Co-existence and niche separation of two subspecies of *Photorhabdus temperata* associated with *Heterorhabditis downesi* in a dune grassland

A thesis submitted to the National University of Ireland, Maynooth for the degree of Doctor of Philosophy

by

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Declaration

This thesis has not been submitted in whole or in part to this or any other university for any other degree and is, except where otherwise stated, the original work of the author.

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Abbreviations

%	percent
<	less than
=	equals
>	greater than
χ^2	Chi-square
$\bar{\chi}$	mean
$\tilde{\chi}$	median
ANOVA	Analysis of Variance
B. bassiana	*Beauveria bassiana*
B. subtilis	*Bacillus subtilis*
BO	Blowout
cm	centimetre
E	Embryonic Dune
E. coli	*Escherichia coli*
EPN	entomopathogenic nematode
et al.	and others
FX	Fixed Dunes
g	gram
g/l	gram/litre
GLM	Generalised Linear Model
GP	Grassy Path
hr(s)	hour(s)
ID	internal diameter
IJ	infective juvenile
ITS	internal transcribed spacer
l	litre
LB/Amp	Luria Broth, Miller/Ampicillin
M	Marram Dunes
M. anisopliae	*Metarhizium anisopliae*
M. luteus	*Micrococcus luteus*
min	minute(s)
ml	millilitre
mm	millimetre
mM	millimolar
MW	Mann Whitney
NA	nutrient agar
NBTA	nutrient bromothymol blue triphenyl tetrazolium chloride agar
OD_{nnn}	optical density
n.nn OD	outside diameter
^{o}C	degrees centigrade
PBS	Phosphate buffered saline
PCR	polymerase chain reaction
PUR	*H. downesi* nematode carrying purple *Photorhabdus temperata* subsp. *temperata* colour variant

pur	purple *Photorhabdus temperata* subsp. *temperata* colour variant
rDNA	ribosomal DNA
rpm	rotations per minute
S.O.C. medium	Super Optimal Broth
sec	second(s)
SFX	Semi-Fixed Dunes
SP	Sandy Path
sp(p)	species
StDev.	Standard Deviation
subsp.	subspecies
x g	Relative centrifugal force
X. bovienii	*Xenorhabdus bovienii*
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
YEL	*H. downesi* nematode carrying yellow *Photorhabdus temperata* subsp. *temperata* colour variant
yel	yellow *Photorhabdus temperata* subsp. *temperata* colour variant
µl	microlitre

Abstract

Entomopathogenic nematodes belonging to the families Heterorhabditidae and Steinernematidae form symbiotic relationships with entomopathogenic bacteria from the genera *Photorhabdus* and *Xenorhabdus*, respectively. Previous studies showed that *Heterorhabditis downesi* was associated with two different colour variants (*pur* and *yel*) of its *Photorhabdus* bacterial symbiont in sand dunes on North Bull Island in Dublin Bay, with evidence of the *pur* variant being more common to the front of the dunes. The main objectives of this study were to confirm the distribution of both colour variants of the nematode-bacterium complex on North Bull Island, identify them, investigate the specificity of the nematode-bacterium relationship and ascertain characteristics of the bacterial variants that might explain their distribution.

A section of the dune system on North Bull Island was surveyed in 2008 and re-surveyed in 2012 (Chapter 2). In 2008 the colour variants were spatially segregated, with nematodes associated with the *pur* variant recovered from the front section of the dunes only and nematodes associated with the *yel* variant recovered from the rear section only. In 2012 nematodes associated with each colour variant were recovered from the front section of the dunes and only nematodes associated with the *yel* variant were recovered from the rear section of the dunes. The nematode-*pur* complex was more abundant than the nematode-*yel* complex in sections where both combinations co-occurred. Three laboratory lines of each nematode and its associated bacterial colour variant were established for further study. A phylogenetic analysis of the nematode ITS region showed that all the nematode isolates were monophyletic, with no difference between the isolates carrying the *pur* or *yel* bacterial symbionts. On the other hand, a phylogenetic analysis of a portion of the

*gyr*B gene of the bacterial isolates showed that the two colour variants were distinct subspecies, *Photorhabdus temperata* subsp. *temperata* (*yel)* and *Photorhabdus temperata* subsp. *cinerea* (*pur*). This confirms *Photorhabdus temperata* subsp. *temperata* as the bacterial partner in the YEL *H. downesi – Photorhabdus* complex. In addition, this is the first report of *Photorhabdus temperata* subsp. *cinerea* in Ireland and also one of the few reports of the co-occurrence of two different symbiotic bacteria subspecies with a single nematode species (Chapter 2). In antibiosis tests against a range of organisms the *P. temperata* subsp. *cinerea* isolates had a greater inhibitory effect than the *P. temperata* subsp. *temperata* isolates. Only one pair of isolates, *yel1* and *pur2,* showed any antibiotic activity against the other subspecies and this pair were mutually inhibitory (Chapter 2). Phenotypic tests indicated that the subspecies had different growth requirements as the *pur* isolates grew better on MacConkey agar than on nutrient agar while there was no difference between the two media for the *yel* isolates. Biochemical characterisation was carried out using API 20E test strips. There was variability both within and between the colour variants, however a notable difference was that only the *yel* isolates were able to utilise trehalose, a major component of insect hemolymph (Chapter 2).

The cross-compatibility of the two different bacterial subspecies with the heterogeneous nematode isolates was tested by injecting *Galleria mellonella* larvae with 10,000 cells of either the *pur1* or *yel3* isolate or both and then infecting them with *H. downesi* nematodes carrying either *pur1* or *yel3* bacteria. The emerging nematodes carried the bacterial subspecies that had been injected into the host, irrespective of which bacterial subspecies the infecting nematode had carried and hybrid lines of nematode-bacterium complex were successfully maintained for the duration of this study (Chapter 3). In the case of cadavers infected with both

bacterial types all the emerging nematodes tended to carry a single subspecies. However, between 20 % and 30 % of the cadavers produced some nematodes carrying the *pur1* and some carrying the *yel3* isolate, (indicating that both bacterial subspecies were colonising the insect host and subsequently colonising the developing nematodes prior to emergence) and where this occurred a higher proportion of the nematodes carried the *yel3* isolate. Furthermore, there was an apparent shift in the colonisation of the nematodes within the host cadaver the proportion of nematodes carrying the *yel3* isolate was higher in later emerging nematodes (Chapter 3).

To test the hypothesis that the distribution of the colour variants was due to host specialisation (*H. downesi* complex differs from front to back of the dune system) the pathogenicity of the homogenous and heterogenous combinations against *Hylobius abietis* larvae was investigated. The LD_{50} was significantly higher for the homogenous YEL/*yel* combination compared to the heterogeneous YEL/*pur* combination. There was no difference in reproduction rate of the nematodes carrying either subspecies in either *H. abietis* or *G. mellonella* and this was backed up by tests of all three isolates of each colour type. All six isolates from North Bull Island were tested against insects from three orders (Lepidoptera, Coleoptera (2) and Diptera), with no differences in mortality or reproduction rate. This is the first report of EPN being able to use the novel host kelp fly (*Coelopa* spp.). While there was no difference in mortality rates between the PUR and YEL strains in this host there did appear to be a difference in the rate of progression of the infection with nematodes carrying the *yel* bacteria emerging earlier than those carrying the *pur* bacteria (Chapter 4).

To test the hypothesis that the bacteria influenced nematode survival in desiccated hosts *G. mellonella* larvae were infected with the homogenous and heterogeneous combinations and stored in dry sand or at 0% relative humidity. There was no difference due to bacteria type in the number of cadavers with emergence from those stored in dry sand for 28 days. There was a highly significant difference due to bacteria type in the number of cadavers with emergence for cadavers stored at 0 % relative humidity for 28 days, with emergence from more cadaver infected with the *pur1* isolate. Overall, more nematodes emerged from cadavers infected with the *pur1* isolate following 28 days storage in dry sand or at 0 % relative humidity (Chapter 5).

Heterorhabditis downesi is associated with two different subspecies of *Photorhabdus temperata* on North Bull Island: *Photorhabdus temperata* subsp. *temperata* and *Photorhabdus temperata* subsp. *cinerea* and the association of the nematode with a particular subspecies appears to vary with the soil conditions prevalent in the front and the rear sections of the dunes: Under normal conditions *P.t. temperata* appears to outcompete *P.t. cinerea* in forming a symbiotic association with the nematode when both subspecies are present in the same insect host, resulting in the *H. downesi-P.t. temperata* association predominating in the rear section of the dunes, where the increased organic content of the soil favours moisture retention. On the other hand the drier conditions that predominate in the sandier soil towards the front of the dune system favour *H. downesi-P.t. cinerea* association.

Chapter 1
General Introduction

1.1 **Symbiosis**

The term “symbiosis” was first used by Professor Heinrich Anton de Bary to describe mutualistic or parasitic associations in the plant kingdom (Paracer & Ahmadjian, 2000). The word literally means “living together” and is of Greek origin. The word symbiosis is used to describe a range of associations between organisms from different species and frequently involves organisms from different domains of life. By convention the term “host” is usually applied to the larger partner. Viral symbiotic associations are found in all three domains of life (Paracer & Ahmadjian, 2000), and microbial symbiosis is considered to have resulted in the evolution of eukaryotes through the incorporation of alpha-proteobacteria and cyanobacteria into other bacterial cells, giving rise to mitochondria in animal cells and chloroplasts in plant cells and protists respectively (Gray & Doolittle, 1982; Sapp, 2004). Symbiotic relationships can be described as mutualistic, where both partners benefit; commensal, where one benefits and the other is unaffected; or parasitic, where one benefits and the other is harmed (Paracer & Ahmadjian, 2000), although in practice the relationship can span a continuum covering all three, particularly in complex relationships involving multiple players. The associations can be intermittent or persistent, for example, the pollination of flowers by birds and insects or the dispersal of seeds by animals and birds is a seasonal event, some species of cleaner shrimp spend less than 2% of their time cleaning the fish they associate with (Jonasson, 1987), and the red-billed ox-pecker utilises 11 different

hosts (Grobler, 1980), on the other hand, most endosymbionts (intracellular or extracellular) are persistent within their host, for example the head louse *Pediculus humanus* and its intracellular endosymbiont *Candidatus* Riesia pediculicola and the earthworm *Eisenia foetida* and its extracellular endosymbiont *Verminephrobacter eiseniae* (Bright & Bulgheresi, 2010). The relationship can be obligate, as in the case of anemone and damefish (Leung & Poulin, 2008) or facultative, for example the secondary symbionts of pea aphids (Oliver *et al.*, 2010). The symbiotic relationship can allow one or both partners to exploit a niche which would otherwise be unsuitable, for example the photobiont (green algae or cyanobacteria) in a lichen is protected from desiccation and exposure to the sun's rays and thus can survive within the lichen thallus in exposed habitat such as tree trunks, rocks and tundra (Lewin, 1982) and associations with sulphur-oxidising bacteria have enabled a wide range of marine animals to live in inhospitable environments such as hydrothermal vents, cold seeps and coastal sediments (Dubilier *et al.*, 2008). The association is not limited to two partners; many associations involve multiple interactions, such as plants with fungi (Parniske, 2008), mammals and the microbial communities that live in their gut (Ley *et al.*, 2008) and insects and their intestinal microbial communities (Broderick *et al.*, 2004). Symbiosis offers organisms a way of overcoming genetic constraints that limit them to a particular niche through the acquisition of new traits via the symbiotic partner (Moran, 2007; Janson *et al.*, 2008).

Numerous examples of bacterial symbiosis with invertebrates are found, covering the continuum of relationships from commensal to mutualistic to parasitic. The bacteria frequently provide a metabolic service to the invertebrate host as in the case of sap-feeding insects such as the Cicadellinae, where the symbiont (*Baumannia*

spp.) provides nutrients that are not available to the insect from its sap diet (Moran, 2007). The nature of the relationship can change over time depending on the individual partnership as in the case of *Drosophila simulans* in California where its *Wolbachia* symbiont has increased the fecundity of the female hosts in approximately 20 years, indicating a switch from a parasitic to a mutualistic relationship (Weeks *et al.*, 2007). The insect gut microbiota also plays a role in protecting the insect host from infection; in larvae of the oriental tea fly tortrix, *Homona magnanima*, the normal gut microorganisms (*Streptococcus* spp. and *Staphylococcus* spp.) suppressed the growth of *Bacillus thuringiensis* in the insect but elimination of the normal gut microorganisms from the larvae allowed *B. thuringiensis* to multiply and kill the larvae (Takatsuka & Kunimi, 2000).

The specificity of the symbiotic relationship is dependent on many factors and can operate at the level of host specificity or symbiont specificity, as in the case of coral-algal symbiosis where some species of coral can host more than one type of *Symbiodinium* algae whereas others are only able to associate with a single type and likewise the algal partner can be associated with just a single host species or with a number of different genera (Baker, 2003). A similar pattern of generalists and specialist is seen in entomopathogenic nematodes (EPN) with some species of nematode being able to carry only its own symbiont while others can carry a different species and conversely the bacterial partner can be found associated with only a single nematode species or with several (Adams *et al.*, 2006; Lewis & Clarke, 2012). Lectins are a group of proteins that were initially discovered in plants and were shown to cause animal blood cells to clump *in vitro* (Sharon & Lis, 2004). Lectins can bind specific sugar residues in membrane glycoprotiens, as well as playing a role in cell–molecule and cell-cell recognition and specificity as well as

having regulatory, defence and immune functions, reviewed in Sharon & Lis (1989, 2004), and although they were first identified in plants they are now known to be involved in many host-symbiont interactions from plants (Dazzo & Hubbell, 1975) to invertebrates (Nordbringhertz & Mattiasson, 1979; Bai *et al.*, 2013) to mammals (Cash *et al.*, 2006). Host-symbiont recognition can involve interactions between surface structures on either the host or the symbiont; for example, bacterial peptidoglycan and host cilia, which are induced to produce mucus as in the case of the bacterium *Vibrio fischeri* and the Hawaiian bobtail shrimp *Euprymna scolopes,* or pili on the nitrogen-fixing bacteria *Azoarcus* required for adherence to the lateral roots of Kallar grass *Leptochloa fusca* (Hurek & Reinhold-Hurek, 2003). Lipopolysaccharides are also involved in host-symbiont recognition and specificity and these can be either host-associated or symbiont-associated. Microbial symbionts mainly express lipopolysaccharides (LPS) and exopolysaccharides (EPS), while host surface sugars are usually in the form of glycosylated proteins (Bright & Bulgheresi, 2010). Quorum sensing also appears to play a role in host-symbiont communication as the production of EPS can be under the control of the quorum sensing system in some bacteria (Bright & Bulgheresi, 2010).

1.2 **Entomopathogenic nematode symbiosis**

1.2.1 Life cycle

The term entomopathogenic nematode is mainly applied to members of two families, Steinernematidae and Heterorhabditidae, which are mutualistically associated with *Gammaproteobacteria* (*Xenorhabdus* and *Photorhabdus*, respectively) (Forst *et al.*, 1997). Although both heterorhabditids and steinernematids have a similar life cycle (Figure 1.1) they are not closely related (Blaxter *et al.*, 1998). In both genera the

only free living stage of the nematode is the non-feeding infective juvenile (IJ), which typically occurs in the soil. The bacterial partner is carried freely in the gut in *Heterorhabditis* spp. (Waterfield *et al.*, 2009), while in contrast the symbiotic bacteria is carried in a specialised vesicle called a receptacle in the case of *Steinernema* spp. (Snyder *et al.*, 2007). Both nematode-bacterium complexes form tri-trophic relationships, mutualistic between the nematode and the bacterium and parasitic within insects (Eleftherianos *et al.*, 2010). The IJ stage is analogous to the dauer stage in *C. elegans* – developmentally-arrested, non-feeding and long-lived (Viney *et al.*, 2005), but has a suite of specialised host-seeking and host-recognition behaviours. In both heterorhabditids and steinernematids the IJ enters an insect and "recovers" – resumes development and within the insect host the nematodes feed on a nutrient soup consisting of the symbiotic bacteria and degraded host tissue (Stock *et al.*, 2012).

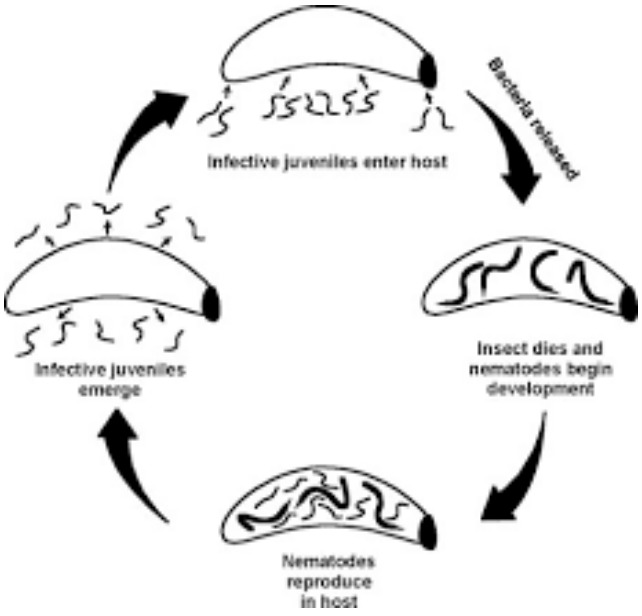

Figure 1.1: Generalised life cycle of entomopathogenic nematodes (Shapiro-Ilan & Grewal, 2008)

Species of both genera are capable of killing a broad range of insects and, together with their bacterial symbionts, utilise the nutrients provided by the insect host for growth and reproduction (Poinar, 1993). Entomopathogenic nematodes invade their prey via the spiracles, the mouth or the anus or, in the case of *Heterorhabditis* spp., through the cuticle using a dorsal tooth, which *Steinernema* spp. lack (Kaya & Gaugler, 1993). There is also evidence that *Steinernema* spp. produce secretions such as proteases which aid their penetration of insect cuticle or gut wall (Peters & Ehlers, 1994; Wang & Gaugler, 1998). Once inside the insect IJ recovery occurs, the nematode releases the symbiotic bacteria, either by regurgitation in the case of heterorhabditids or via the anus in the case of steinernematids (Koppenhöfer & Gaugler, 2009), and resumes its development. In both heterorhabditids and steinernematids the bacteria rapidly colonise the insect and death due to septicaemia caused by bacterial toxins (Boemare *et al.*, 1996) occurs within 48 to 72 hrs, depending on the number of IJs invading, and the nematode and insect species.

