical distribution.

5.3.1.5 Module (6) N content

During the sequence run, the instrument may substitute the conventional nucleotide base for an 'N'. This occurs when the sequencer is unable to produce a base call with sufficient confidence. The N content module represented the percentages of N's that are called at each position of the sequence reads. According to the graph of the forward read (see appendix), no percentages of N's were obtained. This implied that every base at each position within the sequence reads was called with sufficient confidence by the sequence instrument.

5.3.1.6 Module (7) Sequence length distribution

This module generated a graph which showed that the high throughput sequencer produced fragment sizes of similar lengths (301 bp) for both the forward and reverse sequence reads (see appendix for graph).

5.3.1.7 Module (8) Duplicate sequences

The module selected a proportion of sequences and analyzed the subset for duplications. The findings were then extrapolated to the entire sequence library. The duplicated sequence graphs for the forward and reverse paired-end reads showed the extents to which each sequence was duplicated sequence in the library. For the forward read, warnings were obtained (see appendix for graph) because more than 20 % of the sequences were found to be non-unique. This meant that the diversity in one part of the library was exhausted and the same sequences were re-

sequenced. However, the instructor's manual stated this finding was expected as a result of the library type. There were fewer duplicated sequences obtained in the reverse sequence reads.

5.3.1.8 Adapter content

This module measured the content of adapter sequences within the forward and reverse paired-end sequence reads. The module issued warnings for the forward reads because the adapter content of the sequence reads exceeded 10 %. The green lines indicated that a high percentage of NextEra adapter sequences were present in the forward and reverse paired-end reads (see appendix for graph) According to the instructor's manual, the high percentage of adapter sequence content present within the sequence reads indicated that the process of trimming was required.

5.3.2 FastQC modules for trimmed data

5.3.2.1 Module (1) Basic statistics and Module (2) Per Base Sequence Quality

The number of sequence reads were reduced by a value of 42 814. The molecular G+C content increased to 46 % with read length fragments of 301 bp. The second module Per Base Sequence Quality was regarded as the most important module for assessment. This graph showed that the quality of the forward paired-end sequence reads improved after the trimming process. The quality scores obtained for each read were above 28 (see appendix for graph). This implied that the quality of the reads across the nucleotide bases for each sequence read at the 5' and 3' ends were of exceptional quality. The quality scores obtained for the reverse reads were all above the threshold of 20. These overall findings implied that the trimming process removed the bases with poor qualities. Furthermore, this implied that good quality sequences were obtained along the entire length of the sequence reads from the 5' to the 3' regions.

5.3.2.2 Module (8) Sequence length distribution

This module showed that the trimming process produced a large proportion of sequence reads with small fragment sizes. The sequence length distribution modules for both the forward and

reverse paired-end reads produced warnings (see appendix for graphs). This meant that the fragment sizes were not entirely uniform after the process of trimming.

5.3.2.3 Adapter content

The third most critical module observed was the cumulative percentage of adapter sequences within the forward and reverse paired-end sequence reads. The module reproduced graphs which showed no trace of adapter sequences within the forward and reverse paired-end reads (see appendix for graph). This finding implied that all the NextEra adapter sequences were removed from all the sequence reads after the trimming process.

5.3.3 Genome assembly metrics (SPAdes3.5)

Table 5.1: The genome assembly metrics of the species *Xenorhabdus* sp. strain GDc328.

Genome Assembly Metrics	Contiguous fragments
# Contiguous fragments (>=0 bp)	300
# Contiguous fragments (>=1000 bp)	195
Total length (>=0 bp)	4155465
Total length (>=1000 bp)	4092977
# Contiguous fragments	275
Largest contiguous fragment (bp)	204218
Total length (bp)	4147562
(Guanine and Cytosine) GC (%)	44.6%
N50 (bp)	54354
N75 (bp)	26936
L50	20
L75	47
# N's per 100 Kbp	0.00

All statistics are based on contiguous fragments of size greater than or equal to 500 bp, unless noted (eg. "# contigs (> = 0 bp) and total length (> = 0 bp)" include all contiguous fragments.

5.3.4 Genome Annotation

Table 5.2: The summary genome annotation of *Xenorhabdus* sp. strain GDc328 performed by the NCBI PGAP v 2.10.

Genome Annotated features	Number (#)
Total number of genes	3959
Protein Coding DNA Sequences (CDS)	3608
Pseudo genes	259
Clustered regularly interspaced short palindromic repeats (CRISPR) arrays	1
Ribosomal ribonucleic acids (rRNAs)	17 (5S, 16S and 23S)
Transfer RNAs	72
Non-coding RNAs	3
Frame-shifted genes	74

Table 5.3: The genes of *Xenorhabdus* sp. strain GDc328 which encoded the associated proteins involved in flagella biosynthesis.

Name of Protein	Gene	Gene locus	Accession number
Flagella biosynthesis protein	*fliR*	Contig 15	LGYQ01000171 (AFK69_15560)
Flagella biosynthesis protein	*fliQ*	Contig 15	LGYQ01000171 (AFK69_15565)
Flagella biosynthesis protein	*fliP*	Contig 15	LGYQ01000171 (AFK69_15570)

Flagella motor switch protein	*fliN*	Contig 15	LGYQ01000171 (AFK69_15580)
Flagella motor switch protein	*fliM*	Contig 15	LGYQ01000171 (AFK69_15585)
Flagella basal-body associated protein	*fliL*	Contig 15	LGYQ01000171 (AFK69_15590)
Flagella biosynthesis chaperone	*fliJ*	Contig 15	LGYQ01000171 (AFK69_15600)
Flagella motor switch protein	*fliG*	Contig 15	LGYQ01000171 (AFK69_15615)
Flagella assembly protein	*FliH*	Contig 15	LGYQ01000171 (AFK69_15610)
Flagella M-ring protein	*FliF*	Contig 15	LGYQ01000171 (AFK69_15620)
Flagella hook-basal body	*FliE*	Contig 15	LGYQ01000171 (AFK69_15625)

Table 5.4: The effectors of *Xenorhabdus* sp. strain GDc 328 which functioned in toxin-antitoxin (TA) systems.

Name of Protein	Gene locus	Accession number
Toxin-antitoxin system	Contig 84	LGYQ01000063
Antitoxin (*DinJ*)[1]	Contig 77	LGYQ01000060 (AFK69_01205)
Toxin (YafQ)[1]	Contig 77	LGYQ0100060 (AFK69_01200)
Bifunctional antitoxin[2]	Contig 63	LGYQ01000176 (AFK69_16205)
RelE-like toxin[2]	Contig 63	LGYQ01000176 (AFK69_16210)
Bacterial addiction toxin molecules[3]	Contig 90	LGYQ01000279 (AFK69_19290)
Addiction molecule antitoxin[3]	Contig 90	LGYQ01000279 (AFK69_19295)
Toxin[4]	Contig 16	LGYQ01000121 (AFK69_07425)
Antitoxin[4]	Contig 16	LGYQ01000121 (AFK69_07420)

The superscript numbers (1, 2, 3 and 4) succeeding the protein names represented the associated toxin-antitoxin pairs.

Table 5.5: The proteins of *Xenorhabdus* sp. strain GDc328 which functioned in warm and cold temperature adaptation.

Name of Protein	Gene locus	Accession number
Molecular chaperone (HSP) (GroEL)	Contig 6	LGYQ01000139 (AFK69_10285)
Molecular chaperone HSP (GroES)	Contig 6	LGYQ01000139 (AFK69_10280)
HSP33-like Chaperone	Contig 5	LGYQ01000145 (AFK69_11590)
Heat shock protein (HspQ)	Contig 20	LGYQ0100072 (AFK69_15775)
Heat shock protein (Htpx)	Contig 15	LGYQ01000171 (AFK69_15545)
Heat shock protein GrpE	Contig 59	LGYQ01000220 (AFK69_18150)
Cold shock protein	Contig 83	LGYQ01000154 (AFK69_13225)
Cold shock induced protein	Contig 3	LGYQ01000132 (AFK69_08960)
Cold shock protein	Contig 3	LGYQ01000132 (AFK69_08835)
Cold shock protein	Contig 4	LGYQ01000159 (AFK69_14545)
Cold shock protein	Contig 42	LGYQ01000179 (AFK69_16360)
Cold shock protein	Contig 69	LGYQ0100056 (AFK69_00960)
Starvation inducible regulatory protein CspD	Contig 4	LGYQ01000159 (AFK69_14330)
Trehalose-6-phosphate hydrolase	Contig 5	LGYQ01000145 (AFK69_11495)
PTS system Trehalose	Contig 5	LGYQ01000145 (AFK69_11490)

Table 5.6: The genes characteristic of *Xenorhabdus* bacteria.

Name of protein	Gene	Gene loci	Accession number
Osmolarity sensor	*EnvZ*	Contig 5	LGYQ01000145 (AFK69_11300)
Osmolarity regulator	*OmpR*	Contig 5	LGYQ01000145 (AFK69_11305)
Global	*LysR*	Contig 15	LGYQ01000171 (AFK69_15640)

Transcriptional regulator			
RNA polymerase sigma factor	*RpoS*	Contig 37	LGYQ01000174 (AFK69_16000)

Table 5.7: The protein sequence homologies of different *Xenorhabdus* species of bacteria.