In *Heterorhabditis* IJs develop into hermaphrodites which lay eggs and give rise to males, females and hermaphrodites in the next generation. In contrast, *Steinernema* IJs develop into adult males and females in the case of most species (Ciche *et al.*, 2006), although a hermaphrodite *Steinernema* species has been reported (Griffin *et al.*, 2001). In both *Steinernema* and *Heterorhabditis*, in a process known as *endotokia matricida*, in each generation some eggs hatch within the maternal body and consume it (Johnigk & Ehlers, 1999; Baliadi *et al.*, 2004). Nematodes of both genera go through one to three generations within the host cadaver, until low nutrient supply, low bacterial concentration and high nematode concentration trigger a switch to IJ production, where juvenile development arrests at the third stage. The developing IJs are colonised by a small number of the bacterial symbiont (Ciche &

Ensign, 2003; Martens *et al.*, 2003). Large numbers of IJs which have developed either from eggs laid by the mother or through *endotokia matricida* (Ciche *et al.*, 2006) emerge from the depleted cadaver in search of new hosts. The number of IJs produced depends on the size and species of the host insect and can range from tens of thousands to several hundred thousand per insect (Stuart *et al.*, 2006).

1.2.2 Benefits of *Photorhabdus* and *Xenorhabdus* for *Heterorhabditis* and *Steinernema*

Photorhabdus and *Xenorhabdus* employ a range of mechanisms to kill the host and provide an accessible supply of nutrients for nematode development and reproduction. The bacteria face a number of challenges: firstly from the insect immune system, secondly from the endogenous microbial community associated with the insect and finally from exogenous bacterial and fungal organisms and scavenging insects. Colonisation of the insect is very rapid, for example *Photorhabdus luminescens* C9 can multiply to more than 1 x 10^9 cells per insect from an initial bacterial load of less than 500 cells per insect in 24 hours following nematode infection in the wax moth *Galleria mellonella* (Hu & Webster, 2000). The ability of the entomopathogenic bacteria to outgrow their competitors in the insect cadaver may be a factor in the lack of putrefaction of the cadaver (Jarosz, 1996), which is characteristic of successful colonisation of the insect by the nematode-bacterium complex.

A number of different types of compounds have been identified which have been shown to have a role in the complex interactions between bacteria, nematode and insect. For example culture broth from *P. luminescens* was shown to have haemolysin, phospholipase C, protease, lipase and nuclease activity (Bowen *et al.*, 1998), while *P. temperata* M1021 was shown to have catalase, protease and lipase

activity (Jang *et al.*, 2012). The genomes of *P. luminescens* TT01 (Duchaud *et al.*, 2003), *Xenorhabdus nematophila* ATCC19061 and *Xenorhabdus bovienii* SS-2004 (Chaston *et al.*, 2011), have been sequenced and this has led to the identification of genes predicted to be involved in the pathogenicity of these bacteria. Among the gene products in the *P. luminescens* TT01 sequence are large numbers of proteases, haemolysins, toxins, lipases and antibiotics and while similar genes have been identified in the *Xenorhabdus* sequences there are also differences in, for example, antibiotic and toxin production (Chaston *et al.*, 2011).

Inhibition of phospholipase A_2, resulting in a reduced immune response in the insect, has been shown for *P. temperata* subsp. *temperata*, *P. luminescens* subsp. *luminescens, X. poinarii, X. beddingii,* and *X. nematophila* (Kim *et al.*, 2005). Both *Photorhabdus* and *Xenorhabdus* produce lipases which act in a variety of ways. In *X. nematophila* lipase XlpA contributes to nematode production (Richards & Goodrich-Blair, 2010), while secreted lipase from *P. temperata* strain K122 was shown to be toxic to *G. mellonella* (Clarke & Dowds, 1995). Proteases cause general degradation of the insect cadaver, releasing nutrients for the nematode development. They can also inhibit specific components of the insect immune system, such as lysozyme, cecropin and attacin. Proteases have been identified from *P. luminescens* (Abu Hatab *et al.*, 1998; Jarosz, 1998), and from *Xenorhabdus poinarii* (Abu Hatab *et al.*, 1998) and *X nematophila* (Caldas *et al.*, 2002; Park *et al.*, 2007).

Bacterial enzymes classed as haemolysins are toxic proteins that cause cell lysis and are detected on blood agar by the appearance of a transparent zone around the colony. A number of haemolysins, including α-Xenorhabdolysin (C1) from *X. nematophila* strain F1, as well as haemolysins from *Xenorhabdus japonica*, *X. bovienii* and *Xenorhabdus beddingii*, all of which showed cytolytic activity against

insect haemocytes, have been identified (Brillard *et al.*, 2001). Other haemolysins produced by the symbiotic bacteria include XhlA, identified from *X. nematophila* (Cowles & Goodrich-Blair, 2005) and PhlA haemolysin, identified from *P. luminescens* strain W14 (Brillard *et al.*, 2002). In a study of three strains of *P. luminescens,* NC-19, Hm and W-14, all isolated from *H. bacteriophora* nematodes, high molecular weight proteins with insecticidal properties were identified which were toxic orally or by injection to the larvae of tobacco hornworm, *Manduca sexta,* a model lepidopteran insect frequently used in EPN studies, (Bowen & Ensign, 1998). Four toxin complexes (Tc) have been identified from *P. luminescens* culture supernatant and two of the complexes, Tca and Tcd, showed oral toxicity to insects (ffrench-Constant & Bowen, 2000). Similar compounds are also present in *Xenorhabdus* spp. as Tc-like genes have been identified in *X. nematophila* strain Xu (Waterfield *et al.*, 2001), and toxin A24tox has been identified from *X. nematophila* strain A24 (Brown *et al.*, 2004).

A wide range of antibiotic activity has been reported from both *Photorhabdus* and *Xenorhabdus*, with members of both genera being able to inhibit a range of test organisms (Akhurst, 1982; Boemare & Akhurst, 1988). Common test organisms used by Akhurst (1982) and others (Simões & Rosa, 1996; Gualtieri *et al.*, 2009; Lanois *et al.*, 2010) include *Bacillus subtilis, Escherichia coli, Micrococcus luteus* and *Staphylococcus aureus.* These and other microorganisms form part of the microbial community in the insect gut in Lepidoptera such as *M. sexta* (van der Hoeven *et al.*, 2008) and the gypsy moth, *Lymantria dispar* (Broderick *et al.*, 2004), while Coleoptera, many of which are important plant pests, tend to have a more diverse microbial community as in for example the European cockchafer, *Melolontha melolontha*, (Egert *et al.*, 2005), and weevils, *Otiorhynchus* spp.,

(Hirsch *et al.*, 2012). Antibiotic compounds that have been identified include for example 3,5-dihydroxy-4-isopropylstilbene (ST) and anthraquinones (AQ) isolated from *P. luminescens* (*X. luminescens*) strain HK (Richardson *et al.*, 1988) and *P. luminescens* strain C9 (Li *et al.*, 1995). Stilbenes occur in plants and *Photorhabdus luminescens* is the only known non-plant stilbene producer. Stilbenes have antibacterial, antifungal and insecticidal activity and may also function as signal molecules in nematode growth and development (Joyce *et al.*, 2008). Anthraquinones have antibacterial properties and also deter ants and birds from scavenging on the host cadaver (Pankewitz & Hilker, 2008). Among the antibiotic compounds produced by *Xenorhabdus* are nematophin (Li *et al.*, 1997), which has antibacterial and antifungal activity and two xenocoumacins (McInerney *et al.*, 1991), both of which had antibacterial activity and one of which also had antifungal activity. Other *Xenorhabdus* antibiotic compounds include xenorhabdins, xenorxides, benzylideneacetone, iodinine, phenethylamides and indole derivatives, reviewed in Bode (2009).

Another important category of compounds produced in both genera are the bacteriocins, proteins with antibiotic action against closely related strains or species of the same bacteria. Both interspecific and intraspecific activity as well as activity against the other genus was observed in different species and strains of *Xenorhabdus* and *Photorhabdus* isolated from four different *Steinernema* species and three different species of *Heterorhabditis*, respectively, and members of each genus showed activity against the other (Akhurst, 1982). A number of bacteriocins have been identified including lumicins, isolated from four different *Photorhabdus* species (Sharma *et al.*, 2002), and xenorhabdicin from *X. nematophilus* (Thaler *et al.*, 1995).

In addition to the insect-pathogenic compounds the bacteria also produce compounds which have nematicidal properties. Secondary metabolites, 3,5-dihydroxy-4-isopropylstilbene (ST) and indole, from *P. luminescens* strain MD, were found to be nematicidal to some species of nematodes including some EPN and plant-parasitic nematode *Meloidogyne incognita* (Hu *et al.*, 1999).

There is considerable variability in the contribution of the bacteria to insect death and nematode reproduction in both genera. Not all species of symbiotic bacteria are pathogenic to insects, for example *X. poinarii* was unable to kill *G. mellonella* larvae when whole cells were injected into the insect haemocoel (Akhurst, 1986) and neither whole cells nor liquid culture supernatant of *X. japonica* were pathogenic to *Spodoptera litura* when injected into the insect haemocoel (Yamanaka *et al.*, 1992). The mortality of *M. sexta* injected with Tc toxins from three *P. luminescens* strains varied considerably with the Tc toxins from one strain producing 30% mortality compared to 100% mortality for the other two strains (Bowen *et al.*, 1998), indicating the variability of these compounds among different strains of the same species of bacteria. Much of the investigation of bacterial products has focused on a small number of species and strains so it is unclear how widespread the mechanisms discussed here are among the members of each genus.

1.2.3 Benefits of *Heterorhabditis* and *Steinernema* for *Photorhabdus* and *Xenorhabdus*

The nematode IJ provides a protective environment for the symbiont in the soil and transports it in to the insect, giving the bacteria access to the insect haemolymph (Forst & Clarke, 2002). The bacteria can be isolated from the nematode or an infected insect and cultured on suitable media in the laboratory. The bacteria are transported into the insect body cavity by the nematode. Once inside the insect the

symbiotic bacteria are released and start to multiply. Both *Heterorhabditis* and *Steinernema* have been shown to be able to evade the insect immune system through a number of mechanisms. Three species of *Heterorhabditis*, *H. bacteriophora, H. downesi* and *H. gerrardi*, were able to reduce the ability of haemocytes in *M. sexta* larvae to phagocytose their respective bacterial symbionts (Eleftherianos *et al.*, 2010). All three species nematodes with or without their symbiotic bacteria caused a reduction in phenoloxidase (PO) activity, nodulation and haemocyte aggregation, all key components of the insect immune response, indicating that the nematode had a role in this reduction. *Heterorhabditis marelatus* IJs were able to avoid encapsulation in Colorado potato beetle, *Leptinotarsa decemlineata,* (Armer *et al.*, 2004). Similarly, Brivio *et al*. (2002) found that *S. feltiae* strain UK caused a reduction in PO activity in *G. mellonella* by inhibiting haemolymph protease activity, thus interfering with the PO activation pathways, and showed that this was due to interactions with LPS-like molecules from the nematode cuticle and LPS-binding proteins in the insect haemolymph.

Heterorhabditis and *Steinernema* are also able to destroy antimicrobial compounds induced on infection in insects. In *G. mellonella*, *Heterorhabditis bacteriophora* secreted an extracellular proteinase that was shown to be active against cecropin-like antibacterial peptides from *G. mellonella* and cecropin B from *Hyalophora cecropia* (Lepidoptera) (Jarosz, 1998). S*teinernema carpocapsae* (*Neoaplectana carpocapsae*) strain MEX was able to destroy proteins of the immune system of *H. cecropia* which were shown to be active against the nematode's symbiont, *X. nematophila* (Gotz *et al.*, 1981). A transcript analysis of IJs of *H. bacteriophora* (strain Az29 and strain Az36), indentified a putative surface antigen with similarity to surface antigens in the parasitic nematodes *Trichinella spiralis, Necator americanus* and *Ancylostoma*

caninum, which were found to be involved in the ability of the parasite to evade the host immune response (Hao *et al.*, 2012). A similar analysis of *S. carpocapsae* strain Breton found significant numbers of genes predicted to encode immune suppression proteins such as proteases (Hao *et al.*, 2010). Differences occur in proteolytic abilities of different strains of the same nematode; two axenic strains of *S. carpocapsae* (Breton and Az27) produce different rates of mortality in *G. mellonella* and the more virulent strain (Breton) had a higher rate of protease activity, possibly related to toxicity (Simões *et al.*, 2000). Table 1.1 summarises some of the characteristics distinguishing *Heterorhabditis* and *Photorhabdus* from *Steinernema* and *Xenorhabdus* spp.

Table 1.1: Some of the characteristics that distinguish *Heterorhabditis* and *Photorhabdus* from *Steinernema* and *Xenorhabdus* species.

Nematode	*Heterorhabditis* (19 species described by 2012)	*Steinernema* (75 species described by 2012)
Infective juveniles	free living in soil, non-feeding, has circular tooth, develop from eggs laid by female or via *endotokia matricida*, protects symbiont in soil, transports symbiont into insect, reduces insect immune system response to symbiont, destroys insect antimicrobial compounds	free living in soil, non-feeding, no circular tooth, produce proteases, develop from eggs laid by female or via *endotokia matricida*, protects symbiont in soil, transports symbiont into insect, reduces insect immune system response to symbiont, destroys insect antimicrobial compounds
First generation adults	Hermaphrodites	Males and females
Symbiotic bacteria	*Photorhabdus* (3 species and 12 subspecies described by 2012)	*Xenorhabdus* (21 species described by 2012)
Symbiotic relationship	Mutualistic with nematode host, nematode may be associated with more than one species or subspecies of bacteria. Parasitic with insect hosts	Mutualistic with nematode host, bacteria may be associated with more than one species of nematode. Parasitic with insect hosts
Bacterial location	Free in the intestine, released by regurgitation	In specialised vesicle in intestine, released via the anus
	Biochemical activity*	
Bioluminescence	+	-
catalase	+	-
phospholipase	Phospholipase A activity Phospholipase C activity	Phospholipase A activity
proteases	+	+
lipases	Lip-1	XlpA
nucleases	+	+
haemolysin	PhlA haemolysin	Xenorhabdolysin
toxins	Tca, Tcd	A24tox
Pigments	Anthraquinones	-
Antibiotics	Hydroxystilbenes Indoles	Xenomacoumacins Xenorhabdins Xenorxides Indoles
Bacteriocins	Lumicins	Xenorhabdicins
Insecticidal activity	Hydroxystilbene	Xenorhabdin 2
Nematicidal activity	Hydroxystilbene Indoles	Nematophin Indoles

***** not an exhaustive list

+ activity reported for at least some strains

- no activity reported

1.2.4 EPN Diversity

There are many more species of *Steinernema* than *Heterorhabditis* and *Steinernema* tend to be more abundant in the environment (Hominick, 2002). New species of both genera of nematode and their associated symbionts are being described on an ongoing basis; in 2006 over 40 species of *Steinernema* and over 10 species of *Heterorhabditis* had been recognised (Adams *et al.*, 2006). By 2012 this had increased to more than 60 species of *Steinernema* and more than 20 species of *Heterorhabditis,* (Campos Herrera *et al.*, 2012; Lewis & Clarke, 2012) with 21 species of *Xenorhabdus* and three species and 12 subspecies of *Photorhabdus (Tailliez et al., 2006; Tailliez et al., 2010).* Although *Heterorhabditis* and *Steinernema* are both entomopathogenic nematodes they are not closely related (Blaxter *et al.*, 1998; Blaxter, 2011; Chaston *et al.*, 2011) (Figure 1.2). On the other hand, *Photorhabdus* and *Xenorhabdus* are sister groups within the family *Enterobacteriaceae* (Tailliez *et al.*, 2010; Chaston *et al.*, 2011) (Figure 1.2). The ancestors of *Heterorhabditis* and *Steinernema* each separately formed relationships with the gram-negative bacteria and through a process of convergent evolution gave rise to the mutualistic associations between *Heterorhabditis* and *Photorhabdus* and *Steinernema* and *Xenorhabdus* that we see today (Poinar, 1993).

The first entomopathogenic nematode to be described was *Steinernema kraussei (Aplectana kraussei*) by Steiner in 1923 (Poinar & Grewal, 2012). *Heterorhabditis bacteriophora* Poinar was first described in 1976 (Poinar, 1976). The first description of the symbiotic bacteria associated with either genus was of *X. nematophila* (*Achromobacter nematophilus*) from *S. Carpocapsae* in 1965 by Poinar and Thomas (Poinar & Grewal, 2012), and the first description of *Photorhabdus* was that of *P. luminescens* (*X. luminescens*) from *H. bacteriophora* (Poinar, 1976).

Heterorhabditis downesi Stock, Griffin & Burnell, 2002, was first isolated from grassland in the southeast of Ireland (Griffin *et al.*, 1991; 1994). It was originally called *Heterorhabditis* "Irish Group" but was later described as a new species on the basis of morphological characteristics, with K122 considered as the type isolate (Stock *et al.*, 2002).

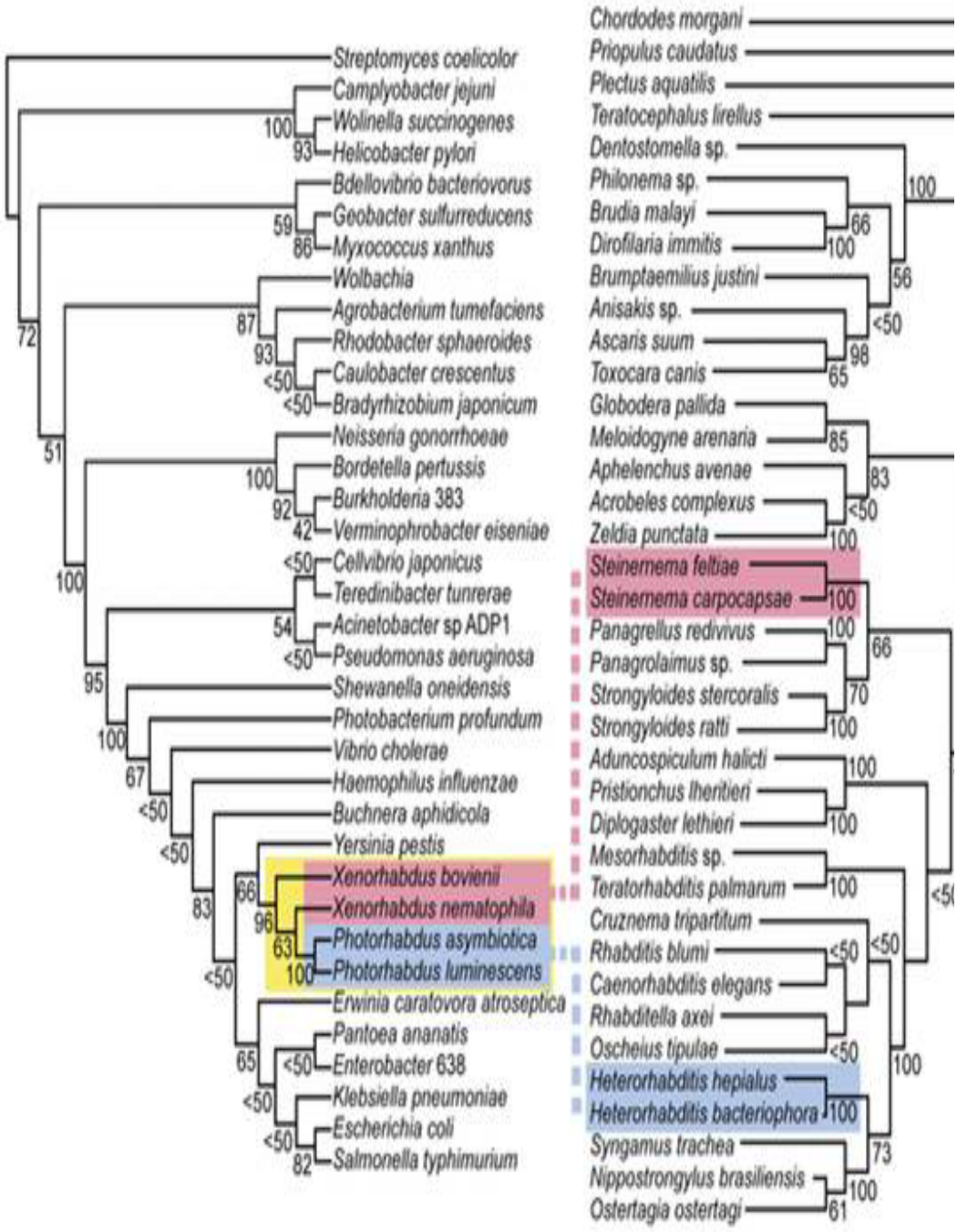

Figure 1.2: A comparison of the relationships between *Heterorhabditis* and *Steinernema* and their symbiotic bacteria *Photorhabdus* and *Xenorhabdus* based on 16S RNA (bacteria) and 18S inter-ribosomal RNA (nematodes). From Chaston *et al.* (2011).

In 1993 *X. luminescens* was reclassified as *P. luminescens* on the basis of low DNA relatedness to other *Xenorhabdus* spp. as well as phenotypic characterisation (Boemare *et al.*, 1993a). A major reclassification of the genus *Photorhabdus* occurred in 1999 (Fischer-Le Saux *et al.*), with the description of three species and four subspecies: *P. luminescens* subsp. *luminescens*; *P. luminescens* subsp. *akhurstii*; *P. luminescens* subsp. *laumondii*; *P. temperata*; *P. temperata* subsp. *temperata*; and *P. asymbiotica*. In that study bacteria isolated from *H. megidis* strains XINach (type strain) from Russia, *H. megidis* strains HL81 and HW79 from the Netherlands and an Italian *Heterorhabditis* species, strain ItH211 were identified as *Photorhabdus temperata* subsp. *temperata*. Nielsen & Lübeck (2002) used universally primed-PCR (UP-PCR) and DNA cross-hybridisation to characterise the symbionts of *Heterorhabditis* species and identified strain K122 (*H. downesi* K122, Ireland), strain HSH2 (*H. megidis*, Germany), strain HL81 (*H. megidis*, The Netherlands) and strain S172 (*H. megidis*, Denmark) as *P. temperata* subsp. *temperata.* Also Ehlers & Niemann (1998), using a primer specific to variation within the variable region of the 16s rRNA, found that the bacterial symbiont of *H. downesi* strains K122 and M145 from Ireland was the same as the bacterial symbiont of *H. megidis* strains HSH1, HSH2, HSH3, HN1 from Germany and HL81, HF85, FR86 and others from The Netherlands as well as *H. megidis* isolates from Finland, Switzerland and USA.

The *gyrB* gene has been suggested as a more useful tool than 16S rRNA to group bacterial isolates into subspecies and species because it has a higher frequency of nucleotide substitution (Yamamoto & Harayama, 1995; Dauga, 2002). In an analysis of *P. asymbiotica* Akhurst et al (2004) found that the use of 16S rRNA grouped subspecies into recognised groups but failed to place these within the correct species groups, on the other hand, use of *gyrB* sequences produced groups as previously

described by Fischer-Le Saux (1999) who used DNA-DNA hybridisation and PCR-RFLP methods. The *gyrB* gene has also been used as part of a multigene approach (Peat *et al.*, 2010; Tailliez *et al.*, 2010). This multigene approach provides more robust phylogenetic trees than any of the trees obtained from analysis of a single sequence including *gyrB* and 16S rRNA, and a polyphasic approach using multiple genes for sequence analysis as well as phenotypic and biochemical characterisation is recommended for detailed species description (Adams *et al.*, 2006). However, for the identification at subspecies level the analysis of the *gyrB* gene is considered sufficient (Akhurst *et al.*, 2004; Maneesakorn *et al.*, 2011). The phylogenetic relationship of a number of *Photorhabdus* and *Xenorhabdus* species is shown in Figure 1.3 (Tailliez *et al.*, 2010).

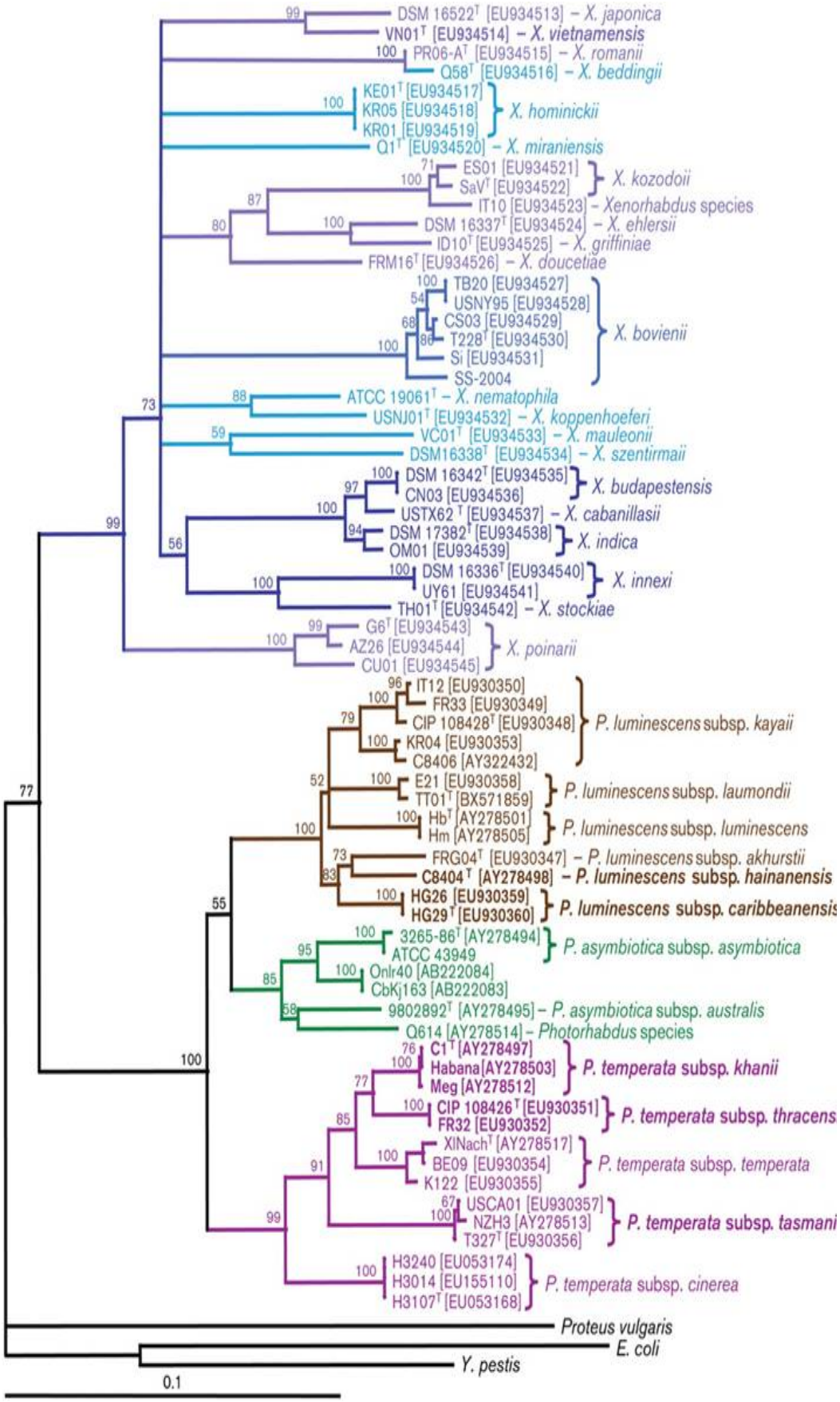

Figure 1.3: The phylogenetic relationship of *Xenorhabdus* species and *Photorhabdus* species based on gyrB sequences. From Tailliez *et al.* (2010).

As with bacterial identification, molecular techniques are now routinely used to identify nematode species. Among the most widely used genetic markers are the ribosomal genes (Stock, 2009). These occur in high copy number and are comprised of a number of units that evolve at different rates and contain both variable and conserved regions. This means that different sections of the genes can be analysed depending on the objectives of a particular study. The internal transcribed spacer 1 (ITS1) unit has been used by many groups to identify nematodes at species and intraspecific level (Emelianoff *et al.*, 2008b; Malan *et al.*, 2008; Nguyen *et al.*, 2008; Maneesakorn *et al.*, 2011). The widespread use of the ITS1 region has resulted in a substantial database of sequences being available for phylogenetic analysis of new isolates. The phylogenetic relationship of a number of *Heterorhabditis* species is shown in Figure 1.4 (Nguyen *et al.*, 2008). This shows that *H. downesi* and *H. Megidis* are sister taxa.

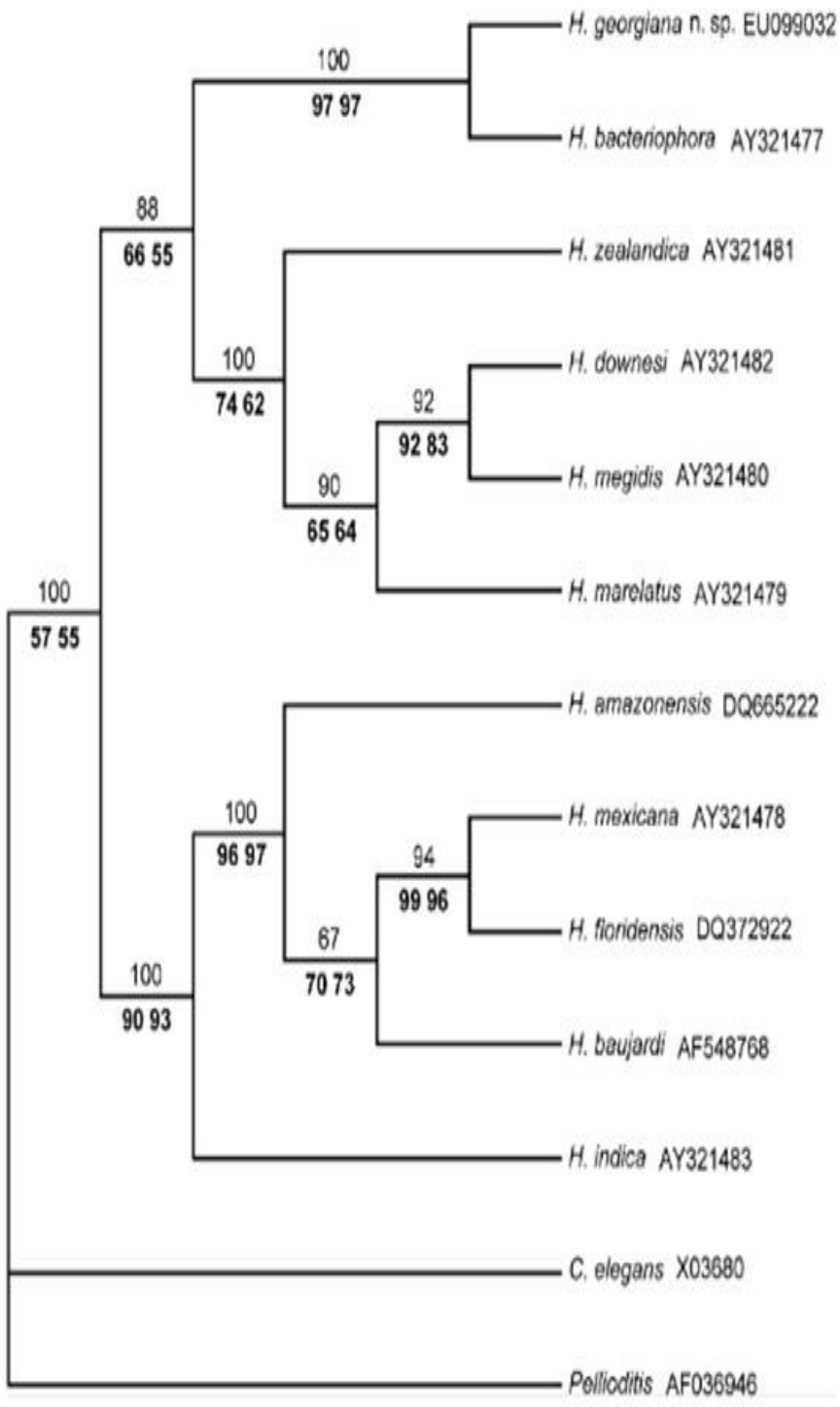

Figure 1.4: Phylogenetic relationship of *Heterorhabditis* species based on ITS rDNA sequences. From Nguyen *et al*. (2008)

1.2.5 Association of *Heterorhabditis* with *Photorhabdus*

The conventional view of the EPN nematode-bacteria symbiosis has been that each nematode species carries a single bacterial species (Adams *et al.*, 2006). However, the relationships between *Heterorhabditis* spp. and *Photorhabdus* spp. are more complex (Table 1.2). *Heterorhabditis downesi* is associated with *Photorhabdus temperata* subspecies *cinerea (P.t. cinerea)* and *Photorhabdus temperata* subspecies *temperata (P.t. temperata)*. *Heterorhabditis bacteriophora* is associated with three *P. luminescens* subspecies and two *P. temperata* subspecies, and *H. megidis* is associated with three *P. temperata* subspecies. These different *Heterorhabditis-Photorhabdus* associations tend to occur at geographically distant locations, for example *H. megidis-P.t. temperata* occurs in Europe and *H. megidis-P.t. khanii* occurs in the USA; and *H. bacteriophora-P. luminescens* subsp. *kayii* (*P.l. kayii*) occurs in Italy and *H. bacteriophora-P. luminescens* subsp. *laumondii* (*P.l laumondii*) occurs in Australia (Tailliez *et al.*, 2010). Where more than one *H. bacteriophora-Photorhabdus* association has been recorded from the same region there has been evidence of genetic variation between the nematode strains. Molecular analysis of *H. bacteriophora* isolates from southern France showed that two strains of *H. bacteriophora* carrying different subspecies of *P. luminescens* had differences in their ITS region (Emelianoff *et al.*, 2008b). In the relationship between *Steinernema* and *Xenorhabdus* there is a broadly one to one relationship with a single nematode species being associated with only one bacterial species, although a single bacterial species can be carried by more than one species of nematode. Table 1.3 lists currently described *Steinernema-Xenorhabdus* associations.

Table 1.2: List of described species of nematodes in the genera *Heterorhabditis* and their *Photorhabdus* bacterial symbionts.

Nematode	Bacteria
H. amazonensis	unknown
H. atacamensis	unknown
H. bacteriophora	*P. luminescens* subsp. *caribbeanensis, P. luminescens* subsp. *kayaii, P. luminescens* subsp. *laumondii, P. luminescens* subsp. *luminescens, P. temperata* subsp. *khanii*, *P. temperata* subsp. *thracensis*.
H. baujardi	unknown
H. brevicaudis	unknown
H. downesi	*P. temperata* subsp. *cinerea*, *P. temperata* subsp. *temperata*
H. floridensis	unknown
H. georgiana	*P. luminescens* subsp. *akhurstii*
H. gerradi	*P. asymbiotica* Kingscliff
H. heliothidis	*P temperata* subsp. *khanii.*
H. indica	*P. luminescens* subsp. *akhurstii*
H. marelatus	*P. temperata* subsp. *tasmaniensis*
H. megidis	*P. temperata* subsp. *cinerea*, *P. temperata* subsp. *khanii, P. temperata* subsp. *temperata*,
H. mexicana	unknown
H. poinari	unknown
H. safricana	unknown
H. sonorensis	*P. luminescens* subsp. *sonorensis*
H. tayserea	unknown
H. zealandica	*P. temperata* subsp. *tasmaniensis*
Heterorhabditis sp.	*P. luminescens* subsp. *caribbeanensis, P. luminescens* subsp. *hainanensis, P. luminescens* subsp. *kayaii, P. temperata* subsp. *khanii, P. temperata* subsp. *thracensis*

Data in the table is drawn from Tailliez *et al* (2010), Campos Herrera *et al*. (2012), Lewis & Clarke (2012) and Orozco (2012)

Table 1.3: List of described species of nematodes in the genera *Steinernema* and their *Xenorhabdus* bacterial symbionts.