Protein	*Xenorhabdus* sp. strain GDc328 (query)	*Xenorhabdus* species	Accession number (protein subject)	% Maximum identity
Osmolarity regulatory protein (ompR)	A0A068QX46	*Xenrohabdus nematophila*	A0A077QKB6	95.7
Lys-R family transcriptional factor	A0A068QRG3	*Xenorhabdus doucetiae*	W1J5M6	89.7

5.4 DISCUSSION

5.4.1 Assembly quality metrics

The contiguous fragment N50 value may be regarded as the most widely used metric to assess the quality of an assembly (Meader et al., 2010). The contig N50 value which was generated by the SPAdes v 3.5 algorithm showed to be highest value amongst the three different assemblers which were used (results not displayed). This metric was defined by Salzburg and York (2005) as the weighted median contiguous fragment size in which 50 % of the genome was explained by contigs with sizes of the N50 value or larger. The high N50 value was indicative of a good quality assembly. The N50 value and number of contiguous fragments (see table 5.1) of *Xenorhabdus* sp. strain GDc328 was similar to other whole genome shotgun sequence projects of species which comprised the *Xenorhabdus* genus. The study conducted by (Murfin et al., 2015) showed that the genome assemblies of 9 different strains of *X. bovienii* produced contiguous fragment numbers in the range of 400 and N50 values ranging from 30-55 Kbp. Furthermore, out of the total number of contiguous fragments produced by the SPAdes algorithm (see table 5.1), 195 contiguous fragments ranged from sizes of 1000 bp or larger. This finding substantiated the quality of the SPAdes3.5 genome assembler because the algorithm assembled small sized overlapping reads into large contiguous fragments of DNA.

5.4.2 Annotation

The whole genome shotgun technique has been applied to different species and strains which comprise the *Xenorhabdus* genus. Amongst these were *X. nematophila*, *Xenorhabdus poinarii*, *X. bovienii*, and *Xenorhabdus zsentirmaii*. The sizes of the genomes varied between 4.5 Mb and 4.8 Mb. The molecular G+C content of the genomes were in the ranges of 44.2 % to 45 % (Stock, 2015). These studies were coherent with the findings of the whole genome shot gun project of *Xenorhabdus* sp. strain GDc328. The species under investigation comprised a complete genome size of 4.09 Mb (see table 5.2).The size of this genome was relatively consistent with existing *Xenorhabdus* genomes which have been sequenced (Stock, 2015; Bisch et al., 2016). The selective pressures which are exerted from the ecological niche may affect the content and size of the genome (Bisch et al., 2016).

The study conducted by Bisch et al., (2016) showed that two strains of *X. boveinii* (*Xb* CS03 and *Xb* SS-2004 differed in genome sizes. The authors related this finding to the manner in which the two strains exploited their respective insect hosts. The strain *Xb* CS03 comprised a larger genome size than *Xb* SS-2004. The genome structure of the former strain revealed a large proportion of antimicrobial encoded genes. The authors suggested that this species specialized in competitor inhibition as opposed to the rapid infection and evasion of the insect hosts thereby relying considerably on the virulence products of their cognate EPN partners. Contrastingly, the genome of the strain *Xb* SS-2004 was smaller in size. The structure of the genome revealed large proportions of genes which encoded for host invasion and exploitation. These genes included toxins, invasins and extracellular enzymes. The size of *Xenorhabdus* sp. GDc328 was closer to the size of the strain *Xb* SS-2004. The species under investigation in this research project also exhibited similar virulent characteristics without their symbiotic EPN partners (see previous chapter). The number of pseudogenes (see table 5.2) implied that the ecological niches were stable and nutrient-plentiful (Bisch et al., 2016).

According to the study conducted by Bisch et al., (2016), pseudogenization may be attributed to relaxed selective pressures elicited from the ecological niches of the obligate species of bacteria. The authors stated that the accumulation of pseudo genes were hallmarks for the initial stages of genome reduction. The authors also stated that the sizes of the bacterial populations of highly host restricted species occur in small numbers. Two phenomena may contribute to the small sized genomes of the species. These concepts include genetic drift and horizontal gene transfer.

The comparative genome study conducted by Ogier et al., (2014) demonstrated that gene acquisition for virulence factors of the many species within the *Xenorhabdus* genus occurred through the process of horizontal gene transfer. The species which comprise the *Xenorhabdus* genus possesses two systems which function in toxin molecule delivery. These systems include the flagella system and the outer membrane vesicle system (Ogier et al., 2014). The flagella system has been identified in *Xenorhabdus* sp. strain GDc328 (see table 5.3). The system was encoded for by the different genes which were positioned on contiguous fragment 15 of the bacterial genome. The study conducted by Blair and Clarke (2007) showed that deletion or mutations of the genes which encoded the system resulted in attenuation of virulence of the

bacterial species against insect hosts. According to Murfin et al., (2015), the flagella system of *Xenorhabdus* species may be evolutionary related to the type III secretion systems. In other *Xenorhabdus* species such as *X. nematophilus*, the flagella system exported virulent determinants into the insect host which consisted of non-flagella products into the insect host (Murfin et al., 2015).

5.4.3   Toxin-antitoxin (TA) systems

The toxin-antitoxin system comprises two genes which are closely linked. The first encodes the stable toxin whereas the second encodes a labile antitoxin. According to Ogier et al., (2014), the TA system stabilizes genomic regions. The authors observed that when the TA locus was lost, the disappearance of the antitoxin gene triggered cell death within the bacterial population. The roles of the TA system were elucidated by Ogier et al., (2014). These included bacterial stress response and cell quality control. The draft genome sequence of *Xenorhabdus* sp. strain GDc328 revealed co-localized pairs of toxin and antitoxin genes (see table 5.4). The study conducted by Bisch et al., (2016) showed that 40 % of genes which comprised the draft genome sequence of the species *X. boveinii* encoded for TA systems. However, the strain demonstrated non-virulent characteristics without their symbiotic EPN partners. The genome of the virulent strain of *X. boveinii* comprised a smaller proportion of genes (20 %) which encoded for the TA systems. This finding also concurred with the study conducted by Ogier et al., (2014). The authors observed the proportion of genes which encoded the Toxin-antitoxin (TA) system differed between obligate intracellular and free-living prokaryotic species. Furthermore, pairs of co-localized antitoxin and toxin genes were not identified in non-virulent bacterial strains. Four pairs of the co-localized antitoxin and toxin encoded genes were identified in *Xenorhabdus* sp. strain GDc328 (see table 5.4). One important gene identified in the draft genome sequence was the homologue of the *E.coli* derived RelE toxin gene. The gene locus was identified on contiguous fragment 63.

5.4.4   The RelE toxin

During the stress-related conditions such as nutrient deficits, the toxin gene may become over expressed. The expression of this gene encodes a toxin protein which interferes with cellular targets. The corresponding antitoxin becomes truncated by protease mediated degradation. The

antitoxin protein may neutralize the toxin protein produced from the same TA model. The TA models function in bacterial persistence, drug tolerance and resistance. Extensive research has been conducted on the TA models produced by the bacterial species *E. coli*. Amongst these modules, much focus was attributed to the RelBE model. According to Rathore and Gautam (2014), the RelE toxin was regarded as a member of the RelBE model of TA systems. The RelE toxin gene was regarded as the global inhibitor of translation. The protein possesses conserved target sites, wide specificities and functionalities. To regulate the expression of genes, the RelE toxin competes with the release factors and enters sites which enable the interaction between the toxin and messenger ribonucleic acid (mRNA). When the stress levels become reduced and nutrient conditions restored, the corresponding antitoxin neutralizes the toxin molecules. The growth of the bacterial cells and protein translation subsequently resumes (Rathore and Gautam, 2014). The role of the gene was implicated in bacterial persistence. The proteins which contributed to temperature tolerance were also identified in the draft genome sequence of *Xenorhabdus* sp. strain GDc328.

5.4.5   Temperature related genes

The draft genome sequence of *Xenorhabdus* sp. strain GDc328 comprised proteins which functioned in temperature tolerances (see table 5.5). Selvan et al., (1996) stated that the synthesis and expression of heat shock proteins was induced from environmental stresses. The authors observed that the heat shock treatment was positively correlated to the synthesis of heat shock proteins. According to Shimuta et al., (2004), the heat shock protein complex functioned in the maintenance of the structural integrity of cellular proteins. This may be conducted by two approaches. The first approach included the folding of the proteins and the second involved the degradation of proteins which have become denatured. The study conducted by Shimuta et al., (2004) identified the GroEL-GroES molecular chaperone complex in the genome of *E. coli*. This microorganism was taxonomically classified within the same family (Enterobacteriacae) as *Xenorhabdus* species. This complex was also identified in the draft genome sequence of *Xenorhabdus* sp. strain GDc328. The function of the complex was elucidated by Shimuta et al., (2004). The complex prevented the denaturing of proteins by excess heat through the stimulation protein folding. Also, the complex functioned in the process of protein refolding during the event of protein misfolding. Apart from the molecular chaperone proteins, the authors identified

proteases. These complexes functioned in the degradation of denatured proteins. Furthermore, the authors identified a novel *hspQ* gene. This gene was also identified in the draft genome sequence of the species under investigation in this research project (see table 5.5). Shimuta et al., (2004) inferred that the key function of the gene entailed the quality control of proteins by the exclusion of denatured proteins. The *GrpE* gene was one other important co-chaperone identified in the draft genome sequence of the bacteria. Harrison (2003) stated that this gene functioned as an important nucleotide regulation factor. The author observed stated that the gene was regulated thermally through the process of heat shock treatment.