Nematode	**Bacteria**	**Nematode** (continued)	**Bacteria** (continued)
S. abasi	*X. indica*	*S. kushidai*	*X. japonica*
S. aciari	unknown	*S. leizhouense*	unknown
S. affine	*X. bovienii*	*S. litorale*	unknown
S. akhursti	unknown	*S. loci*	unknown
S. amazonense	unknown	*S. longicaudum*	*X. beddingii*
S. anatoliense	*X. nematophilia*	*S. masoodi*	unknown
S. apuliae	*X. kozodoii*	*S. minutum*	unknown
S. arenarium	*X. hominickii*	*S. monticolum*	*X. hominickii*
	X. kozodoii	*S. neocurtillae*	unknown
S. ashiuense	unknown	*S. oregonense*	*X. bovienii*
S. asiaticum	unknown	*S. pakistanense*	unknown
S. australe	unknown	*S. puertoricense*	*X. romanii*
S. backanense	unknown	*S. pui*	unknown
S. beddingi	unknown	*S. puntauvense*	*X. bovienii*
S. bicornutum	*X. budapestensis*	*S. rarum*	*X. szentirmaii*
S. boemarei	*X. kozodoii*	*S. riobrave*	*X. cabanillasii*
S. brazilense	unknown	*S. ritteri*	unknown
S. carpocapsae	*X. nematophilia*	*S. robustispiculum*	unknown
S. caudatum	unknown	*S. sangi*	*X. vietnamensis*
S. ceratophorum	*X. budapestensis*	*S. sasonense*	unknown
S. cholashanense	unknown	*S. scapterisci*	*X. innexi*
S. colombiense	unknown	*S. scarabaei*	*X. koppenhoeferi*
S. costaricense	unknown	*S. seemae*	unknown
S. cubanum	*X. poinarii*	*S. serratum*	*X. ehlersii*
S. cumgarense	*unknown*	*S. siamkayai*	*X. stockiae*
S. diaprepesi	*X. doucetiae*	*S. sichuanense*	*X. bovienii*
S. eapokense	unknown	*S. silvaticum*	unknown
S. everestense	unknown	*S. tami*	unknown
S. feltiae	*X. bovienii*	*S. texanum*	unknown
S. glaseri	*X. poinarii*	*S. thanhi*	unknown
S. guangdongense	unknown	*S. thermophilum*	*X. indica*
S. hebeiense	unknown	*S. tielingense*	unknown
S. hermaphroditum	*X. griffiniae*	*S. unicornum*	unknown
S. ichnusae	*unknown*	*S. websteri*	*X. nematophilia*
S. intermedium	*X. bovienii*	*S. weiseri*	*X. bovienii*
S. jollieti	*X. bovienii*	*S. xechuanense*	unknown
S. karii	*X. hominickii*	*S. yirgalemense*	unknown
S. khoisanae	unknown	*Steinernema* sp.	*X. miraniensis*
S. kraussei	*X. bovienii*	*Steinernema* sp.	*X. mauleonii*

Data in the table is drawn from Tailliez *et al.* (2010), Campos Herrera *et al.* (2012), Lewis & Clarke (2012).

In symbiotic systems the holobiont (the host and its symbiotic bacteria) might reasonably be considered as the unit of selection in evolution (Feldhaar, 2011). Both partners benefit from acquisition of new traits from the other (Moran, 2007). Thus we might expect to find evidence of co-evolution between species of nematode and their associated bacteria. In a study of five *Heterorhabditis* species and their associated bacteria, a cophylogenetic analysis of 21 *Heterorhabditis-Photorhabdus* associations found cospeciation, duplication and host switching events, however, the analysis was limited to ITS and gyrB sequences only and did not include all the recognised *Heterorhabditis* species and *Photorhabdus* subspecies as the bacterial or nematode partner had not always been identified and there was no overall support for co-evolution (Maneesakorn *et al.*, 2011). A cophylogenetic analysis of 30 *Steinernema-Xenorhabdus* associations, using a multilocus approach, also found evidence of cospeciation, host-switching and sorting events but no overall support for co-evolution between *Steinernema and Xenorhabdus* (Lee & Stock, 2010).

1.2.6 Specificity of the nematode-bacteria symbiosis

The ability of the symbiont of one nematode species to support the growth of a different nematode species is limited. The specificity of the symbiotic relationship functions at three levels in EPN: IJ recovery, which prompts the IJs to exit the dauer stage, the supply of nutrients to support nematode growth and development, and the colonisation of the nematode by the symbiont (Grewal *et al.*, 1997).

Recovery of both *Heterorhabditis* and *Steinernema* is prompted by food signals from the symbiotic bacteria (Grewal *et al.*, 1997; Han & Ehlers, 1998). In the case of *P. luminescens* strain NP1/1, a small heat-stable molecule from *M. sexta* haemolymph was also shown to be involved in inducing IJ recovery (Ciche & Ensign, 2003). Nematodes of both genera are able to develop on non-natural symbiotic bacteria *in*

vitro (Gerritsen & Smits, 1993, 1997; Grewal *et al.*, 1997). It has been shown that *H. bacteriophora* was able to develop and retain the symbionts from two strains of *H. megidis* (Han & Ehlers, 2001). In some cases specific growth requirements have been uncovered. For example *H. downesi* K122 cannot grow on *P.t. temperata* K122 *exbD* mutants that are deficient in the ability to scavenge iron in the insect haemolymph whereas iron is not a requirement for the growth of *H. bacteriophora* on its symbiont, *P. luminescens* although it is required for virulence by the *H. bacteriophora-P. luminescens* complex (Watson *et al.*, 2010). *Steinernema* are less dependent on their natural symbiont for growth and development and will grow without a symbiont (Sicard *et al.*, 2003; Mitani *et al.*, 2004).

1.2.7 Colonisation

A number of specific factors regulating symbiosis in the nematode-bacterium relationship have been elucidated as a result of the sequencing of the genome of *P. luminescens* strain TT01 (Duchaud *et al.*, 2003). The purine metabolic gene, *purL*, has been shown to play a role in the persistence of *P. temperata* in its host *H. bacteriophora* GP11, possibly through biofilm formation (An & Grewal, 2011b). In *P. luminescens* TT01 the *pbgPE1* gene, part of the *PbgPE* operon has been shown to have a role in the ability of the bacteria to colonise the nematode gut (Bennett & Clarke, 2005). *Photorhabdus luminescens* strain TT01 has been shown to switch from a P form, associated with pathogenicity in the insect host, to an M form, associated with colonising the nematode, through the expression of *mad* genes that regulate the expression of fimbriae on the cell surface, and *mad* deficient mutants were unable to colonise the *H. bacteriophora* host (Somvanshi *et al.*, 2010; Somvanshi *et al.*, 2012). Other *Photorhabdus* genes involved in IJ colonisation in the *H. bacteriophora–P. luminescens* strain TT01 nematode-bacterium complex have

been identified through screening colonisation incompetent TT01 mutants, and include genes in the *pbgPE* operon, *galE*, *galU, proQ*, *asmA*, and *hdfR,* and the *pbgPE, galE*, and *galU* genes are predicted to be involved in the production of LPS by the bacterium (Easom *et al.*, 2010). While many genes involved in the regulation of the colonisation events have been identified in *P. luminescens* strain TT01 it is unclear how universal these are among all *Photorhabdus* species and it is also likely that nutritional dependencies such as the reliance of *H. downesi* K122 on its symbiont for iron metabolism (Watson *et al.*, 2005) play a major role in the specificity of nematode-bacterium complexes.

Factors regulating symbiosis in the nematode-bacterium relationship have also been found through the sequencing of *X. nematophila* strain ATCC19061 and *X. bovienii* strain SS-2004 genomes (Chaston *et al.*, 2011). For example, two *X nematophila* genes, *nilB* and *nilC* encode membrane-localised proteins required for the colonisation of its host, *S. carpocapsae* (Heungens *et al.*, 2002), and expressing these genes in other *Xenorhabdus* spp. allowed them to colonise *S. carpocapsae* (Cowles & Goodrich-Blair, 2008). In *X. nematophila* strain AN6 Mrx fimbriae are involved in IJ colonisation (Snyder *et al.*, 2011).

1.3 **Ecology and use of EPN in biocontrol**

Entomopathogenic nematodes are known to kill a wide range of insects in the laboratory, although relatively little is known about their natural host range (Poinar, 1979; Peters, 1996). Their broad host range is due to the combined actions of the nematodes and their symbiotic bacteria in overcoming the insect immune mechanisms. Both *Steinernema* and *Heterorhabditis* species have been recovered from naturally infected insects (Peters, 1996; Lewis & Clarke, 2012). At least

thirteen species of *Steinernema* and four *Heterorhabditis* species have been recovered have from such natural infections in the field (Peters, 1996). Insect orders in which these natural infections occurred include Coleoptera, Diptera, Hymenoptera and Lepidoptera. In some cases infected insects were recovered during the course of general surveys for EPN, whilst in other cases the recovery occurred during the course of field trials against particular pest insects. Larvae of the wax moth *G. mellonella* are frequently used to recover EPN in the field as well as from soil samples in the laboratory (Bedding & Akhurst, 1975). Many studies have used insects from orders including Lepidoptera, Coleoptera and Diptera as bait to recover EPN from soil survey sites (Hominick, 2002).

Early work in the laboratory suggested that EPN had a broad host range, for example *S. carpocapsae* has been shown to infect more than 250 insect species in 13 orders (Poinar, 1979; Hodson *et al.*, 2011). However, the ability to kill an insect in the laboratory was not always matched in the field and indeed it was shown that differences occurred between strains as well as between species with no single species or strain being the most effective against all insects (Bedding *et al.*, 1983; Barbosa Negrisoli *et al.*, 2013), and it is probable that no single strain or species would out-perform all others across a range of traits (Shapiro-Ilan *et al.*, 2003). It is now recognised that both generalists and specialists occur. For example, *H. bacteriophora* and *S. carpocapsae* are considered generalists, with *H. bacteriophora* being naturally associated with Coleoptera and Lepidoptera and *S. carpocapsae* being naturally associated with these two orders as well as Hymenoptera and Diptera (Peters, 1996). On the other hand, *S. glaseri* has only been recovered from scarabaeid beetles, while *S. scapterisci* has only been recovered from mole crickets (*Scapteriscus* spp). The ability of EPN to utilise a range of natural host species

would facilitate populations to persist in the soil. There is also evidence that many EPN will infect already dead insects (San-Blas & Gowen, 2008) as well as insects already infected by their own or other EPN species (Hay & Fenlon, 1995). Both of these strategies would help EPN to persist during natural fluctuations in host availability and utilise insects or life stages that they might not normally infect (Půža & Mráček, 2010). As discussed earlier (Section 1.2.2), the more diverse range of microorganisms in the intestinal community of Coleoptera compared to Lepidoptera may partly explain the specificity of action against certain pests (Egert *et al.*, 2003; Hirsch *et al.*, 2012).

EPN display a range of foraging behaviours, ranging from ambushing to cruising (Campbell & Kaya, 2002; Lewis, 2002). Ambush foragers nictate, where the IJ raises most of its body off the soil, this allows them to attach to passing insects (Campbell & Gaugler, 1993). Extreme ambush foragers spend several hours nictating, interspersed with short periods of crawling to relocate on the surface of the search area (Campbell & Gaugler, 1993). Cruise foragers on the other hand never nictate and instead actively search over a wider area for hosts in the soil (Campbell & Gaugler, 1993; Griffin *et al.*, 2005). Some nematodes display an intermediate foraging strategy, with active searching behaviour interspersed with short periods of nictating (Lewis, 2002). Ambush foragers are more likely to find mobile hosts on the soil surface whereas cruise foragers are more likely to find sedentary hosts within the soil (Campbell & Gaugler, 1993) and those nematodes with an intermediate foraging strategy can locate hosts which themselves range for mobile to inactive. Host colonisation is the final event in a series of steps including dispersal, attraction to host habitat, host finding and host penetration and at each step the nematode responds to a range of chemical and physical cues (Lewis *et al.*, 2006). These cues

include CO_2, temperature and host faeces (Gaugler *et al.*, 1980; Byers & Poinar Jr, 1982; Grewal *et al.*, 1993). Hallem *et al.* (2011) found that CO_2 had a major role in attraction of *H. bacteriophora* to *G. mellonella.* Furthermore, plants damaged by below-ground herbivorous insects have been shown to recruit EPN: *H. megidis* responded to the roots of Strawberry plants (Boff *et al.*, 2001) and *Thuja* (van Tol *et al.*, 2001) infested with black vine weevil larvae *Otiorhynchus sulcatus*. Rasmann *et al.* (2005) found that European varieties of maize produce (E)-β-carophyllene in response to root damage caused by western corn rootworm *Diabrotica virgifera virgifera* and *H. megidis* responded to this plant signal, resulting in infection of the rootworm by the nematode.

Effective use of EPN in biological control requires an understanding of the ecology of the target pest and the EPN species. Laboratory and field trials have shown that many EPN are effective against a wide range of insect pests, see for example chapters 7 -17 in Grewal *et al.* (2005) and Georgis *et al.* (2006). For example, *S. carpocapsae*, which performs well against a wide range of hosts in the laboratory including scarab beetles is ineffective against these pests in the field. Gaugler *et al.* (1997) suggest that the reasons for this is that the beetle larvae are sedentary and live below the surface of the soil while the nematode employs an ambusher foraging strategy, waiting near the soil surface for suitable hosts, the nematode is not strongly attracted to the scarab beetle as a host, the scarab immune response is effective against the nematode and if the nematode does succeed in infecting and killing the scarab beetle the rate of nematode reproduction is low. On the other hand, both *H. bacteriophora* and *S. glaseri* appear to be well adapted to scarab pests as they both employ a cruiser foraging strategy and respond to host cues from scarab beetle larvae, they can overcome the scarab immune system and both reproduce well in

scarab beetle larvae. Furthermore, it is also necessary to take biotic interactions with other soil dwelling organisms including endemic EPN, predators and scavengers and the impact on off-target species as well as abiotic factors such as soil type, soil moisture into account in assessing the suitability of a particular nematode species for use in biological control (Kung *et al.*, 1990, 1991; Kaya *et al.*, 2006).

A number of studies have shown that EPN do not tolerate direct desiccation well. Survival in air at reduced RH is usually measured in minutes (Menti *et al.*, 1997; O'Leary *et al.*, 2001) while survival in hygroscopic liquids is also reduced (Mukuka *et al.*, 2010). Survival in soil of *S. carpocapsae* and *S. glaseri* was shown to decrease with decreasing RH with *S. carpocapsae* (All strain) and *S. glaseri* surviving for 2 days and 4 hrs respectively at 25% RH compared to 16 days for both nematodes at 97% RH (Kung *et al.*, 1991). Soil type and soil moisture were also shown to have an effect on the survival and infectivity of EPN, with nematode persistence being different for four species, (*S. scarabaei* AMK001 strain, *S. glaseri* NC1 strain, *H. zealandica* X1 strain and *H. bacteriophora* GPS11 strain), tested in three different soil types at five different water potentials (Koppenhöfer & Fuzy, 2006, 2007). In these studies the authors found that *H. zealandica* persisted better in dry loamy sand (2.3% moisture) conditions than *H. bacteriophora* (72% compared to 40% survival after 56 days). Sandy soil does not retain water well due to its large particle size and Molyneux & Bedding (1984) found that the *H. bacteriophora* D1 and *S glaseri* KG strains did not infect insects in sand with a low moisture content (equivalent to -100kPa water potential) whereas the same strains did infect in loamy sand at the same moisture content (equivalent to -1000 kPa).

1.4 **Distribution**

Entomopathogenic nematodes are found in a wide range of ecosystems from the tropics to temperate, sub arctic and arctic regions and on all continents except Antarctica (Griffin *et al.*, 1990; Hominick, 2002). There is evidence that some *Heterorhabditis* species are found mainly in sandy soils, in both coastal and inland sites (Griffin *et al.*, 1991; 1999; 2000). However this association is not universal as *H. bacteriophora* occurs in loamy soils, for example in the Azores (Rosa *et al.*, 2000). Three *Heterorhabditis* species, *H. bacteriophora, H. downesi* and *H. megidis* have been isolated from Europe. *H. bacteriophora* occurs from Germany (Glare *et al.*, 1993), and France (Emelianoff *et al.*, 2008b) in the north to Turkey (Susurluk *et al.*, 2001; Hazir *et al.*, 2004), Spain (Morton & García-del-Pino, 2009) and Portugal (Valadas *et al.*, 2013) in the south. *Heterorhabditis downesi* appears to have a more northerly distribution occurring in Ireland (Griffin *et al.*, 1991; 1994), the UK (Hominick *et al.*, 1995), Denmark (Griffin *et al.*, 1999), and Hungary (Griffin *et al.*, 1999; Tóth, 2006), while *H. megidis* appears to have a wider distribution than either of the other two *Heterorhabditis* species, occurring in the UK (Hominick *et al.*, 1995), across Northern Europe as far as Estonia (Hominick *et al.*, 1995; Miduturi *et al.*, 1996; Griffin *et al.*, 1999; Sturhan, 1999) and as far south as Greece (Menti *et al.*, 1997). Some *Steinernema* species are found in coastal locations (Emelianoff *et al.*, 2008b) although for example in Spain and the Azores they tend to occur at higher altitudes than *Heterorhabditis* species and are frequently associated with woodlands (Rosa *et al.*, 2000; Campos-Herrera *et al.*, 2007)

To date the only *Heterorhabditis* species that has been detected in Ireland has been *H. downesi* and it occurs only in sandy coastal sites such as sand dunes and sandy grasslands (Griffin *et al.*, 1991; Griffin *et al.*, 1994; Rolston *et al.*, 2005; Rolston *et*

al., 2006). Insects infected with Irish *H. downesi* isolates turn yellow-orange, green-purple or red or yellow and in at least one site two colour variants of *H. downesi* were detected (Rolston *et al.*, 2005). *Steinernema* are more common than *Heterorhabditis* in continental Europe, (reviewed in Hominick (2002)), and this is the case in Ireland also. Three species of EPN have been recorded for Ireland to date: *Heterorhabditis downesi, Steinernema affine* and *S. feltiae*, with a greater frequency of *Steinernema* than *Heterorhabditis* (Blackshaw, 1988; Griffin *et al.*, 1991; Griffin *et al.*, 1994; Rolston *et al.*, 2002; Dillon, 2003).