5.4.6   Cold shock proteins

According to Horn et al., (2007), the induction of the cold shock protein (Csps) may be triggered by the decrements in temperature. The Csps belong to a class of small sized proteins which share high sequence and structural homologies. The proteins exhibited their functions by regulating different biological processes of the bacteria during cold stresses. However, cold shock proteins may also be synthesized during the normal temperature conditions for other cellular regulatory functions. The study conducted by Horn et al., (2007) identified 9 classes of cold shock proteins from the species *E. coli*. These included Cold shock protein A-I. Amongst the identified protein families, the Cold shock protein CspD was identified in the draft whole genome sequence of *Xenorhabdus* sp. strain GDc328. According to the Horn et al., (2007), the synthesis of the CspD was stimulated by the cold shock response. The authors suggested a pattern of protein expression subsequent to the cold shock response. The authors divided the pattern of protein expression into different stages. The first stage was regarded as an acclimation phase. The acclimation phase was characterized by the production of the newly synthesized cold shock proteins. Also, this stage was also accompanied by the simultaneous inhibitory effect on the synthesis of non-cold shock proteins. After the acclimation phase was complete, the production of the cold shock proteins were reduced to constant levels. The synthesis of non-cold shock proteins were restored with a novel acclimatized pattern which was adapted to lower temperature regimes (Horn et al., 2007).

5.4.7   The characteristic genes of *Xenorhabdus* sp. strain GDc328

The study conducted by Kim and Forst (2005) investigated the role of the EnvZ-OmpR complex in different species which comprised the *Xenorhabdus* genus. The Universal Protein / Basic

Local Alignment Analysis Tool (UniProt / BLAST) revealed that the sequence of the OmpR protein obtained from the draft genome sequence of the *Xenorhabdus* sp. strain GDc328 shared 95.7 % homology to the species *X. nematophila*. According to Kim and Forst (2005), this system was implicated in regulating the time of flagella and exoenzyme production within the species *X. nematophila*. Furthermore, the authors observed elevated expression levels of the flagella master switch regulatory (*flhDC*) operon in *ompR* mutant strains. One of the major family transcriptional regulators was identified in the genome sequence of *Xenorhabdus* sp. strain GDc328. This was the LysR transcriptional regulator. The study conducted by Hinchliffe et al., (2010) elucidated the role of this transcriptional factor within the different *Xenorhabdus* species. The authors stated that LysR regulated the expression of the *flhDC* operon which was largely responsible for the virulence of the different *Xenorhabdus* species. The UniProtKB BLAST algorithm revealed that the sequence of the Lys-R transcriptional factor from *Xenorhabdus* sp. strain GDc328 shared the highest homology (89.7 %) to the Lys-R protein regulator from the species *Xenorhabdus doucetiae*.

One other important gene was identified on contiguous fragment 37. This was the ribonucleic acid (RNA) polymerase sigma factor gene (*RpoS*). The study conducted by Kim and Forst (2005) implicated this gene in the symbiosis which exists between the EPN and the bacteria. This finding was substantiated by the study conducted by Vivas and Goodrich-Blair (2001). The authors disrupted the *RpoS* gene of *X. nematophilus*. The virulence of the mutant strain was determined. The authors observed that pathogenesis of the bacteria was unaffected but the strain was unable to colonize the gut of their cognate nematode partner. The authors then inferred that the gene was implicated in the mutualism which existed between the EPN and bacterial partner.

5.5   CONCLUSION

The draft genome sequence of the *Xenorhabdus* sp. strain GDc328 elucidated significant genes within the areas of virulence, temperature tolerance and symbiosis. The mechanism of gene acquisition may be largely attributed to horizontal gene transfer. The acquisition of the genome sequence of the bacteria may provide insight into the genetic improvements of the EPN-bacterial complex against insect pests for the optimization in EPN-product formulation for the South African market.

CHAPTER 6: CONCLUSION AND FUTURE WORK

6.1   CONCLUSION

In this research project, the novel entomopathogenic nematode (EPN) species was isolated and molecularly characterized from South African soil. The national centre for biotechnological information basic local alignment tool (NCBI BLAST) parameters revealed an 85 % maximum identity to the species *S. khoisanae*. However, the phylogenetic analysis showed that the species under investigation shared a common ancestor with the species *S. khosianae* but was regarded as a novel species based on extended branch lengths. Its associated symbiotic partner shared high sequence similarity of 99 % to and taxonomically grouped with the species *Xenorhabdus* sp. strain GDc328. The entomopathogenic nematodes (EPNs) have not yet been commercialized in South Africa for agricultural purposes. It was critical to use current tools and knowledge to investigate the biological control potential of endemic EPN isolates. The biological control potential of *Steinernema* spp. was rigorously evaluated through different investigations.

Host infectivity by the EPN-bacterial complex was evaluated at three different factors. These were time, temperature and population density. Host mortality significantly increased with time. Host mortality significantly increased with a decrease in temperature at smaller population densities of infective juveniles. However at larger population densities of infective juveniles, the differences obtained in host mortality at each temperature regimen were not significantly different. This may have suggested that the large numbers of infective juveniles may have overcome the effects of temperature. However, there were no statistical differences in host mortalities obtained between the two population densities. This was also supported by the statistical insignificant interaction between population density and temperature. However, since differences in host mortality was observed the most between the two population densities at the temperature regimen of 30° C, it was concluded that interaction was present between population density and temperature but the level of interaction was not sufficient to be regarded as statistically significant.

The subsequent biological process evaluated was the recovery of the EPNs. This process was measured in relation to two factors. These factors were temperature and population density. The

percentages of recovery were high between both population densities. The mean recoveries were not statistically different between the two population densities of infective juveniles. This was expected because recovery was recorded based on whether progeny infective juveniles emerged from the insect cadavers. Also, there were no significant differences between mean recoveries at the different experimental temperature regimens. This contributed to the non-significant interaction between population density and temperature. The last biological process measured was the progeny yield of infective juveniles. The last two biological processes were related as both processes were dependent on the ability of the infective juveniles to exit their developmental arrest, develop into first generation adults and reproduce to obtain the infective juvenile progeny. This investigation was also evaluated in relation to the factors of population density and temperature. A higher number of infective juveniles were produced at the lower population density. Also higher progeny yields were produced at the temperature regimen of 20° C. The mean yields obtained between the two population densities and temperature regimens were statistically significant. This contributed to the strong significant interaction between population density and temperature.

The investigations above were performed using the EPN-bacterial partnership. The high performances of the species at the temperature regimen of 20° C and decrements in performance at the temperature regimen of 30° C for all biological processes were explained by two possible outcomes. The first outcome involved the climatic origin of species *Steinernema*. These species have temperate origins which may have explain their performances at the temperature regimen of 20° C. The extents of infectivity, recovery and progeny production at the higher temperature regimen may have suggested the flexible ability of the EPNs to adapt to rapid temperature changes resulting from acute acclimatization. Alternatively, these findings may have been explained through the process of inadvertent temperature selection through propagation at 25° C for 18 months. This propagation may have changed the genetic constitution of the EPNs which resulted in the differences in performances at higher and lower thermal limits. The EPNs demonstrated good biological control potential. However, since the EPNs were tested as a bi-partite complex, it was interesting to understand whether the bacteria were able to achieve host infections without their symbiotic nematode partners. Also the ability of the bacteria to infect insect hosts at the two experimental temperatures without prior acclimatization was evaluated.

Host infectivity by the bacteria was investigated at different bacterial doses, time intervals and temperature regimens. The bacteria were sufficient to achieve host infection without the contributions from the nematodes. A directly proportional relationship was observed between host mortality and bacterial dose. The times taken to achieve host mortality were statistically significant. However, this significance was only obtained at two investigations whereby temperature remained the constant factor. These temperature regimens were 20° C and 25° C. At the temperature regimen of 30° C, the significant differences in the times taken to achieve host mortality were not obtained. This was not entirely attributed to the time factor but to possible interaction from temperature at which the investigation was performed. These findings were similar to the previous investigations where the large proportion of host mortality was observed between 24-48 hours of infection. Interaction was also present between bacterial dose and time at the temperature regimen of 20° C. The pattern observed at this regimen was an increase in the time taken to achieve host mortality as the bacterial doses decreased. The interaction between bacterial dose and time were not significant at 25° C and 30° C. A certain level of interaction may have been present but not sufficient to be regarded as statistically significant. The last factor of temperature significantly affected host mortality. Larger extents of host mortality were observed at 30° C and low extents were obtained at 20° C. These findings differed from the findings of the previous temperature-related investigations of the EPN-bacterial complex.

The bacteria were more efficacious at the temperature regimen of 30° C when administered into the larvae without the EPNs. However, the EPN-bacterial complex were less effective at the temperature regimen of 30° C and more effective in killing at 20° C. It was concluded that the adaptation of the EPN-bacterial complex occurred as a partnership which may have contributed to the differences observed in infectivity between the two investigations. Since the bacteria were removed from the partnership and propagated individually, external factors re-shaped the genetic constitution of the bacteria which contributed to the increased efficacies observed at the temperature regimen of 30° C. It was concluded that some of the toxin-complexes involved in host infection were preferentially expressed according to temperature. One of the toxin complexes was the insecticidal toxin complex (Tc). The subsequent investigation elucidated some of the virulence factors of the bacteria through the whole genome sequencing and

annotation technique. Also the genes and proteins involved in temperature change were elucidated.