1.5 **North Bull Island**

North Bull Island lies in Dublin Bay, longitude -6.15, latitude 53.37 (National Grid Reference O 231371) (Figure 1.5). It is of conservation importance nationally and internationally as a site with a diversity of coastal habitats, lagoon, mudflats, saltmarsh, dune complex and beach, which support a wide range of flora and fauna. It is an important overwintering site for waders and wildfowl and contains rare plant and animal species. The site is covered by a number of regional, national, European and other international designations. It has been designated a Special Area Amenity Order site and a Statutory Wildfowl Sanctuary, it contains two Statutory Nature Reserves, it is a Natural Heritage Area, a Special Area of Conservation under the E.U. habitats Directive and a Special Protection Area under the E.U. Birds Directive. It is also a World Biosphere Reserve, a Ramsar Convention site, a Biogenetic Reserve (McCorry & Ryle, 2009).

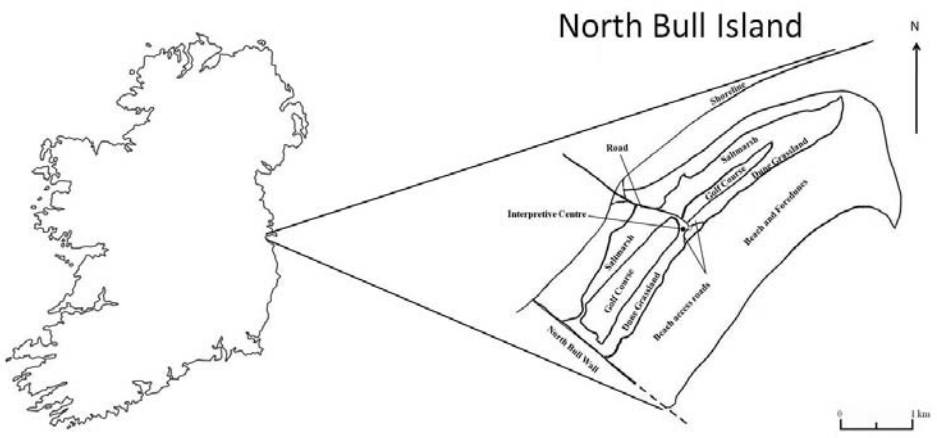

Figure 1.5: North Bull Island lies in Dublin Bay, at the mouth of Dublin Harbour.

The island began as a sandbar, uncovered only at low tide. Construction of the South Wall and North Bull Wall at the mouth of Dublin Port during the 18th and 19th century caused increased sedimentation to the north of the channel and resulted in the formation of the island (Flood, 1977). North Bull Island is approximately 4.85 km long by 0.80 km wide, running parallel to the shore along a southwest to northeast axis. The dunes were reported to have a maximum height of 10.21 m above sea level and an average height of 6 – 7 m in 1977 (Harris, 1977). At the landward side of the island there are extensive salt flats. There are two access routes onto North Bull Island. The first is via a bridge which links the North Bull Wall to the shore. The second is via a causeway constructed in 1964. Construction of the causeway gave rise to the development of mudflats along its northern edge between the island and the shore.

The dune complex consists of 4 main habitat types: embryonic dunes, marram dunes, semi-fixed dunes and fixed dune, each with a characteristic community of plants (Moore, 1977). The first to be encountered as one moves from the sea towards the land are the embryonic dunes, dominated by pioneer species such as *Elytrigia juncea*

and *Leymus arenarius.* The next habitat type is marram dunes where the dominant species is the dune builder *Ammophila arenaria,* which stabilises windblown accumulations of sand. *Elytrigia juncea* disappears as sand accumulates but *L. arenarius* continues in the marram dunes. Thirdly, semi-fixed dunes, with *Agrostis* spp. and *Festuca* spp. appear but *A. arenaria* remains as the dominant grass species. In this habitat type flowering plants appear, including *Hypochaeris radicata, Senecio* spp. and *Leontodon* spp. Finally fixed dunes develop, where *A. arenaria* no longer dominates and grasses include *Poa* spp. An abundant herbiage also develops, including *Anthyllis vulneria, Galium verum, Viola tricolor* as well as an extensive cover of lichens and mosses. Fixed dune habitat dominated by dune grassland species such as *Lolium perenne, Bellis perennis and Trifolium repens* is generally absent due to a number of factors. These include the construction of two golf clubs which occupy up to half the width of the island on the landward side. The habitat in these areas has been greatly modified by the application of fertiliser and regular mowing and is now characteristic of amenity grassland (Moore, 1977). In addition, dune grassland is typically composed of consolidated and flattened dunes which are frequently used for agricultural grazing, this leads to a species-rich close sward (Fossitt, 2000). Absence of grazing and intensive recreational use on North Bull Island has lead to rank grassland with bare areas caused by tramping in the zone immediately bordering the seaward perimeter of the golf courses.

The natural hosts of *H. downesi* and *S. feltiae* on North Bull Island is unknown, however, the dune system supports a very diverse invertebrate fauna, including protozoa, nematodes, annelids, molluscs and crustaceans, however, arthropods comprise over 90 % of the recorded invertebrate fauna for the island (Healy, 1975). A number of studies have been carried out on the arthropod fauna of the dune system

and the saltmarsh (Table 1.4). Grassland generalist insect species make up approximately 50% of the arthropod species recorded from the dunes and dune grassland, while the drier marram-covered dunes are dominated by dune specialists (Speight, 1997; O'Connor & Speight, 2002). In the region between the strand line and the embryonic dunes a number of the dune and grassland species forage at night. Kelp fly, *Coelopa frigida,* (Diptera) which is found in beds of rooting seaweed along the strand line around the coast of Ireland and the UK has also been recorded (Healy, 1975). Over 132 invertebrate species were recorded in a three year period ranging in depth from soil surface to 20 cm in the region between the strand line and the embryonic dunes (Healy, 1975). (See also Speight (1975) for an account of Syrphidae (Diptera) from Ireland, including species occurring on North Bull Island). Rolston (2005) noted the frequent occurrence of the black marram beetle *Otiorhynchus atroapterus* throughout the dune system. In the course of the present study adult *Psilothrix* spp. (Coleoptera) were observed in the front section of the dune system and larvae of the cinnabar moth *Tyria jacobaeae* and adult *O. atroapterus* were observed throughout the dune system (Maher, personal observation).

Table 1.4: Arthropod species associated with zones with the dune system on North Bull Island.

Grassland generalist species		
Species	Order	
Platycheirus clypeatus	Diptera	Speight, 1997
Philaenus spumarius	Hemiptera	Speight, 1997
Neophilaenus lineatus	Hemiptera	Speight, 1997
Myrmica ruginodis	Hymenoptera	Speight, 1997
Bombus muscorum	Hymenoptera	Speight, 1997
Philoscia muscorum	Isopoda	Speight, 1997
Armadillidium vulgar	Isopoda	Speight, 1997
Zygaena filipendulae	Lepidoptera	Speight, 1997
Dune specialist species		
Species	Order	
Dilta hibernica	Archaeognatha	Speight, 1997
Demetrias atricapillus	Coleoptera	Speight, 1997
Dromius melanocephalus	Coleoptera	Speight, 1997
Broscus cephalotes	Coleoptera	Speight, 1997
Phthiria pulicaria	Diptera	Speight, 1997
Sphaerophoria rueppellii	Diptera	Speight, 1997
Philonicus albiceps	Diptera	Speight, 1997
Thereva nobilitata	Diptera	Speight, 1997
Neoleria maritima	Diptera	O'Connor & Speight, 2002
Psammochares plumbeus	Hymenoptera	Speight, 1997
Armadillidium album	Isopoda	Speight, 1997
Embryonic dune species		
Species	Order	
Laemostenus terricola	Coleoptera	Speight, 1997
S*ilpha tristis*	Coleoptera	Healy, 1975
Thanatus rugosus	Coleoptera	Healy, 1975
Coelopa frigida	Diptera	Healy, 1975
Philoscia muscorum	Isopoda	Healy, 1975
Armadillidium vulgare	Isopoda	Healy, 1975

1.6 **Aims of the project**

Two colour variants of the *H. downesi* - *P. temperata* nematode - bacterium complex, designated *yel* and *pur*, have been detected in the sand dunes on North Bull Island (Rolston *et al.*, 2002). Surveys detected a pattern of spatial distribution of the two colour phenotypes in the dune system with *pur* tending to occur towards the front of the dunes (Rolston *et al.*, 2005; Rolston *et al.*, 2006). The main aim of this project was to test the hypothesis that differences in the bacterial symbiont associated with the *H. downesi* nematodes in the front and the rear sections of the dunes were driving the spatial distribution of the nematode – bacterium complexes in the dune system on North Bull Island and to identify the traits responsible.

Specific objectives were to:

- Confirm the distribution of the two colour phenotypes in the dune system on North Bull Island. Identify the nematode and their bacterial symbionts by sequencing the ITS1 (nematode) and gyrB (bacteria) regions of the organisms. Investigate the biochemical and antibiosis profiles of the bacterial colour phenotypes and identify differences between the *yel* and *pur* phenotypes which might help to explain the spatial distribution of the nematode – bacterium complexes (Chapter 2).
- Test the specificity of the nematode – bacterium relationship by investigating whether the nematode associated with each bacterial colour phenotype can carry the other phenotype. Investigate the cost to the nematode of carrying the bacterium by assessing the number of bacteria of each type carried and the influence of each type of bacteria on IJ survival **(**Chapter 3).

- Investigate the adaptation of the bacterial colour phenotypes to a range of hosts. Differences in the ability of the nematode – bacterium combinations to kill and reproduce in a range of host could be due to differences in the ability of the bacterial phenotypes to overcome the insect host immune system and its intestinal microbial community and utilise the host tissue. As the host range of the dune system will vary from front to back differences in host adaptation between the two bacterial colour phenotypes may help to explain the trend for the nematodes carrying the *pur* bacteria to be more prevalent in the front section of the dune system (Chapter 4).
- Investigate the role of the bacterial partner in the adaptation of the nematode – bacterium complex to the harsh abiotic conditions present in the sand dunes by testing the ability of different nematode – bacterium combinations to survive and emerge from desiccated insect hosts. Differences in the ability of the nematode – bacterium complex may also help to explain the trend for the nematodes carrying the *pur* bacteria to be more prevalent in the front section of the dune system where desiccating conditions are more prevalent (Chapter 5).

Chapter 2
Recovery of *Heterorhabditis downesi* from North Bull Island and characterisation of their bacterial symbiont

2.1 **Introduction**

Heterorhabditis downesi are entomopathogenic nematodes which are mutualistically associated with bacteria of the genus *Photorhabdus.* By 2007 at least eleven species of *Heterorhabditis* had been described (Nguyen & Hunt, 2007), and additional species are being described on an ongoing basis (Malan *et al.*, 2008; Nguyen *et al.*, 2008; Lewis & Clarke, 2012). Three species of *Photorhabdus, P. luminescens, P. temperata* and *P. asymbiotica,* have been described, along with a number of subspecies of *P. luminescens,* and *P. temperata* (Tailliez & Boemare, 2009; Tailliez *et al.*, 2010; Orozco, 2012). To date, only a single *Heterorhabditis/Photorhabdus* complex, *Heterorhabditis downesi/Photorhabdus temperata* subsp. *temperata*, has been recorded in Co. Wexford, Ireland. Griffin *et al.* (1991) recorded the occurrence of *H. downesi* at a single site in Ireland. This was a sandy coastal location. A subsequent survey in Ireland and Britain recorded *H. downesi* only at sandy coastal locations, including Portmarnock, Co. Dublin, approximately nine kilometres north of North Bull Island (Griffin *et al.*, 1994). In mainland Europe *H. downesi* has been recorded in Denmark and Hungary (Griffin *et al.*, 1999). In a study of entomopathogenic nematodes on North Bull Island two colour variants of *Photorhabdus* sp. were found to be associated with the nematode *H. downesi,* based on the colour of infected insect cadavers, (Rolston *et al.*, 2005). These were

designated purple (*pur*) and yellow (*yel*). Studies of *H. downesi* in Ireland, Britain and mainland Europe reported the occurrence of four colour phenotypes, red, yellow-orange, green-purple, and grey (Griffin *et al.*, 1994; Tóth *et al.*, 2008). However, the work by the Rolston et al (2005) was the first to report the co-occurrence of two colour phenotypes within the same survey site. The two colour phenotypes appeared to be spatially segregated within the dune system with the purple phenotype tending to dominate in the front 50 m section.

North Bull Island is a low dune-covered island lying just off the coast in the northern half of Dublin Bay (see Section 1.5 for a full description). A number of gradients occur within the sand dune system, for example there is a progression from open sand to closed grassland as one moves from seaward to landward side of the dune system (Jeffrey, 1977). Associated with this gradient in soil cover there is a change from loosely compacted sand which loses moisture quickly to soil with a build-up of organic material both within the soil profile and overlaying the soil surface, which aids moisture retention. The combined effect of the increase in organic material and the leaching of minerals which occurs over time results in a decrease in pH and a decrease in salinity with distance into the dunes. Thus the dune system is comprised of a number of different microhabitats, with different plant and animal communities occurring across the dune system, both above and below the soil surface, with the number and range of species increasing with distance into the dune system, associated with the maturity of the dune system.

In this chapter the continued presence of *H. downesi* in the dune system on North Bull Island will be confirmed by soil sampling. The soil sampling will seek to determine the spatial segregation of the colour phenotypes and investigate whether

the colour phenotypes have a spatial distribution or association with particular habitat type in the dune system.

A molecular approach will be used to confirm the identity of the *Heterorhabditis* sp nematodes on North Bull Island as *Heterorhabditis downesi* (see Section 1.2.4.). Nuclear ribosomal DNA (rDNA) has been widely used in the identification of nematodes (Joyce *et al.*, 1994; Hominick *et al.*, 1997; Powers *et al.*, 1997). DNA sequence analysis of the internal transcribed spacer 1 (ITS1) of the ribosomal genes has proved to be a useful molecular tool for the identification of *Heterorhabditis* at species level (Adams *et al.*, 1998; Maneesakorn *et al.*, 2011).

The symbiotic partners of nematodes of the genus *Heterorhabditis* are entomopathogenic bacteria of the genus *Photorhabdus* (see Section 1.2.5). A polyphasic approach will be employed to characterise the symbiont of *H. downesi*. Molecular analysis of the *gyrB* gene will be used to identify the symbiont (see Section 1.2.4.). This method has been used to investigate the phylogeny of the genus and has proved useful at the intrageneric level to identify closely related species and subspecies (Akhurst *et al.*, 2004; Tóth & Lakatos, 2008; Boemare & Tailliez, 2009; Maneesakorn *et al.*, 2011). Morphological, biochemical and physiological characteristics will be investigated to describe the two colour phenotypes found on North Bull Island. This polyphasic procedure may elucidate differences between the two phenotypes.

The change in habitat discussed above is also accompanied by a change in the community of microorganisms in the soil. Entomopathogenic nematodes also encounter the gut biota of the insects they invade. The entomopathogenic bacteria associated with *Heterorhabditis* and *Steinernema* are known to produce a range of

antimicrobial compounds which are effective in suppressing competition from other microorganisms and nematodes (Derzelle *et al.*, 2002; Ansari *et al.*, 2005; Eleftherianos *et al.*, 2007) (See Section 1.2.2). The antibiotic activity of the two phenotypes will be investigated using a range of bacterial and fungal targets. The hypothesis is that *yel* phenotype will be more effective in this regard as it would be expected to encounter a broader range of antagonistic organisms in the more organic soil in the rear half of the dune system and in the wider range of host insects present in the rear half of the dune system.

The objectives of this chapter are:

i) To investigate the distribution of *H. downesi* in the dune system on North Bull Island and to recover fresh isolates of each colour phenotype for study.

ii) To identify the nematodes and their symbiotic bacteria using molecular techniques.

iii) To look for differences between the *pur* and *yel* bacterial phenotypes, especially in antibiotic production.

2.2 Materials and Methods

2.2.1 Detection of nematodes on North Bull Island

2.2.1.1 Sampling Method

North Bull Island was sampled for entomopathogenic nematodes (EPN) during the period September 2008 to October 2008. Sampling was conducted in three transects. Each transect was 130 m long and ran from the beach across the dunes to the fescue-dominated grassland and consisted of fourteen sampling locations. Transect 1 began 100 m northeast of the more northerly beach access road. Transect 2 was located 500

m northeast of transect 1 and transect 3 was located 500 m southwest of transect 1 (Figure 2.1). The first sampling location in each transect was 2 m above the strand line at the base of the first embryonic dune. The sampling locations were at 10 m intervals running into the dune system along a bearing of 330^{o}, perpendicular to the long axis of the island. Samples (3.7 cm^{3}) were taken to a depth of approximately 10 cm with a bucket type soil corer (Figure 2.2). Thirty soil subsamples were taken at each sampling location, with samples being taken 10 m to either side of the transect along a line perpendicular to the transect. At each sampling location the subsamples were pooled in a plastic bag to make a single sample of approximately 500 g.

In 2012 transect 1 was resampled. On this occasion three indivdual samples were taken to a depth of approximately 10 cm with a bucket type soil corer, with one sample being taken along the transect and one sample being taken 10 m to either side of the transect along a line perpendicular to the transect. Samples were placed in individual 50 ml tubes (Sarstedt, Numbrecht, Germany).

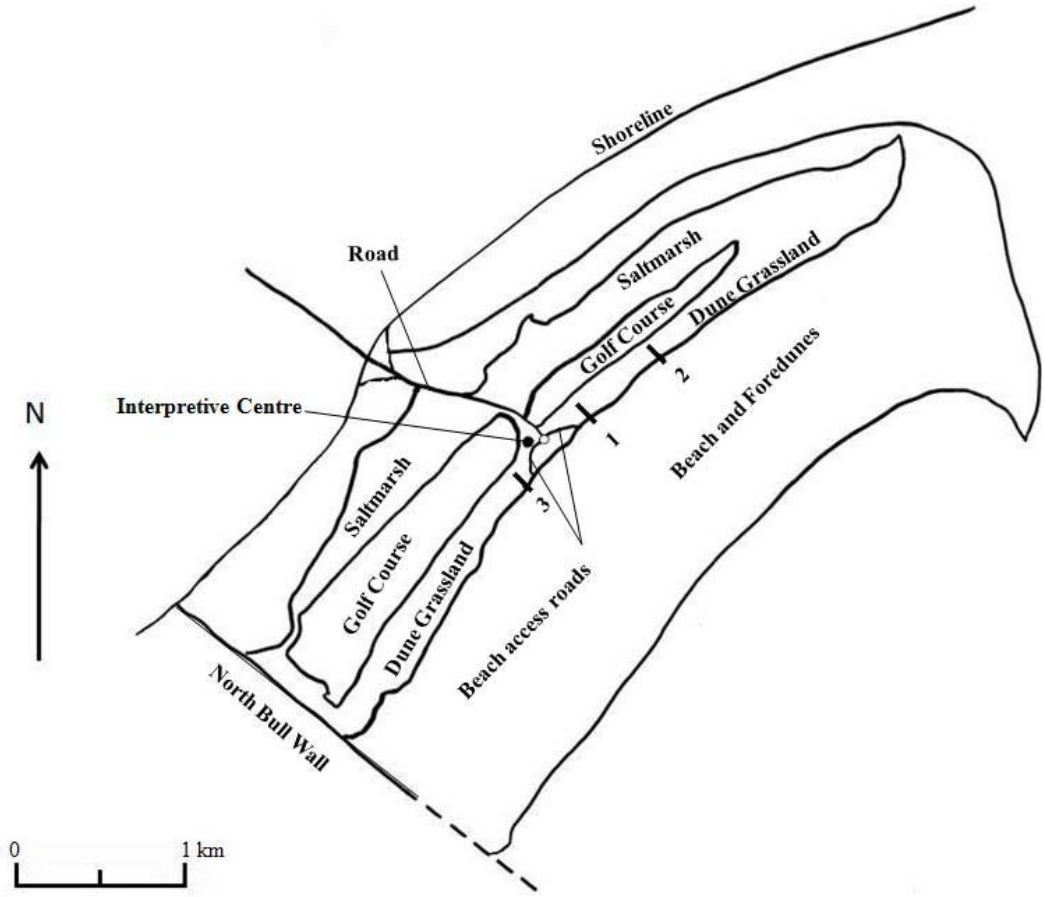

Figure 2.1: North Bull Island. 1, 2 and 3 indicate the location of the transects sampled in this study. Transect 1 and transect 2 overlapped with the areas sampled in 2001 and 2002 in the Rolston (2005) study (see Appendix Figure A. 1)

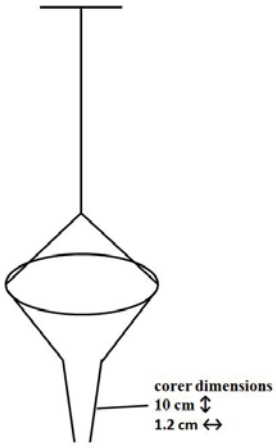

Figure 2.2: Bucket type soil corer used for taking soil samples on North Bull Island. The corer was washed with 70% ethanol and allowed to air dry between sampling locations.

2.2.1.2 Soil baiting

In the laboratory the soil type was classified as sandy or sandy/organic by visual inspection. Each pooled soil sample (500 g approximately) was subdivided and placed into three separate 250 ml plastic food tubs with a diameter of 9 cm ("Econo" type 731, Huhtamaki, Espoo, Finland).

To recover the nematode-bacterium complex each tub was baited with five final instar larvae of the greater wax moth (*Galleria mellonella)*. Wax moths were obtained from the Mealworm Company (Sheffield, UK), and were stored in wood shavings in batches of 250 at 15 ^{o}C for up to four weeks. The bait insects were placed on the soil and the tubs were lidded, inverted and stacked in random order in stacks of three in a plastic tray and covered with tinfoil to reduce evaporation. The baited tubs were incubated at 20 ^{o}C. In the case of soil samples taken in 2012 a single *G. mellonella* larva was place on the soil in each tube and the tubes were

inverted and incubated at 20 ^{o}C. The bait insects were removed after four days and stored individually in 5.5 cm Petri dishes (Figure 2.3) on moist tissue paper at 20 ^{o}C for a further four days. The baiting was repeated once.

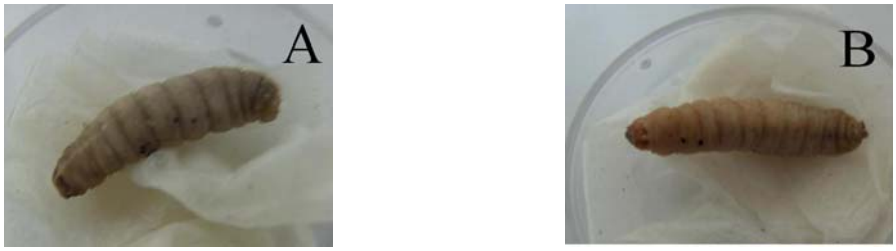

Figure 2.3: Nematode-infected bait insects recovered from soil from North Bull Island. Colour phenotypes were designated PUR (A) and YEL (B)

2.2.1.3 Nematode recovery from bait insects

Nematode infected cadavers were attributed to *H. downesi* or *Steinernema* sp. on the basis of cadaver bioluminescence and colour. Bioluminescence was assessed by observing cadavers in a darkened room for 10 min. Colour phenotypes were designated PUR (Figure 2.3A) (cadaver achromatic darkening to purple) or YEL (Figure 2.3B) (cadaver straw coloured darkening to yellow). Fourteen days after baiting cadavers exhibiting evidence of a *H. downesi* infection (no putrefaction, body remaining soft, cadavers luminescent, cadaver colour PUR or YEL were placed on individual White traps (Kaya & Stock, 1997) and stored at 20 ^{o}C to collect emerging infective juvenile (IJ) nematodes. White traps for individual cadavers consisted of a lid of a 3.5 cm Petri dish covered with a 55 mm filter paper (FisherBrand FB59015, Fisher Scientific, Ireland) in a 9 cm Petri dish (Figure 2.4). The Petri dish was filled to approximately half its depth with tap water. Infected cadavers were placed on the

filter paper and emerging IJs were allowed to accumulate in the water for five days before harvesting.

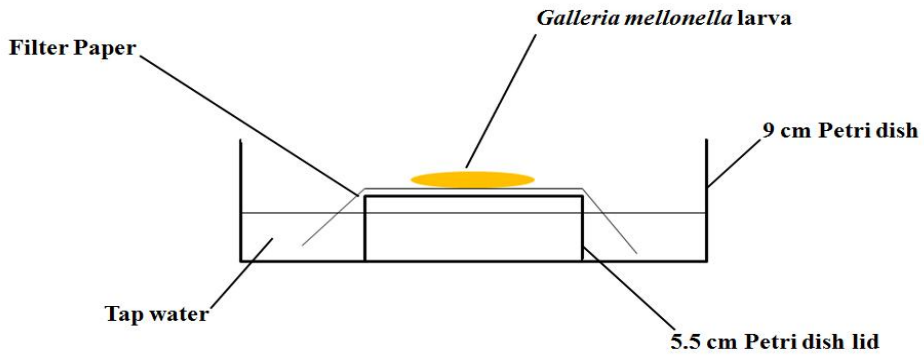

Figure 2.4: White trap used for single infected cadavers

2.2.1.4 Nematode harvesting

White traps were harvested by pouring the IJ suspension into a 50 ml glass beaker. Each White trap was rinsed three times to collect all the IJs. The IJs were washed three times by sedimentation by filling the glass beaker with tap water, allowing the IJs to settle to the bottom of the beaker, pouring off the supernatant and refilling the beaker with tap water.

2.2.1.5 Surface sterilisation

IJ suspension was transferred to a 50 ml graduated cylinder and surface sterilised with 0.16 mM Hyamine 1622 solution (Sigma-Aldrich) (Enright & Griffin, 2004). The graduated cylinder was sealed with Parafilm (Pechiney Plastic Packaging, Menasha, USA) and inverted three times and then IJs were allowed to settle for 15 min. The supernatant was removed and the IJs were washed three times by resuspending in sterile tap water, inverting three times, allowing the IJs to settle, pouring off the supernatant and resuspending in sterile tap water again.

2.2.1.6 Nematode counting and storage

Prior to storage nematode counts were carried out. The nematode suspension was made up to 50 ml with sterile tap water in a graduated cylinder which was sealed with parafilm and inverted three times. A 100 µl sample was removed and pipetted in a line of drops into a Petri dish. Live IJs were counted with the aid of a dissecting microscope by moving from one drop to the next. Three 100 µl samples were counted and the number of IJs in the total volume was calculated based on the mean of three counts. The nematode suspension was adjusted to approximately 1000 IJs/ml with sterile tap water and stored at 9 ^{o}C in 25 ml aliquots in 125 ml plastic food tubs with a diameter of 9 cm ("Econo" type 721, Huhtamaki, Espoo, Finland). Infective juveniles were stored for at least seven days at 9 ^{o}C before being used.

2.2.1.7 Culturing of nematodes

Isolates were reared in final instar *G. mellonella* larva. The lid and base of a 9 cm Petri dish were each lined with a 90 mm filter paper. Ten final instar *G. mellonella* larvae were placed in the dish and 1 ml of IJ suspension (1000 IJs/ml) was pipetted evenly onto the lid and base, equivalent to a dose of 200 IJs per insect. The dishes were incubated at 20 ^{o}C. After four days any insects exhibiting signs of an EPN infection were placed on moist filter paper in a 9 cm Petri dish and stored at 20 ^{o}C. After a further ten days the cadavers were placed on a White trap consisting of a 5.5 cm Petri dish lid covered with a 70 mm filter paper (FisherBrand FB59017, Fisher Scientific, Ireland) in a 14.5 cm Petri dish. The Petri dish was filled to approximately half its depth with tap water. Infected cadavers were placed on the filter paper and the White trap was stored at 20 ^{o}C (Figure 2.5). Emerging IJs were allowed to accumulate in the water for five days before harvesting as described in Section 2.2.1.4. The nematode suspension collected from bulk infections with ten *G.*

mellonella was made up to 250 ml in a graduated cylinder then counted and concentration adjusted to 1000 IJs/ml as described in Section 2.2.1.6 using normal tap water and stored for up to 1 month before use in experiments. Nematodes were cultured in this manner at least every three months.

Figure 2.5: White Trap for routine culturing of nematodes.

2.2.1.8 Habitat survey

The flora at each sampling location was recorded. The soil temperature at a depth of 10 cm was recorded using a HI98509 Checktemp1® thermometer (Hanna Instruments, Bedfordshire, UK). Soil pH was measured using a pH meter (model 3510, Jenway, Dunmow, England). Samples were prepared by adding 20 ml deionised water to 10 g of soil in a 50 ml tube (Sarstedt, Numbrecht, Germany) and mixing thoroughly (Robertson *et al.*, 1999). The mixture was allowed to equilibrate for 30 min at room temperature before reading. Three replicate samples were prepared for each sampling location. The habitat along each transect was classified according to Fossitt (2000), modified according to Rodwell *et al* (2000). The categories used were: Embryonic Dunes, Marram Dunes, Semi-Fixed Dunes, Fixed Dunes, Sandy Path, Grassy Path and Blowout. All categories recorded at each

sampling location were used to produce a composite habitat type with thirteen designations.

2.2.2 Nematode identification

Heterorhabditis sp. nematodes were identified on the basis of sequence analysis of the internal transcribed spacer 1 (ITS1) region of the ribosomal genes (rDNA).

2.2.2.1 DNA extraction from nematodes

DNA extraction was carried out on bulk samples of each of the nematode isolates listed in Section 2.2.1.3. DNA was extracted according to the Animal Tissue Protocol using a DNeasy® Blood and Tissue Mini Spin Column Kit (Qiagen, Crawley, UK). All buffers (ATl, AL, AW1, AW2, AE), proteinase K, spin columns and collection tubes were supplied with the kit. Details of reagent constituents are available in the DNeasy® Blood and Tissue Handbook. Sufficient Buffer AL was premixed with an equal volume of molecular grade ethanol (Sigma-Aldrich) (AL/EtOH) according to the manufacturer's instructions for the number of reactions being carried out. Buffers AW1 and AW2 were made up with molecular grade ethanol according to the manufacturer's instructions. A Sigma 1-15 microcentrifuge (Sigma Laboratory Centrifuges, Osterode, Germany) was used for all centrifugation steps.

One ml of surface sterilised nematode suspension (10,000 IJs /ml) was transferred to a 1.5 ml microcentrifuge tube (Fisher Scientific, UK) and collected by centrifugation at 17,968 x *g* for 5 min. The supernatant was discarded and the nematodes were placed at -70 ^{o}C for 15 min. The nematodes were semi-defrosted and ground with a micro pestle (Sigma-Aldrich, St. Louis, USA). The DNeasy protocol was then followed. Briefly, Buffer ATL (180 µl) and proteinase K (20 µl) were added, the

sample was mixed by vortexing for 5- 10 sec then lysed at 56 ^{o}C for 1 hr with shaking at 100 rpm with 5 - 10 sec vortexing every 15 min. AL/EtOH (400 µl) was added and the sample was mixed by vortexing, transferred to a spin column in a 2 ml collection tube and centrifuged at 6000 x *g* for 1 min. The collection tube and flow-through were discarded and the spin column was transferred to a fresh collection tube. Buffer AW1 (500 µl) was added and the sample was centrifuged at 6000 x *g* for 1 min. The collection tube and flow-through were discarded and the spin column was transferred to a fresh collection tube. Buffer AW2 (500 µl) was added and the sample was centrifuged at 17,968 x *g* for 3 min. The collection tube and flow-through were discarded and the spin column was transferred to a 1.5 ml microcentrifuge tube. Buffer AE (100 µl) was added and the sample was incubated at room temperature for 1 min then centrifuged at 6000 x *g* for 1 min. This step was repeated once. The spin column was discarded and the concentration of the DNA in the eluate was quantified using a NanoDrop® ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, USA). A portion of the eluate was adjusted to a concentration of 20 ng/µl. Both portions of DNA were stored at -20 ^{o}C until used. Three replicate extractions were carried out for each isolate.

2.2.2.2 PCR amplification of nematode DNA

Amplification of the nematode DNA extract was carried out using primers specific for the ITS1 region of the rDNA (Vrain *et al.*, 1992). All reactions were carried out using an Eppendorf Mastercycler Gradient machine (Eppendorf, Cambridge, UK). All reagents were kept on ice. All materials were from Sigma-Aldrich unless indicated otherwise. Sufficient master mix for three reactions per isolate was made up in a 1.5 ml microcentrifuge tube. Individual reactions were carried out in 0.5 ml thin-walled PCR tubes (Fisher Scientific, Ireland) using 45 µl of master mix and 5 µl

of DNA extract (20 ng/µl). A negative control to which no DNA was added was also included. The contents of the tubes were mixed by vortexing and then briefly centrifuged. Table 2. gives details of the PCR mixture, along with suppliers for the ingredients, Table 2.2 lists the cycling conditions used and Table 2.3 gives details of primers used.

For each reaction 5 µl PCR product was mixed with 1 µl of 5 x tricolour loading buffer (Bioline, UK) and analysed on a 1 % agarose gel: agarose (Life Technologies) 1 g dissolved in 100 ml 1 x TAE buffer (40 mM Tris-acetate, 1mM EDTA) (see Appendix Table A 1 for Tris-acetate and EDTA buffer composition) stained with 1 x SYBR Safe DNA Gel stain (Life Technologies). Gels were run at 100V for 1 hr in 1 x TAE buffer. Gels were visualised on a Transilluminator TFX-35M (Vilber Lourmat, Marne-la-Vallée, France) and photographed using a Kodak Electrophoresis Documentation and Analysis System (EDAS) system (Eastman Kodak Company, Rochester, NY, USA)

Table 2.1: PCR mixture for amplification of ITS1 region of rDNA from nematode isolates from North Bull Island. Reactions were carried out in 50 µl volumes. Reagents were supplied by Sigma-Aldrich unless otherwise indicated.

Reagent	Volume (µl)	Final concentration	Supplier
Buffer (10x)	5	1x	
MgCl2 (25mM)	4	2 mM	
dNTP Mix (10mM)	1	200 µM	Metabion
Forward primer (10mM)	5	1µM	Eurofins MWG Operon
Reverse primer (10mM)	5	1 µM	Eurofins MWG Operon
Taq Polymerase	0.25	0.025 U/µl	
DNA template (20 ng/µl)	5	2 ng/µl	
Molecular grade water	24.75		

Table 2.2: PCR cycling conditions for amplification of ITS1 region of rDNA from nematode isolates from North Bull Island (Hominick *et al.*, 1997). Lid temperature was set to 95°C.

Stage	Cycles	Temperature	Duration
Initial denaturation	1	94°C	2 min
Denaturation	40	94°C	30 sec
Annealing		45°C	1 min
Extension		72°C	90 sec
Final extension	1	72°C	5 min
Hold	1	4°C	indefinite

Table 2.3: Primer sequences for nematode ITS1 rDNA (Vrain *et al.*, 1992)

Primer	Sequence
18S (Forward)	5'-TTGATTACGTCCCTGCCCTTT-3'
28S (Reverse)	5'-TTTCACTCGCCGTTACTAAGG-3'

2.2.2.3 Cloning PCR product

Cloning reactions were set up using a TOPO TA Cloning® Kit (Life Technologies). One Shot® TOP10 chemically competent *Escherichia coli* (*E. coli*) cells, pCR®2.1-TOPO cloning vector, salt solution (1.2 M NaCl, 0.06 M $MgCl_2$) and S.O.C. medium were included in the cloning kit. Details of cloning kit reagents are available in the TOPO TA Cloning® Kit User Manual. Ampicillin stock solution (50 mg/ml) was prepared by dissolving ampicillin in distilled water and filter sterilised using a 0.2 µm Filtropur S syringe filter (Sarstedt, Numbrecht, Germany). LB/ampicillin (LB/Amp) broth and agar were prepared using LB broth or LB agar (BD, Franklin Lakes, NJ, USA) made up to 1 L according to the manufacturer's instructions, autoclaved at 121 °C for 20 min and allowed to cool to 55 °C before adding

ampicillin (50 μg/ml). LB/Amp agar was poured into 9 cm Petri dishes and allowed set. LB/Amp agar plates and LB/Amp broth were stored at 4 ^{o}C until required. LB/Amp/X-gal agar plates were prepared by spreading LB/Amp agar plates with X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) (Promega, Madison, WI, USA) (1.6 mg) and the plates were warmed at 37 ^{o}C for 30 min before use.