The draft whole genome sequence of the bacteria elucidated major complexes and systems involved in virulence. Different genes which comprised the flagella system of the bacteria were identified. Also, the bacterial genome was comprised of toxin-antitoxin (TA) genes. These genes were co-localized and positioned throughout different parts of the genome. An important gene was identified which functioned in the symbiosis between the EPN and the bacteria. This gene was the ribonucleic acid polymerase sigma factor gene (*RpoS*). Studies showed that the non-expression of this gene did not alter the infectious ability of the bacteria but only affected the re-colonization ability of the bacteria with the nematode. The presence of the gene provided evidence for that the symbiosis shared between the bacteria and the EPN were tightly regulated through genetic mechanisms. Also, this genetic evidence supported the application of the bacteria as a bio-pesticide. Heat and cold shock proteins were also identified within the genome and functioned to facilitate the biological processes of the EPN-bacterial complex and bacteria during temperature transitions.

This research project showed that the novel endemic South African EPN and the bacteria showed incredible potential as biological control agents of endemic insect pests. The knowledge obtained from this research project may contribute to the research discipline of Genetic Improvement Programs (GIPs). The uses of genetic approaches in the enhancement of the EPNs and their symbiotic bacteria have been strongly advocated for the potential improvements in efficiencies as biological control agents of insect pests. Genetic improvements may enhance single or multiple beneficial traits (Shapiro-Ilan et al., 2014). The availability of whole genome sequences of the bacteria and EPN permits direct genetic modification by hybridization studies or through the selection of beneficial traits. Improvements may be required in the areas of host infectivity, protection against environmental extremes and survival. Furthermore, establishing a biological control agent with desired attributes may contribute to cost-efficient product formulation methods for agricultural application.

6.2   FUTURE WORK

This research project may be extended into the whole genome sequencing of the EPNs. This technique may involve the high throughput-sequencing technique, assembly and full functional genome annotation of the EPN species *Steinernema* spp. The whole genome sequence of the symbiotic nematode partner may provide insight into the mechanisms which regulate the different biological processes of infection, establishment, and reproduction during different environmental changes. As stated previously, the genome may harbour the full variety of genes. This establishes the groundwork for comparative genome studies. The knowledge acquired in this area may be extremely valuable because the different mechanisms which contribute to bacterial symbiosis and insect pathogenesis may be elucidated. These findings may contribute to improvements in the areas of bacterial retention, thermal tolerance, desiccation tolerance, persistence and virulence of different EPN species which ultimately leads to efficacious strains for agricultural application.

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APPENDIX

Appendix A: Chapter 2
1. Recipes

Ethanol 70 % (500ml)

Absolute ethanol (100 %)	350 ml
Sterile distilled water	150 ml

0.1 % Sodium Hypochlorite solution (100 ml)

Jik solution	0.1 ml
Sterile distilled water (autoclaved)	99.9 ml

Nutrient Broth Medium

4.0% (W/V) Canola oil

- Weigh out nutrient broth powder and suspend in desired volume of distilled water.
- Mix well and dispense adequate amounts into volumetric flasks.
- Add 4.0% (W/V) Canola oil to each volumetric flask containing nutrient broth.

- Autoclave at 121˚C and 15 psi for 15 min.

NBTA media Adapted from Akhurst (1980)

Composition (1L)

Nutrient agar powder	1L
Triphenyltetrazolium chloride (TTC)	0.04g
0.025g bromothymol blue (BTB)	0.025g
Distilled water (make up to 1L)	

- Mix nutrient agar and BTB.
- Autoclave at 121˚C and 15 psi for 15 min.
- Add TTC, just before pouring into Petri dishes; however ensure the autoclaved medium is less than 50˚C. TTC will break down if added when medium is too hot.
- Swirl to mix.

- Dispense into sterile Petri dishes and leave to solidify at room temperature

Lipid Agar (Solid *In vitro* culture) Method adapted from Kaya and Stock (1997)

Modification: Corn syrup was replaced with honey

Composition (1L)

Honey	10g
Yeast Extract	5g
Nutrient agar	25g
Cod liver oil	2.5ml
$MgCl_2.6h_2 0$	2g

- Everything is mixed together, autoclaved at 121^oC and 15psi for 20 minutes and aseptically poured into Petri dishes.

TAF (Microscopy analysis) (100 ml)

Formaldehyde (40%)	7 ml
Triethanolamine	2 ml
Distilled H_2O	91 ml

2. Protocols

Genomic DNA extraction of EPNs

1. Pellet nematodes in a microfuge tube by spinning at 14000rpm for 10 minutes. Place on ice for 30 seconds. Remove excess water.
2. Re-suspend nematode pellet in 1 ml distilled water and transfer the nematode suspension to a 1.5 ml microfuge tube on ice.
3. Centrifuge at 13000-16000 rpm for 3 minutes than place the tube on ice for at least 30 seconds and discard the supernatant.
4. Add 600µl Cell Lysis Solution (from kit) and invert several times.
5. Add 3µl Proteinase K solution (from kit) and invert 25 times. Incubate at 55˚C for 3 hours to overnight, until the tissue particulates have dissolved. Invert periodically.
6. Add 3µl RNase A Solution (from kit) to the cell lysate, invert 25 times and incubate at 37˚C for 15-30 minutes.

7. Cool the sample to room temperature.

8. Add 200µl Protein Precipitation Solution (from kit) to the RNase A. treated cell lysate.

9. Vortex at high speed for 20 seconds.

10. Centrifuge at 13000-16000 rpm for 3 minutes. A tight protein pellet should form. If this pellet is not visible repeat step 10, followed by incubation on ice for 5 minutes, than repeat step 11.

11. Pour the supernatant containing the DNA into a1.5ml centrifuge tube containing 600µl 100% Isopropanol.

12. Invert gently 50 times.

13. Centrifuge at 13000-16000 rpm for 1 minute, the DNA will be visible as a white pellet.

14. Pour off the supernatant and drain the tube on clean absorbent paper.

15. Add 600µl 70% Ethanol and invert the tube to wash the pellet.

16. Centrifuge at 13000-16000 rpm for 1 minute and carefully pour off the ethanol. Pour slowly as the pellet may be loose.

17. Invert and drain the tube on absorbent paper again and allow to air dry for 10-15 minutes.

18. Add 100µl DNA hydration Solution (from kit).

19. Rehydrate the DNA by incubating the sample 1 hour at 65˚C. Tap the tube to aid dispersing the DNA.

20. Store DNA at 4˚C.

Genomic DNA extraction kit for the Bacteria

1. Dilute the concentration of bacterial cells to $5\text{x}10^6$ CFU/ml.

2. Into a microcentrifuge tube, add the following reagents.
   a. 95µl of 2X Digestion buffer
   b. 5µl of Proteinase K

3. Mix the suspension and incubate the tube at 55°C for 20 minutes.

4. Add 700 µl of Genomic Lysis Buffer to the tube and mix thoroughly by vortexing.

5. Transfer the mixture to a Zymo-Spin™ IIC Column in a collection tube and centrifuge the10 000g for one minute.

6. Add 200 µl of DNA-Pre-Wash Buffer to the spin column in a new collection tube. Centrifuge the tube at 10 000g for one minute.

7. Add 400µl of g-DNA Wash Buffer to the spin column. Centrifuge at 10 000g for one minute.

8. Transfer the spin column to a clean microcentrifuge tube, add 50µl of DNA elution buffer. Incubate for 2-5 minutes at room temperature. Centrifuge at the highest speed for 30 seconds to elute the DNA. Store at -20°C.

Appendix B: Chapter 3
The ANOVA input table data

1. Infectivity
Table 1.1: Insect Mortality (Time and Temperature) at 1000 IJs.

Table represents the input for the Two-way ANOVA test

	30 degrees	25 degrees	20 Degrees
48 Hours	93.33	86.67	100
96 Hours	100	100	100
144 Hours	100	100	100

Table 1.2: Insect Mortality (Time and Temperature) at 500 IJs

Table represents the input for the Two-way ANOVA test

	30 degrees	25 degrees	20 Degrees
48 Hours	100	86.67	60
96 Hours	100	100	86.67
144 Hours	100	100	86.67

Tables 1.3, 1.4, and 1.5: Insect mortality at the two population densities of 500 IJs and 1000 IJs at a given time

Table represents the input data for the One-way ANOVA test at 48 hours

	1000 IJs	500 IJs
30 degrees	93.33	60
25 degrees	86.67	86.67
20 degrees	100	100

Table represents the input data for the One-way ANOVA test at 96 hours

	1000 IJs	500 IJs
30 degrees	100	86.67
25 degrees	100	100
20 degrees	100	100

Table represents the input data for the One-way ANOVA test at 144 hours

	1000 IJs	500 IJs
30 degrees	100	86.67
25 degrees	100	100
20 degrees	100	100

2. IJ Recovery

Table 2.1 represents the recovery of IJs at the different population densities of (500 and 1000 IJs)

Table2.1 represents the input data for the One-way ANOVA test (IJ recovery and Population density)

	1000 IJs	500 IJs
30 degrees	93.33	86.67
25 degrees	86.67	73.33
20 degrees	86.66667	80

Table 2.2 represents the input data(IJ recovery and temperatures) for the One-way ANOVA

	1000 IJs	500 IJs
30 degrees	93.33	86.67
25 degrees	86.67	73.33
20 degrees	86.67	80

3. IJ Yield

Table 3.1 represents the IJ progeny yield obtained at the different population densities (500 IJs and 1000 IJs)

Table 3.1 represents the input data for the One-way ANOVA (IJ yield and population density)