One ligation reaction was set up for each of the PCR products obtained in Section 2.2.2.2. Each reaction consisted of 1 μl pCR®2.1-TOPO cloning vector, 1 μl salt solution and 4 μl fresh PCR product. The reagents were mixed gently by pipetting and then incubated at room temperature at 25 rpm for 20 min. The ligation products were then transformed into One Shot® TOP10 chemically competent *E. coli* cells according to the protocol for transforming One Shot Chemically Competent Cells (Life Technologies). Briefly, 2 μl of the ligation reaction was added to a vial of freshly thawed cells. The reaction was mixed by inverting 3 times and incubated on ice for 20 min then heat-shocked for 30 sec at 42 ^{o}C and immediately returned to ice. S.O.C. medium (250 μl) was added to each vial of transformed cells and vials were incubated horizontally at 37 ^{o}C with shaking (200 rpm) for 1 hr. Aliquots of 20 μl, 35 μl and 50 μl of the transformed cells were spread on the LB/Amp/X-gal agar and incubated overnight at 37 ^{o}C for blue/white screening of clones.

2.2.2.4 Plasmid isolation

Plasmid DNA was purified using a QIAprep® Spin Miniprep Kit (Qiagen). All buffers (P1, P2, N3, PB, PE), LyseBlue, RNase A, spin columns and collection tubes were supplied with the kit. Details of reagent constituents are available in the QIAprep® Miniprep Handbook. RNase A (200 μl) and LyseBlue (20 μl) were added to buffer P1 (20 ml) according to the manufacturer's instructions.

For each isolate three single, well separated, white colonies were selected from the LB/Amp/X-gal overnight plates. Each colony was picked into a separate 50 ml centrifuge tube containing 5 ml of LB/Amp broth and incubated overnight at 37 ^{o}C with shaking (200 rpm). Overnight cultures were transferred to 15 ml centrifuge tubes and the cells were collected by centrifugation at 1462 x *g* for 10 min. The supernatant was discarded and the tubes were inverted and allowed to drain for 10 min. The cells were resuspended by pipetting in Buffer P1/RNase A/LyseBlue (250 µl) and transferred to a 1.5 ml microcentrifuge tube. Buffer P2 (250 µl) was added and the sample was mixed gently by inverting until a homogenous blue solution was obtained. Buffer N3 (350 µl) was added and the sample was mixed by inversion until a homogenous colourless suspension was obtained. The sample was centrifuged for 10 min at 17,968 x *g* and the supernatant was transferred to a spin column in a 2 ml collection tube. The sample was centrifuged for 1 min at 17,968 x *g* and the collection tube and the flow-through were discarded. The spin column was transferred to a clean collection tube. Buffer PE (0.75 ml) was added and the sample was centrifuged for 1 min at 17,968 x *g*. The flow-through was discarded and the sample was centrifuged for an additional 1 min at 17,968 x *g*. The collection tube and flow-through were discarded and the spin column was transferred to a clean 1.5 ml microcentrifuge tube. The DNA was eluted in molecular grade water (Sigma-Aldrich) by adding 50 µl water to the spin column, incubating at room temperature for 1 min then centrifuging at 6000 x *g* for 1 min. The spin column was discarded and the concentration of the DNA in the eluate was quantified using a NanoDrop® ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, USA). Purified plasmids were stored at -20 ^{o}C until required.

2.2.2.5 Plasmid analysis

Gel analysis of the plasmids was carried out to confirm presence of the ITS1 rDNA. Reactions were carried out in 50 µl volumes using 1 µl purified plasmid per reaction Sufficient PCR master mix was prepared for 18 samples using the recipe in Table 2. with the exception that the volume of water was increased to 28.75 µl per reaction. Cycling conditions are given in Table 2.4. PCR products were analysed on a 1 % agarose gel as described in Section 2.2.2.2.

Table 2.4: PCR cycling conditions to confirm presence of nematode ITS1 region of rDNA in purified plasmids from clones. Lid temperature was set to 95°C.

Stage	Cycles	Temperature	Duration
Initial denaturation	1	94 °C	2 min
Denaturation	2 - 35	94 °C	30 sec
Annealing		45 °C	60 sec
Extension		72 °C	90 sec
Final extension	1	72 °C	5 min
Hold	1	4 °C	indefinite

2.2.2.6 Phylogenetic analysis of nematode ITS1 sequences

The plasmid DNA obtained in Section 2.2.2.4 was sequenced by a commercial sequencing company (LGC Genomics, Berlin, Germany). Sequencing was carried out using M13 forward and reverse primers. Vector sequences were identified using VecScreen (http://www.ncbi.nlm.nih.gov/VecScreen/VecScreen.html) and the sequences were manually edited to remove vector sequences. A consensus sequence of the clones for each individual isolate was obtained using CAP3 (http://pbil.univ-lyon1.fr/cap3.php) (Huang & Madan, 1999).

An initial multiple sequence alignment was carried out using the six isolates from North Bull Island in MEGA5 (Tamura *et al.*, 2011) using ClustalW (Thompson *et al.*, 1994; Larkin *et al.*, 2007). Homologous nucleotide sequences in GenBank were identified using the blastn algorithm in BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) (Altschul *et al.*, 1997). Table 2.5 contains a full list of the *Heterorhabditis* spp. sequences retrieved from GenBank (http://www.ncbi.nlm.nih.gov/Genbank/). *Caenorhabditis elegans* was included as an outgroup and a multiple sequence alignment with a total of 29 sequences was carried out in MEGA5 using ClustalW. The resulting alignment was trimmed to eliminate unaligned ends to leave 1012 positions shared by all sequences. Phylogenetic analysis was carried out in MEGA5, with the choice of model determined using the "Find Best DNA/Protein Models (ML)" option in the MEGA5 program. Evolutionary history was estimated using the maximum likelihood method (Kimura, 1980) based on the Kimura 2-parameter nucleotide substitution model with a Gamma distribution used to model non-uniform rates between sites. The parameters used for phylogenetic analysis are shown in Appendix Table A 2. A phylogenetic tree using bootstrap analysis with 1000 replicates was constructed.

Table 2.5: *Heterorhabditis* species and strains used in ITS1 analysis.

Species /strain	GenBank accession no.	Bacterial symbiont (***Photorhabdus*** sp.)	Geographical origin	Reference
Heterorhabditis atacamensis				
D099	HM230723	unknown	Chile	(Edgington *et al.*, 2011)
Heterorhabditis downesi				
H3107^{T}	EU921443	*P. temperata* subsp. *cinerea*	Hungary	(Tóth & Lakatos, 2008)
H3173	EU921444	*P. temperata subsp. temperata*	Hungary	(Tóth *et al.*, 2008)
K122	AY321482	*P. temperata* subsp. *temperata*	Ireland	(Nguyen *et al.*, 2004)
K122	EF043442	*P. temperata* subsp. *temperata*	Ireland	(Regeai & Burnell 2006, unpublished)
Heterorhabditis marelatus				
OH10	AY321479	unknown	USA	(Nguyen *et al.*, 2004)
	EF043441	unknown	USA	(Regeai & Burnell 2006, unpublished)
Heterorhabditis megidis				
AGC	AY321480	unknown	USA	(Nguyen *et al.*, 2004)
Andong	AY293284	unknown	Korea	(Kang *et al.* 2003, unpublished)
HF85	EF043439	*H. megidis*	The Netherlands	(Regeai & Burnell 2006, unpublished)
H3014	EU921441	*P. temperata subsp. cinerea*	Hungary	(Tóth & Lakatos, 2008)
NIHHS-KHH05	AB698759	unknown	Korea	(Kim *et al.* 2012, unpublished)
N-UK76	HQ225905	*P. temperata* subsp. *temperata*	UK	(Maneesakorn *et al.*, 2011)
	KJ938577	unknown	Switzerland	(Campos-Herrera *et al.* 2014, unpublished)
Heterorhabditis safricana				
SF52	EU860188	unknown	South Africa	(Malan & Moore, 2008, unpublished)
SF670	FJ473361	unknown	South Africa	(Malan 2008, unpublished)
SF724	FJ791249	unknown	South Africa	(Malan 2008, unpublished)
	EF488006	unknown	South Africa	(Malan *et al.*, 2008)
Heterorhabditis zealandica				
Florida	AY321481	unknown	USA	(Nguyen *et al.*, 2004)
J37	EU727165	unknown	South Africa	(de Waal & Malan, 2008, unpublished)
NZH3	EF530041	unknown	New Zealand	Nguyen 2007, unpublished
X1	AY170330	unknown	New Zealand	Qui *et al.* 2006 unpublished

2.2.3 Symbiotic bacteria characterisation

2.2.3.1 Isolation of symbiotic bacteria

a) Mass isolation from nematodes

Prior to extracting the symbiotic bacteria the nematodes were treated with 1% NaOCl to desheath them, as described by Campbell & Gaugler (1991a; 1991b). Briefly, 6 ml of nematode suspension (1000 IJs /ml) was allowed to settle, 0.5 ml of the supernatant was removed and replaced by 0.5 ml 12% NaOCl (final concentration 1%), inverted three times and allowed to stand for 5 min. The nematode suspension was filtered (Supor® 200 membrane filter, Pall Life Sciences, USA) under vacuum and rinsed five times with 1 ml sterile Ringers solution per rinse. The nematodes were transferred to a sterile cold glass slide and crushed with a sterile glass rod. The crushed IJs were then streaked onto nutrient agar (NA) (Oxoid Ltd, Basingstoke, UK), nutrient agar supplemented with bromothymol blue (Sigma-Aldrich) (0.025 g/l) and triphenyltetrazolium chloride (Sigma-Aldrich) (0.04 g/l) (NBTA) or MacConkey agar (Oxoid Ltd) and incubated in the dark at 27 ^{o}C for 36 – 48 hr. (See Appendix Table A 3 for culture media composition).

b) Isolation from individual infective juveniles

IJs were surface sterilised by adding 1 ml Hyamine 1622 solution (0.16 mM) (Sigma-Aldrich) to nine ml of nematode suspension (1000 IJs/ml), inverting 3 times and allowing to settle. The supernatant was discarded and the IJs were rinsed by adding 9 ml sterile phosphate buffered saline (PBS), inverting 3 times and allowing to settle. The rinse was repeated twice. Individual IJs were pipetted in 10 µl aliquots with the aid of a dissecting microscope and added to 90 µl PBS in a 1.5 ml microcentrifuge tube. Each IJ was crushed with a sterile micropestle for one minute. A 50 µl aliquot of the product was plated onto Luria-Bertani (LB) agar (Difco)

supplemented with 0.1% sodium pyruvate (Sigma-Aldrich) (LB pyruvate agar) and incubated in the dark at 27 ^{o}C for 48 hr. The experiment was carried out once for each of the potential YEL isolates from the 2012 North Bull Island survey, with 10 IJs of each isolate.

c) Isolation from haemolymph of infected insects

Infections to recover symbiotic bacteria from insect haemolymph were set up as described in Section 2.2.1.7, using 5.5 cm Petri dishes and 3 insects per isolate. Infected cadavers were checked for bioluminescence after 72 hours. Bacteria were recovered from all cadavers positive for *H. downesi* infection as follows: the cadaver was surface sterilised by washing in 95% ethanol, flaming and extinguishing by plunging into sterile distilled water. The cadaver was opened under aseptic conditions and a loopful of haemolymph was streaked onto NBTA or MacConkey agar plates and incubated in the dark at 27 ^{o}C for 36 - 48 hours (Kaya & Stock, 1997).

2.2.3.2 Routine culturing of symbiotic bacteria

Bacterial colonies were stored in the dark at 27 ^{o}C or at 15 ^{o}C and subcultured onto NA or MacConkey agar plates every 4 days or once a month, respectively. Liquid cultures were set up by inoculating single colonies from 36 - 48 hr plate cultures into 50 ml nutrient broth (NB) or MacConkey broth (MB) in a 250 ml flask and growing for 24 - 48 hr in the dark with shaking at 200 rpm at 27 ^{o}C. The optical density (OD) at 660 nm of liquid cultures was recorded as required using a Shimadzu UV mini 1240 Spectrophotometer (Fischer Scientific). Working and long-term stock cultures of 6 individual isolates of the bacteria were established by adding 0.5 ml of 24 hr liquid culture to 0.5 ml NB premixed with glycerol (30%), vortexing briefly and

storing at -20 °C and -80 °C respectively. Bacteria were recovered as required from frozen stocks by plating onto MacConkey agar and incubating in the dark at 27 °C.

2.2.3.3 Characterisation of symbiotic bacteria

a) Morphological characterisation

Bacteria isolated either directly from nematodes or from the haemolymph of infected insects were designated *pur* and *yel* according to the colour phenotype of the infected cadaver, PUR and YEL respectively, (Figure 2.3) and were identified as *Photorhabdus* sp. on the basis of bioluminescence. Bioluminescence was assessed by observing colonies in a darkened room for 10 min. Bacteria were subcultured onto NA plates as described in Section 2.2.3.2 and grown in the dark at 27 °C for 36 – 48 hr until colonies visible to the naked eye were obtained. Stock strains of *Escherichia coli* (Gram-negative) and *Bacillus subtilis* (Gram-positive), obtained from the Preparation Laboratory, Biology Department, NUI Maynooth (NUIM), were used as reference strains for Gram staining. Gram staining, cell morphology and cell motility were compared under a Nikon OPTIPHOT-2 light microscope using a 100X oil immersion lens. Colony morphology was examined under a dissecting microscope.

b) Microbial growth - direct cell count

One isolate of each colour phenotype was chosen to investigate growth rate. One liquid culture per isolate was set up as described in Section 2.2.3.2. An aliquot of cell suspension (1 ml) was drawn after 10 hours growth. A second 1 ml sample was drawn after a further 6 hours growth. At each time point a 1 in 10 serial dilution in ¼ strength sterile Ringers solution was prepared until a suitable cell density for counting was obtained. To immobilise the cells for counting 0.5 ml of each dilution was added to 0.5 ml HCl (0.1 N) (Gerhardt, 1981) in a fresh tube. Samples were

placed on ice until counted. Cells were vortexed briefly before counting on a haemocytometer counting chamber under Nikon OPTIPHOT-2 light microscope using a 40X lens. A 10 µl aliquot of the cell suspension was loaded into each chamber and cells were counted in the four corner squares. The average count for the two chambers was multiplied by the dilution factor and then by 10^4 to allow for the volume of the counting chamber. The dilution factor was calculated as 1/0.5 * dilution to allow for the addition of HCl as described above. Growth rate (mean generation time) (g) was calculated using the following formula:

$$g = (\log_{10} N_t - \log_{10} N_0) / \log_{10} 2$$

where N_0 = number of cells at time t_0

N_t = number of cells at time t_1

t_0 = initial time (hrs)

t_1 = final time (hrs)

From the growth rate the doubling time (hours/generation) (t/g) was calculated as

$$t / g$$

where $t = t_1 - t_0$

This experiment was repeated once with cultures grown in NB and samples taken at 10 hours and 22 hours. The experiment was also run with cultures grown in MB and samples taken at 10 hours and 34 hours.

c) Microbial growth – colony count

Samples for spread plating were drawn from the cultures in NB in Section 2.2.3.2b. Aliquots (100 µl) of each of three dilutions were spread on triplicate NA plates (9 cm) at the initial time point (10 hrs). In addition, a final set of samples were drawn

from the cultures in experiment 2 at 64 hours. The final samples were spread on a selection of NA, NBTA and MacConkey agar plates. All plates were incubated at 27^0 C for 48 hours before counting. Plates which had fewer than 30 colonies or more than 350 colonies were not counted.

d) Biochemical characterisation

Bacteria were subcultured onto NA plates as described in Section 2.2.3.2 and grown for 36 – 48 hours in the dark at 27 ^oC. Bacterial identification kits (API 20 E, API 50 CH) (bioMerieux, France) were used to analyse the biochemical and physiological properties of each isolate. Unless otherwise stated all reagents were supplied by bioMerieux. Test strip trays were prepared using distilled water according to the manufacturer's instructions. The API 20E strips (12 tests) were inoculated with a suspension of bacterial culture made by dispersing 1- 4 single, well-isolated colonies in API 20E NaCl 0.85% medium (5 ml) to 0.5 McFarland. The test strips were incubated at 27^o C and checked daily for 5 days. Tests for tryptophan deaminase, indole production, acetoin production and reduction of nitrates were carried out at the end of the incubation period. The API 50 CH strips (49 tests) were inoculated with a bacterial culture in API 50 CHB/E medium made by dispersing up to 20 single, well-isolated colonies in 1 ml sterile distilled water to 4 McFarland. This was then added to a vial of API 50 CHB/E medium and mixed thoroughly before use. The test strips were incubated at 27^o C and checked daily for 5 days. The cytochrome oxidase activity was tested using tetramethyl-*p*-phenylenediamine dihydrochloride (Sigma-Aldrich). The test was carried out by soaking 1 cm squares of filter paper in a 1% aqueous solution of the reagent, allowing to dry, then transferring a colony from the NA plates to the paper using a sterile glass rod (Collins & Lyne, 1984).