	1000 IJs	500 IJs
30 degrees	124305.6	114285.7
25 degrees	341666.7	135714.3
20 degrees	61111.11	63194.44

Table 3.2 represents the IJ progeny yield obtained at the different temperatures

Table 3.1 the input data for the One-way ANOVA (IJ yield and temperature)

	1000 IJs	500 IJs
30 degrees	124305.6	114285.7
25 degrees	341666.7	135714.3
20 degrees	61111.11	63194.44

Appendix C: Chapter 4
1. Recipes

Nutrient agar

Composition (g/l)

Meat Extract   1.0 g

Peptone   5.0 g

Yeast Extract   2.0 g

Sodium Chloride   8.0 g

Agar   15 g

- Weigh 31 g of nutrient agar powder into 1L distilled water
- Autoclave mixture at 121 ºC for 20 minutes

2. The ANOVA input data table

Bacterial dose response assay (dose and time)

Table represents the input data for the Two-way ANOVA for figure 4.2 (b) (25 ºC)

	24 Hours	48 Hours	72 Hours	96 Hours
stock	90	90	90	90
D1	70	80	80	80
D2	30	90	90	90
D3	0	40	50	50
D4	0	20	20	20
D7	0	0	10	10

Table represents the input data for the Two-way ANOVA for figure 4.2 (a) (20 ºC)

	24 Hours	48 Hours	72 Hours	96 Hours
stock	30	70	70	70
D1	0	50	90	100

D2	0	0	40	70
D3	0	0	10	20
D4	0	0	0	0
D7	0	0	0	0

Table represents the input data for the Two-way ANOVA for figure 4.2 (c) (30 ºC)

	24 Hours	48 Hours	72 Hours	96 Hours
stock	100	100	100	100
D1	100	100	100	100
D2	90	100	100	100
D3	60	70	70	70
D4	20	40	40	50
D7	20	30	30	40

Bacterial dose response assay (Dose and Temperature at a given time)

Table represents the input data for the Two-way ANOVA for figure at 24 hours

	20 degrees	25 degrees	30 degrees
stock	30	90	100
D1	0	70	100
D2	0	30	90
D3	0	0	60
D4	0	0	20
D7	0	0	20

Table represents the input data for the Two-way ANOVA for figure at 48 hours

	20 degrees	25 degrees	30 degrees
stock	70	90	100
D1	50	80	100
D2	0	90	100
D3	0	40	70

		0	20	40
D4		0	20	40
D7		0	0	30

Table represents the input data for the Two-way ANOVA for figure at 72 hours

	20 degrees	25 degrees	30 degrees
stock	70	90	100
D1	90	80	100
D2	40	90	100
D3	10	50	70
D4	0	20	40
D7	0	10	30

Table represents the input data for the Two-way ANOVA for figure at 96 hours

	20 degrees	25 degrees	30 degrees
stock	70	90	100
D1	100	80	100
D2	70	90	100
D3	20	50	70
D4	0	20	50
D7	0	10	40

Appendix D: Chapter 5

Fast QC analysis graphs of the untrimmed sequence data -Forward and Reverse reads were highly similar (Represented the forward reads only)

Model 1

Basic Statistics

Measure	Value
Filename	c2f_S6_L001_R1_001.fastq.gz
File type	Conventional base calls
Encoding	Sanger / Illumina 1.9
Total Sequences	1414200
Sequences flagged as poor quality	0
Sequence length	301
%GC	44

Model 2

Per base sequence quality

Module 3
Per sequence quality score

Quality score distribution over all sequences

Module 4 – Per base sequence content

Sequence content across all bases
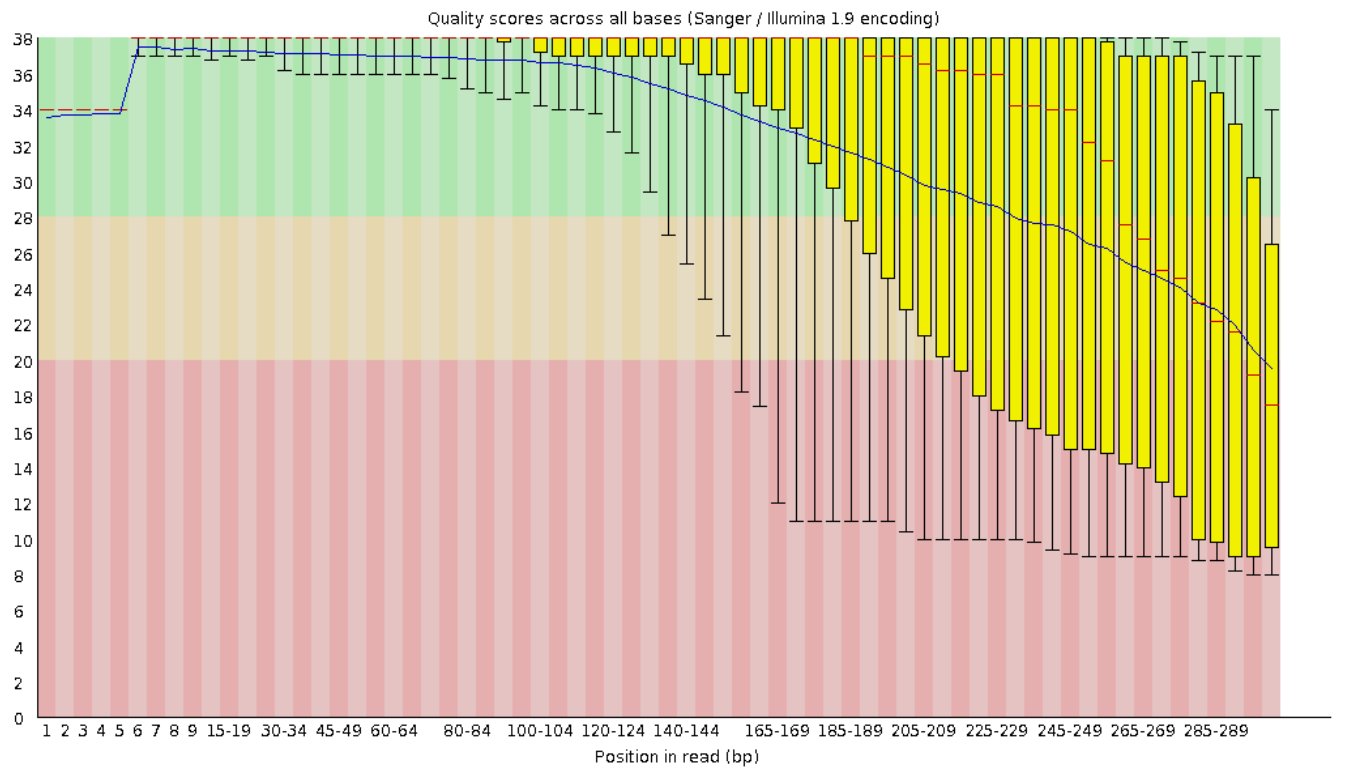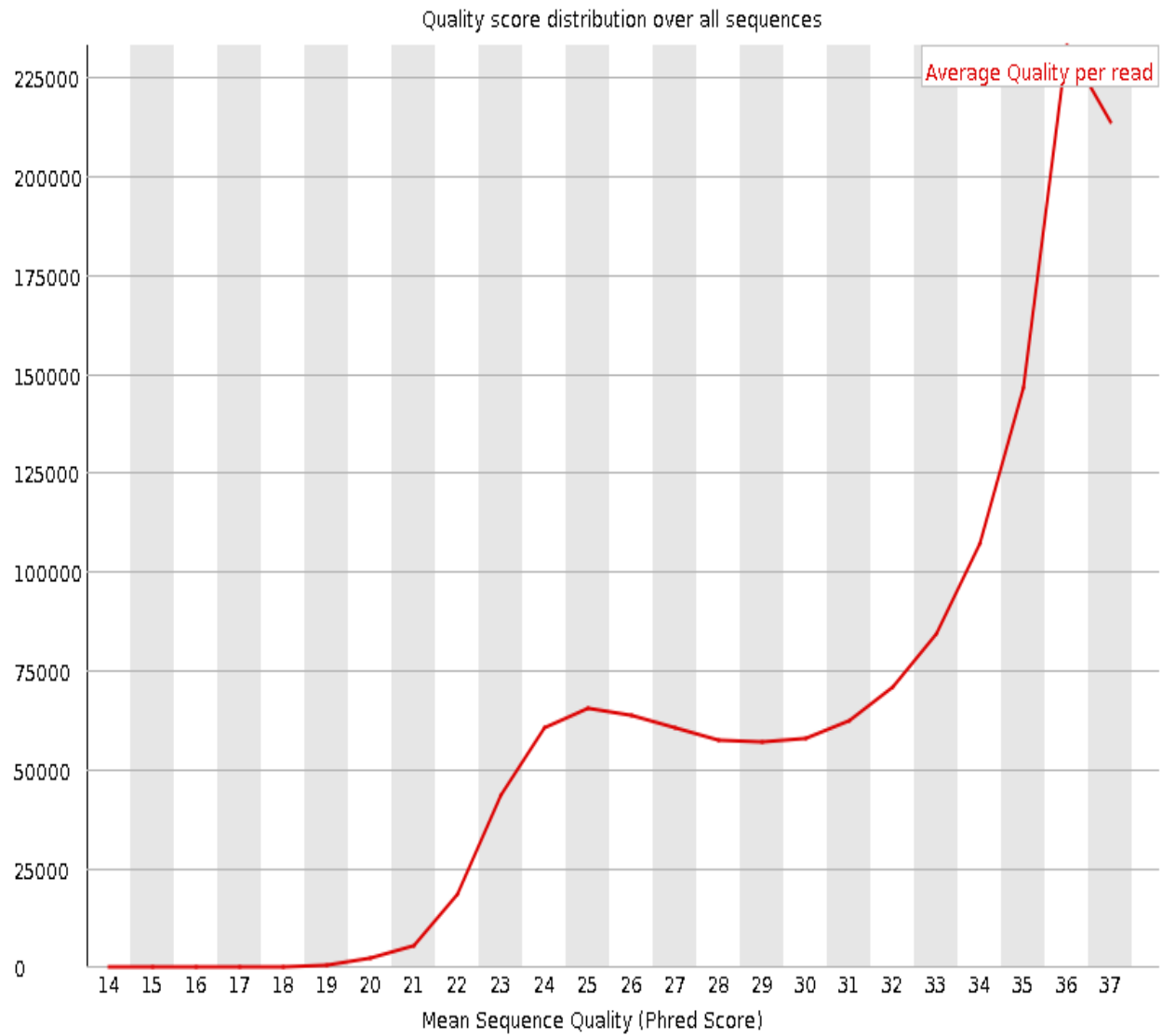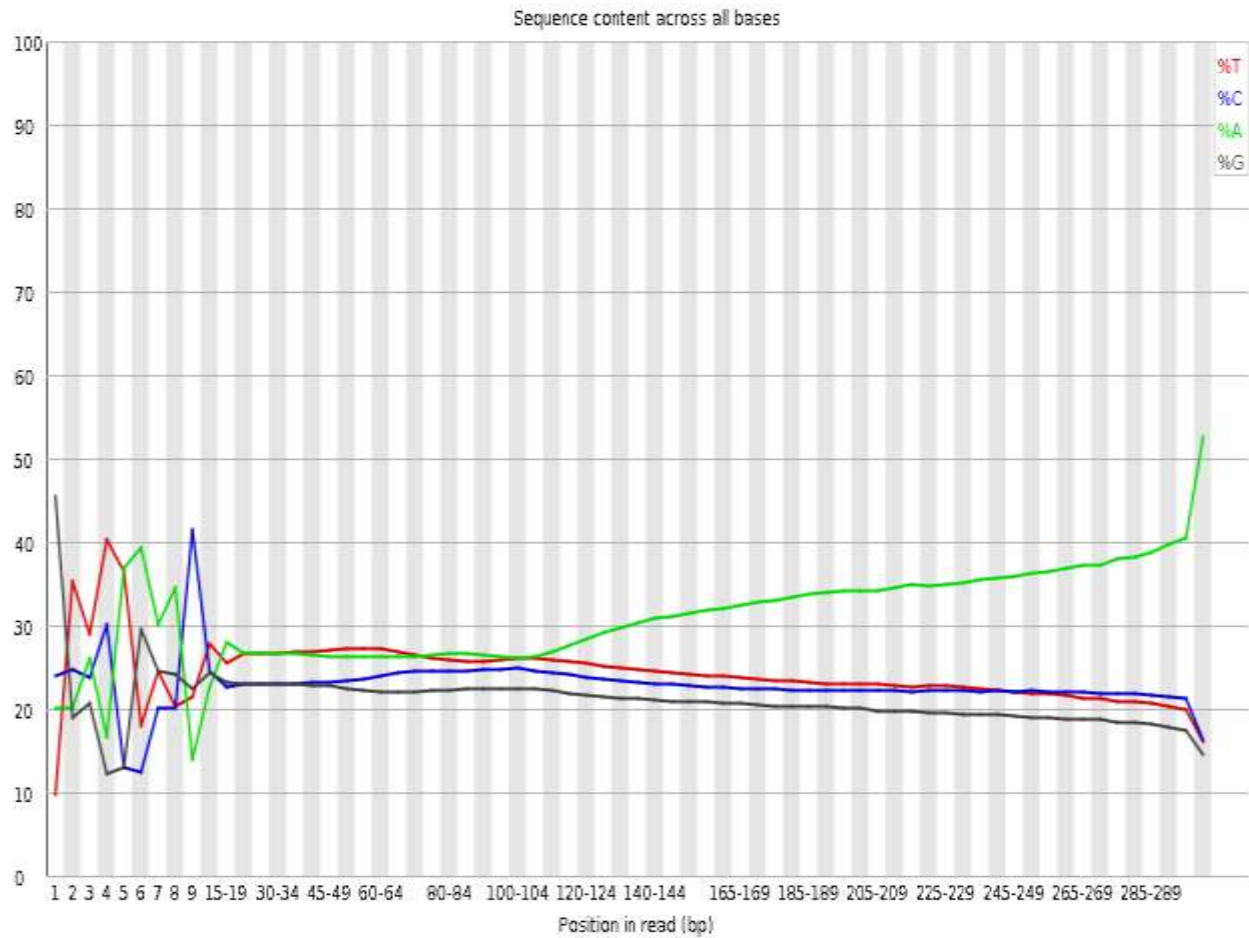
Module 5
Per base G+C content

GC distribution over all sequences

Module 6
N-Content

N content across all bases

Module 7
Sequence length distribution

Distribution of sequence lengths over all sequences
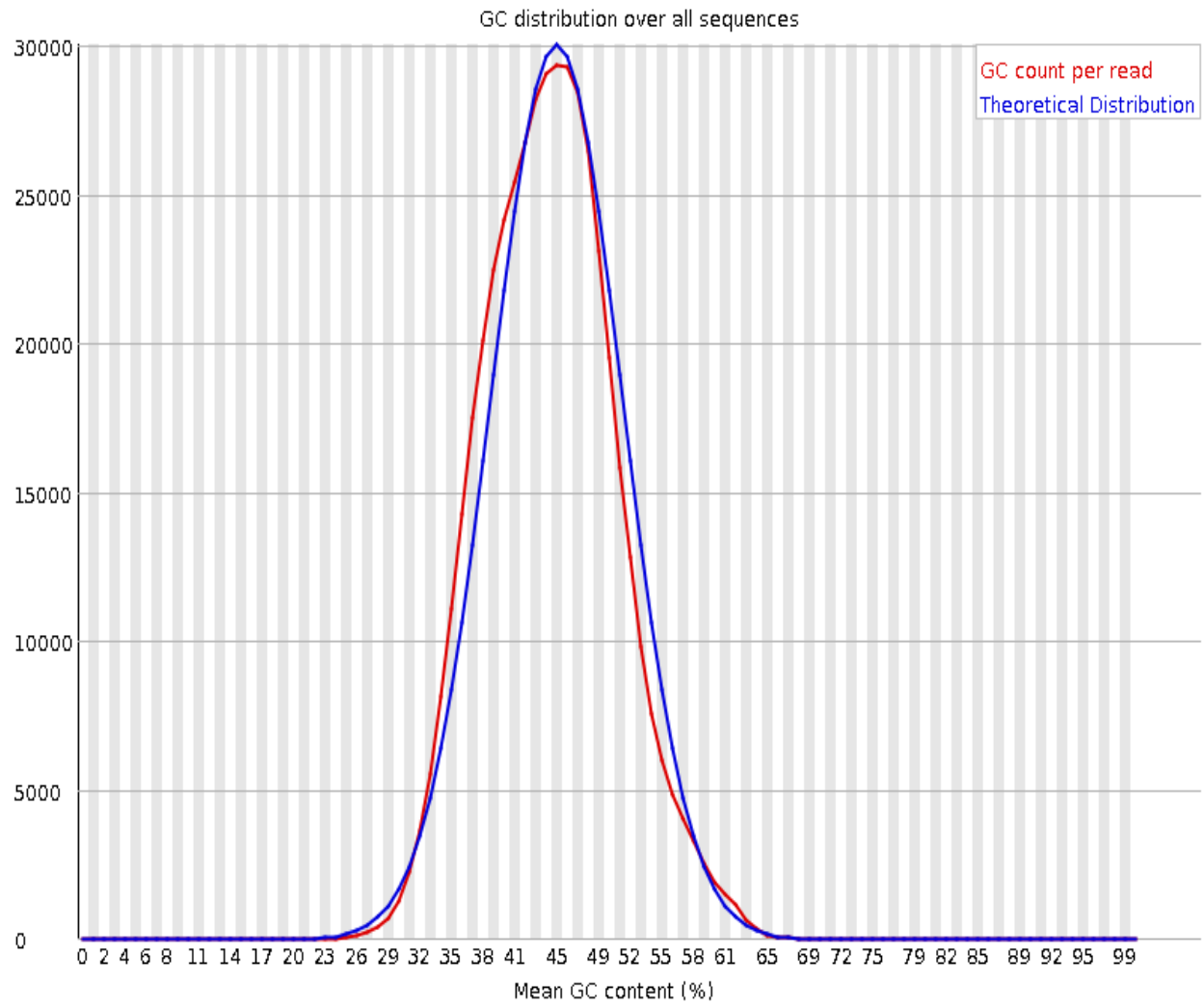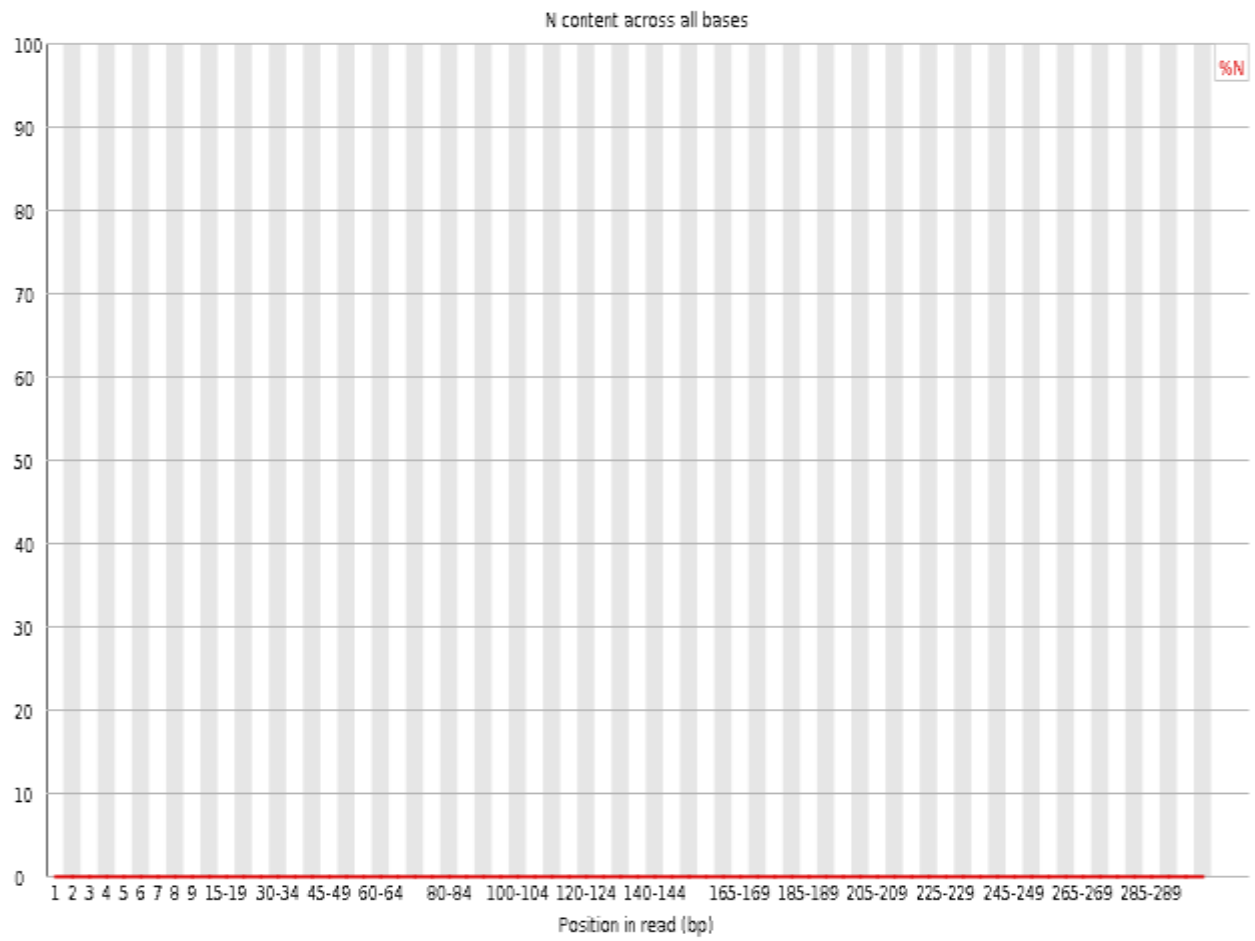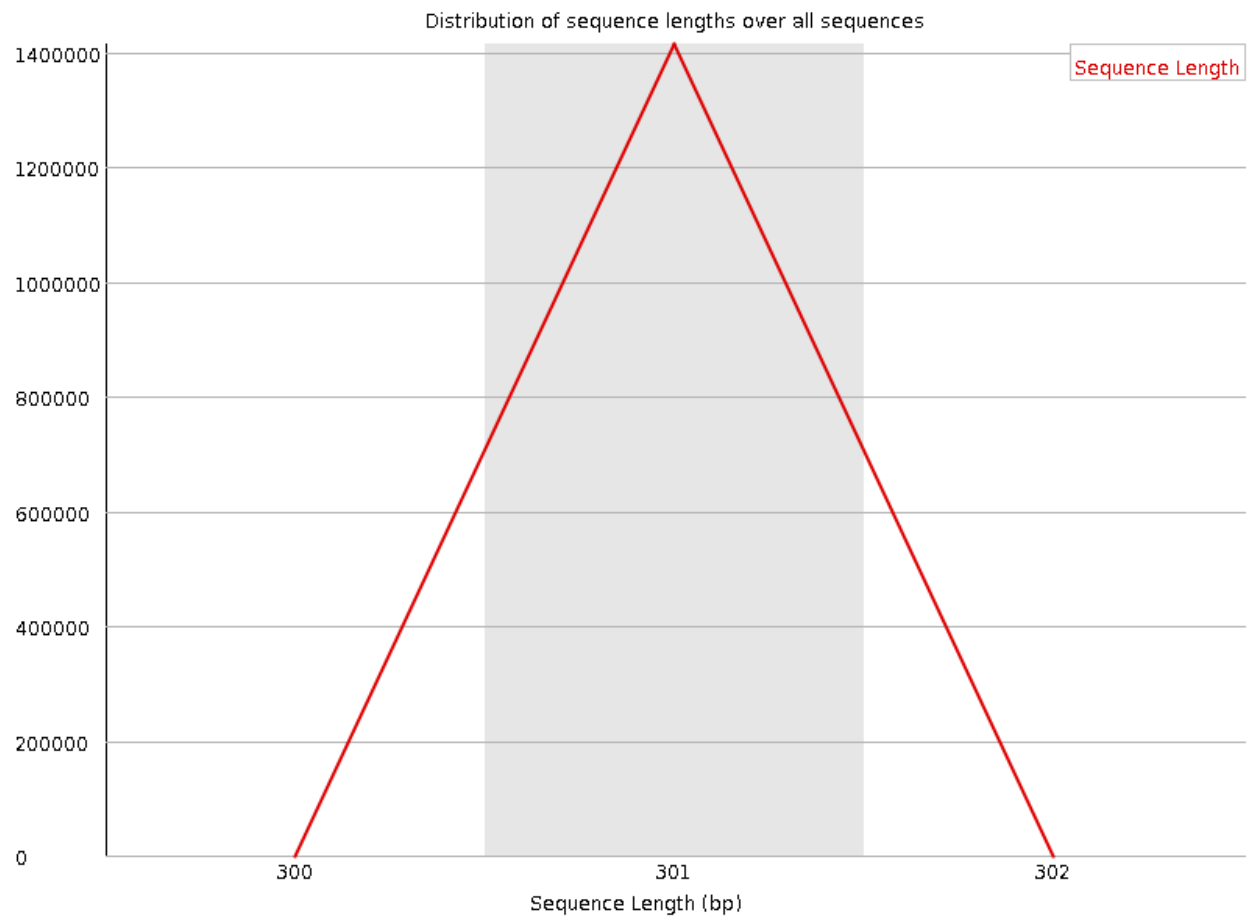
Module 8
Duplicated sequences

Percent of seqs remaining if deduplicated 66.18%
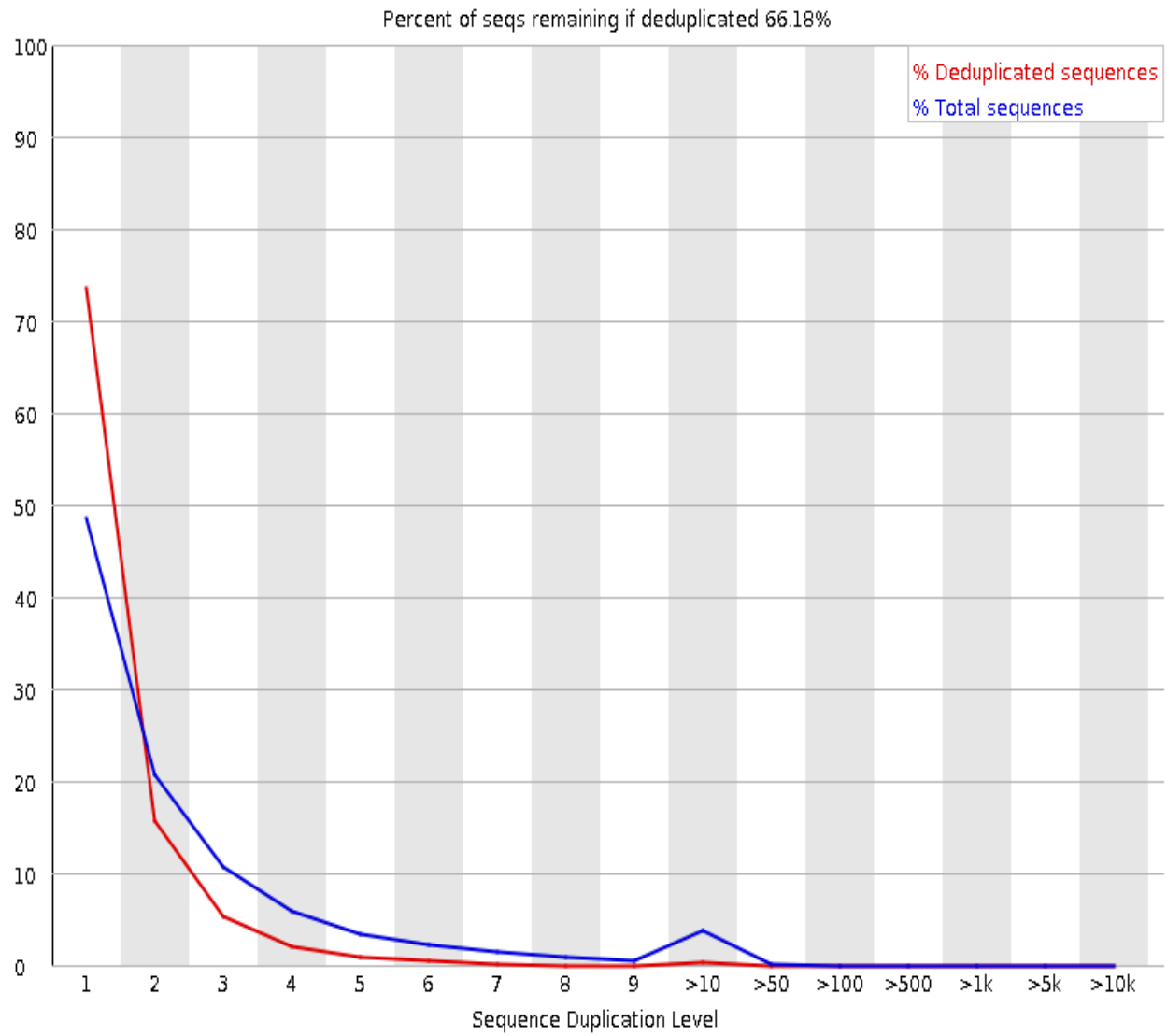
Module 9
Adapter content
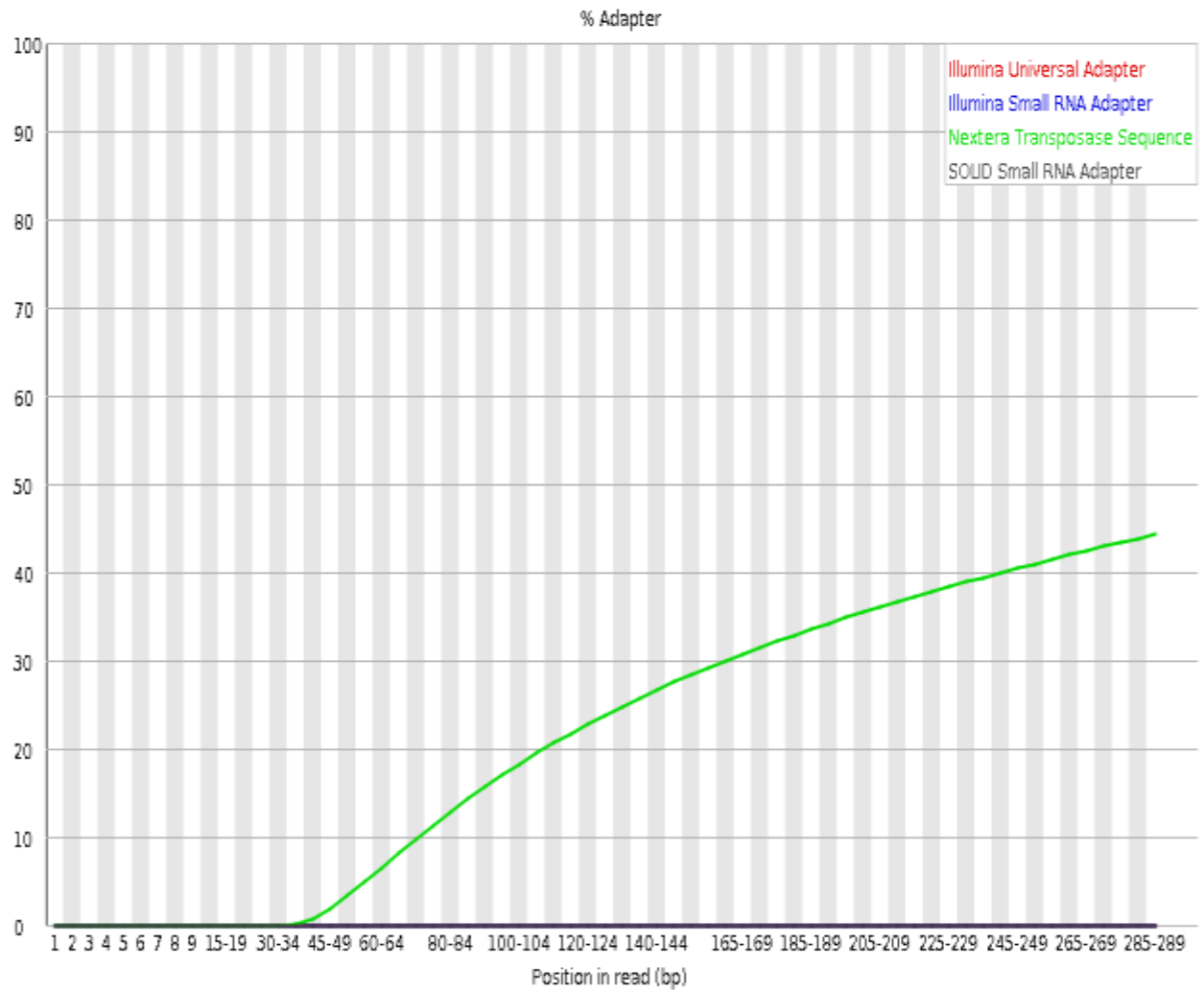
(FASTQC) Trimmed sequence data

Module 1- Basic statistics

Measure	Value
Filename	Bacteria_LA_trimmed_rep1_1P.fastq.gz
File type	Conventional base calls
Encoding	Sanger / Illumina 1.9
Total Sequences	1371386
Sequences flagged as poor quality	0
Sequence length	50-301
%GC	46

Module 2 – Per base sequence quality (trimmed)

Quality scores across all bases (Sanger / Illumina 1.9 encoding)
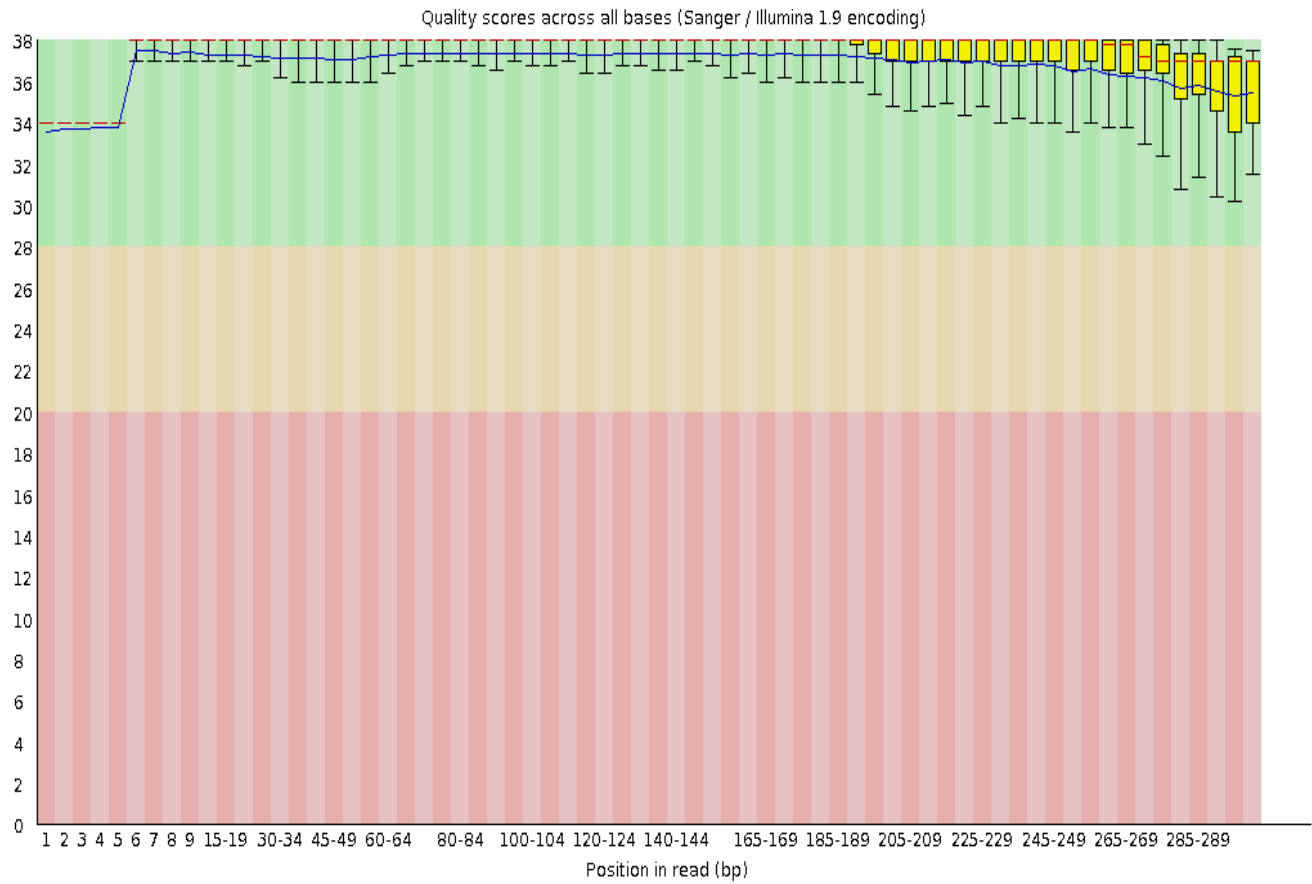
Module (8)- Adapter sequence content

% Adapter