2.2.3.4 Antibiosis

Bacterial isolates were screened for inhibition of bacteria and fungi using a range of organisms. Target organisms and culturing conditions are listed in Table 2.6. Inhibition was assessed by measuring the zone of inhibition at four points around the spot colony and taking the mean of the four measurements (bacterial test organisms) (Figure 2.6) or by measuring the zone of inhibition at 3 points along the bacterial streak (fungal test organisms) (Figure 2.7). Antibacterial tests were based on the method of Boemare & Lanois (1996). Briefly, 50 µl of 24 hr culture was spot inoculated onto NA in a 100 mm glass Petri dish (Fisher Scientific) and incubated in the dark at 27 ^{o}C for 48 hr. Immediately prior to the test the lid of the Petri dish was flooded with chloroform and the dish inverted over the lid for 30 min to kill the colony. The plates were left uncovered in a laminar flow hood for a further 30 min to allow the chloroform to evaporate. Soft agar (7 g agar / L dH2O) was inoculated with the target organism (1 ml / 50 ml agar) and poured onto the chloroform-killed spot colony. A negative control which had no spot colony was included in each treatment. Plates were examined under a Nikon OPTIPHOT-2 light microscope using a 40X lens after 20 hr (*Escherichia coli, Bacillus subtilis*), or 40 hr (*Micrococcus luteus, Xenorhabdus bovienii, Photorhabdus* isolates). For intraspecific tests pur/yel combinations were chosen by drawing lots and each combination was reversed so that all isolates were used as target organisms. Each test had three replicates and each test was run three times with different cultures in each experiment

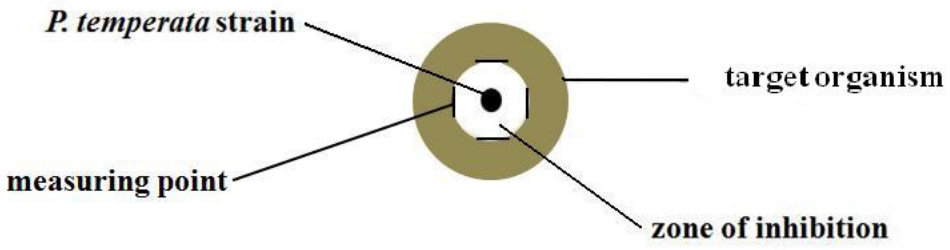

Figure 2.6: Diagram showing zone of inhibition and four measuring points in antibacterial test

For antimycotic tests, the method of Ansari *et al.* (2005) was followed. Briefly, a loopful of *Photorhabdus* culture was streaked in a line 5 cm long and 3 cm from one edge of a 9 cm NA plate and incubated in the dark at 27 ^{o}C for 48 hr. One mycelial plug (1 cm square) was placed in line with the midpoint of the bacterial streak and centered 3cm from the bacterial streak and the wall of the Petri dish. A negative control which had no bacterial streak was included in each treatment. Plates were incubated in the dark at 25 ^{o}C and checked at 7 day intervals for a period of 28 days. Each test had three replicates and each test was run three times with different cultures in each experiment.

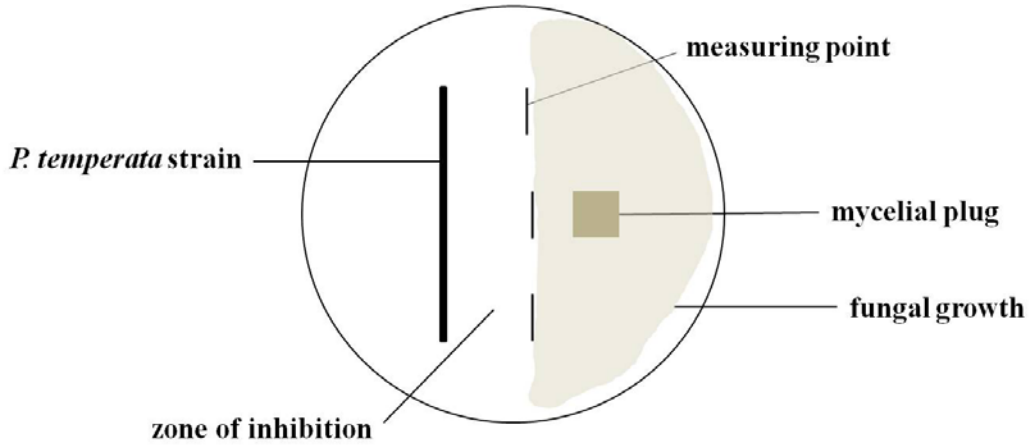

Figure 2.7: Diagram showing experimental setup with *P. temperata* streak, mycelial plug, fungal growth from the inoculating mycelial plug, zone of inhibition and three measuring points in antimycosis test.

Table 2.6: Target organisms and **c**ulturing conditions for used in antibiosis experiments

Test organism	incubation temperature	incubation period
Escherichia coli[1]*	37° C	24 hr
Bacillus subtilis[1]*	37° C	24 hr
Micrococcus luteus[1]*	26° C	48 hr
Xenorhabdus bovienii[2]*	27° C	48 hr
Photorhabdus isolates[3]*	27° C	48 hr
Mucor sp.[1]†	25° C	7 days
Beauveria bassiana[2]‡	25° C	14 days
Metarhizium anisopliae[2]‡	25° C	14 days

1. Obtained from the Preparation Laboratory, Biology Department, NUIM
2. Obtained from colleagues in the Behavioural Ecology Laboratory, NUIM
3. This study.

* Cultures were grown on NA plates and 24 hr before the test a single was picked into 50 ml NB in a 250 ml flask and incubated with shaking at 200 rpm at the appropriate temperature for 24 hr.

† Cultures were grown on Malt Extract agar (Oxoid)

‡ Cultures were grown on Sabouraud Dextrose Agar (SDA) (Oxoid)

2.2.4 Phylogenetic analysis of symbiotic bacteria

2.2.4.1 DNA extraction from symbiotic bacteria

Bacterial DNA was extracted using a Qiagen DNeasy Blood and Tissue 50 spin column kit. All reagents were supplied with the kit unless otherwise stated. Liquid cultures were set up as described in Section 2.2.3.2 in 5 ml of LB broth, Miller, (Luria-Bertani) (BD Difco, NJ, USA) and incubated overnight at 27^{o} C with shaking at 200 rpm. Absorbance at 660 nm was checked and cell suspensions were adjusted to give approximately 2 x 10^{9} cells by diluting in ¼ strength sterile Ringers solution. (See Appendix Figure A. 2 for standard curves.) Cells were collected by spinning in a microcentrifuge at 5000 x *g* for 10 min, the supernatant was discarded and the extraction was then completed following using the DNeasy protocol described in Section 2.2.2.1 with the following variation: cells were lysed for 1.5 hr instead of 1 hr.

2.2.4.2 PCR amplification of symbiotic bacteria

Amplification of symbiotic bacteria DNA was carried out using primers specific for the *gyrB* gene (Tailliez & Boemare, 2009). Amplification reactions and gel analysis of PCR product were carried out as described in Section 2.2.2.2 with the following exceptions: cycling conditions (Table 2.7), PCR mixture (Table 2.8), forward and reverse primers (Table 2.9).

Table 2.7: PCR cycling conditions for amplification of *gyrB* gene from symbiotic bacteria isolated from *H. downesi* nematodes from North Bull Island (Tailliez & Boemare, 2009). Lid temperature was set to 95°C.

Stage	Cycles	Temperature	Duration
Initial denaturation	1	94 °C	5 min
Denaturation	30	94 °C	1 min
Annealing		60 °C	1 min
Extension		72 °C	2 min
Final extension	1	72 °C	7 min
Hold	1	4 °C	indefinite

Table 2.8: PCR mixture for amplification of *gyrB* gene from symbiotic bacteria isolated from *H. downesi* nematodes from North Bull Island (Tailliez & Boemare, 2009). Reactions were carried out in 50 µl volumes. Reagents were supplied by Sigma-Aldrich unless otherwise indicated.

Reagent	Volume (µl)	Final concentration	Supplier
Buffer (10x)	5	1x	
MgCl2 (25mM)	6	3 mM	
dNTP Mix (10mM)	1	200 µM	Metabion
Forward primer (10mM)	1	0.2 µM	Eurofins MWG Operon
Reverse primer (10mM)	1	0.2 µM	Eurofins MWG Operon
Taq Polymerase	0.25	0.025 U/µl	
DNA template (20 ng/µl)	5	2 ng/µl	
dH2O	30.75		

Table 2.9: Primer sequences for *gyrB* gene from symbiotic bacteria isolated from *H. downesi* nematodes from North Bull Island (Tailliez & Boemare, 2009).

Primer	Sequence
gyrBP1 (Forward)	5'-TACACGAAGAAGAAGGTGTTTCAG-3'
gyrBP2 (Reverse)	5'-TACTCATCCATTGCTTCATCATCT-3'

Cloning, plasmid isolation and plasmid analysis was carried out as described in Sections 2.2.2.3 – 2.2.2.5.

2.2.4.3 Phylogenetic analysis of *gyrB* gene sequences

The plasmid DNA obtained in Section 2.2.4.2 was sequenced by a commercial sequencing company (GATC Biotech, Konstanz, Germany). Sequencing was carried out using M13 forward and reverse primers plus four *gyrB* specific primers (Table 2.10) (Boemare & Tailliez, 2009) to obtain overlapping sequences. Sequences were edited as described in Section 2.2.2.6.

Table 2.10: Sequencing primers for *gyrB* gene (Boemare & Tailliez, 2009).

Primer	Sequence
gyrBSP1 (Forward)	5'-GATAACTCTTATAAAGTTTCCG-3'
gyrBSP2 (Reverse)	5'-CGGGTTGTATTCGTCACGGCC-3'
gyrBSP3 (Forward)	5'-CTCTACTTAGTGGAAGGGGA-3'
gyrBSP4 (Reverse)	5'-GCAGTAAATATTTTCCTGGA-3'

An initial multiple sequence alignment was carried out for just the six isolates from North Bull Island using ClustalW (Thompson *et al.*, 1994; Larkin *et al.*, 2007) in MEGA5 (Tamura *et al.*, 2011). Nucleotide sequences were translated and open reading frames in the protein sequence were identified using ORF Finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). Homologous nucleotide sequences

were assembled as described in Section 2.2.2.6. The resulting alignment was trimmed to eliminate unaligned ends to leave 847 positions shared by all the sequences. Table 2.11 contains a full list of the *Photorhabdus* spp. sequences retrieved from GenBank (http://www.ncbi.nlm.nih.gov/Genbank/). Phylogenetic analysis was carried out in MEGA5, with the choice of model determined using the "Find Best DNA/Protein Models (ML)" option in the MEGA5 program. Evolutionary history was estimated using the maximum likelihood method, based on the Kimura 2-parameter nucleotide substitution model (Kimura, 1980) with a Gamma distribution used to model non-uniform rates between sites. The parameters used for phylogenetic analysis are shown in Appendix Table A 4. A phylogenetic tree using bootstrap analysis with 1000 replicates was constructed, using *X. nematophila* as an outgroup.

Table 2.11: *Photorhabdus* species and strains used in *gyr*B analysis.

Species /strain	GenBank accession no.	Nematode host (***Heterorhabditis*** sp.)	Geographical origin	Reference
P. temperata* subsp. *cinerea				
H3107^T	EU053168	*H. downesi*	Hungary	(Tóth & Lakatos, 2008)
H3240	EU053174	*H. downesi*	Hungary	(Tóth & Lakatos, 2008)
H3014	EU155110	*H. megidis*	Hungary	(Tóth & Lakatos, 2008)
P. temperata* subsp. *khanii				
C1^T	AY278497	*H. bacteriophora* NC1*	USA	(Akhurst *et al.*, 2004)
Meg	AY278512	*H. megidis*	USA	(Akhurst *et al.*, 2004)
Habana	AY278503	*Heterorhabditis* sp.	Cuba	(Akhurst *et al.*, 2004)
P. temperata* subsp. *tasmaniensis				
NZH3	AY278513	*H. zealandica*	New Zealand	(Akhurst *et al.*, 2004)
T327^T	EU930356	*H. zealandica*	Australia	(Tailliez *et al.*, 2010)
USCA01	EU930357	*H. marelatus*	USA	(Tailliez *et al.*, 2010)
P. temperata* subsp. *temperata				
HF85	AY278502	*H. megidis*	The Netherlands	(Akhurst *et al.*, 2004)
HL81	AY278504	*H. megidis*	The Netherlands	(Akhurst *et al.*, 2004)
HW79	AY278507	*H. megidis*	The Netherlands	(Akhurst *et al.*, 2004)
X1LIT	AY278516	*H. megidis*	Lithuania	(Akhurst *et al.*, 2004)
X1Nach^T	AY278517	*H. megidis*	Russia	(Akhurst *et al.*, 2004)
H3016	EU053166	*H. megidis*	Hungary	(Tóth *et al.*, 2008)
H3173	EU053169	*H. downesi*	Hungary	(Tóth *et al.*, 2008)
H3179	EU053170	*H. downesi*	Hungary	(Tóth *et al.*, 2008)
H3182	EU053171	*H. downesi*	Hungary	(Tóth *et al.*, 2008)
K122	EU930355	*H. downesi*†	Ireland	(Tailliez *et al.*, 2010)
P. temperata* subsp. *thracensis				
H3210	EU053173	*H. bacteriophora*	Hungary	(Tóth *et al.*, 2008)
CIP108426^T	EU930351	*H. bacteriophora*	Turkey	(Tailliez *et al.*, 2010)
FR32	EU930352	entomopathogenic nematode	France	(Tailliez *et al.*, 2010)
***P. temperata* sp.**				
H111	EF029048	*H. megidis*	Hungary	(Tóth *et al.*, 2008)
H267	EF029049	*H. downesi*	Hungary	(Tóth *et al.*, 2008)
H295	EF029050	*H. megidis*	Hungary	(Tóth *et al.*, 2008)
***Photorhabdus* sp.**				
H3086	EU053167	*H. downesi*	Hungary	(Tóth *et al.*, 2008)

* The naming convention used by Akhurst et al (2004) is followed here.

2.2.5 Statistical analysis

Routine statistical analysis, including tests for normality, was carried out using Minitab statistical software, version 16.1.1 (Minitab Inc., 2010). Normality of data was tested using the Anderson-Darling method. Comparisons between two groups were carried out using a 2-Sample t-test or a paired t-test where appropriate. If the data were not normally distributed a Mann-Whitney U test was used. Analysis of variance was carried out using a one-way ANOVA or when groups had an uneven number of observations the general linear model with an unbalanced nested design was used to compare isolate and colour phenotype. Analysis of variance was followed by pair-wise multiple comparisons using Tukey's test. In experiments where nematode type and bacterial type were factors, results were analysed using a two-way ANOVA. Where no significant interactions occurred treatment means for each factor were compared using a one-way ANOVA and post-hoc Tukey's test for three or more treatments or 2-sample T-test for two treatments. When data could not be normalised treatment medians were compared using a Kruskal-Wallis test, followed by multiple pairwise comparisons using a Mann-Whitney U-test test with Bonferroni 95% confidence intervals, obtained by calculating $(1 - \alpha / g)$ where α is the level of significance and g is the number of pairwise comparisons carried out. In all tests a P value of < 0.05 was taken as significant unless indicated otherwise.

2.3 **Results**

2.3.1 Entomopathogenic nematodes on North Bull Island

2.3.1.1 Detection of entomopathogenic nematodes

Three transects from the seaward side to the landward side of the dunes on North Bull Island were sampled during September and October 2008. Two species of entomopathogenic nematode were recovered: *Heterorhabditis downesi* and *Steinernema feltiae.* A total of 35/42 (83.3%) of the soil samples were positive for nematodes when baited with *G. mellonella* (Table 2.12). Overall, *H. downesi* was detected in 13/42 (31%) of the soil samples and *S. feltiae* was detected in 34/42 (81%) of the soil samples (Table 2.12).

Table 2.12: Number of soil samples positive for entomopathogenic nematodes from North Bull Island. Two species were detected, *H. downesi* and *Steinernema feltiae.* Each transect consisted of 14 soil samples. Each sample was baited twice and each baiting consisted of 15 *G. mellonella.* (n = 90 bait insects per sampling location)

Transect	positive for EPN	*H. downesi*	*S. feltiae*
1	12	7	12
2	11	1	10
3	12	5	12
Total	35	13	34

A higher proportion of bait insects were killed by *S. feltiae* than *H. downesi*, 131/1260 (10.4%) and 22/1260 (1.7%) respectively (Table 2.13). Nematodes were more likely to be detected in the first baiting than in the second, 107/630 and 46/630 respectively (Table 2.14). This was true for both *H. downesi* (20/630 and 2/630 for baiting 1 & 2 respectively) and for *S. feltiae* (87/630 and 44/630 for baiting 1 & 2 respectively). Two sampling locations, 0 m and 10 m, were positive for in the second baiting only and only for *S. feltiae.*

Table 2.13: Number of dead bait insects recovered from soil samples from North Bull Island. Each sample was baited twice and each baiting consisted of 15 *G. mellonella.* (n = 30 bait insects per sampling location in each transect, giving a total n of 90 bait insects per distance (10 m) into the dunes)

	Number of dead bait insects (/30)			Number of dead bait insects (/	
Distance (m)*	transect 1	transect 2	transect 3	*H. downesi*	*S. feltiae*
0	1	0	0	0	1
10	0	0	1	0	1
20	5	3	11	3	16
30	2	1	0	1	2
40	11	3	2	4	12
50	3	1	1	1	4
60	5	3	2	2	8
70	6	1	4	2	9
80	4	6	6	4	12
90	17	2	3	2	20
100	5	5	5	0	15
110	4	0	2	0	6
120	8	5	5	3	15
130	0	4	6	0	10
total	71	34	48	22	131

* The first sampling location in each transect (0 m) was 2 metres above the strand line.

Table 2.14: Detection of nematodes in first and second baiting. Each sample was baited twice and each baiting consisted of 15 *G. mellonella.* Data is pooled for three transects. (n = 30 bait insects per sampling location in each transect, giving a total n of 90 bait insects per distance (10 m) into the dunes.

	H. downesi		*S. feltiae*	
Distance (m)*	bait 1	bait 2	bait 1	bait 2
0	0	0	0	1
10	0	0	0	1
20	3	0	9	7
30	1	0	2	0
40	4	0	6	6
50	1	0	4	0
60	2	0	5	3
70	1	1	8	1
80	3	1	6	6
90	2	0	12	8
100	0	0	13	2
110	0	0	5	1
120	3	0	9	6
130	0	0	8	2
total	20	2	87	44

* The first sampling location in each transect (0 m) was 2 metres above the strand line.

2.3.1.2 Occurrence of *H. downesi* colour phenotypes - 2008

Two colour phenotypes of *H. downesi* were detected, designated PUR and YEL on the basis of cadaver colour (see Section 2.2.1.2, Figure 2.3) and pigmentation of the symbiotic bacteria on NA plates. The PUR phenotype was detected in all three transects whereas the YEL phenotype was detected in transect 1 only, and there was no overlap of the two colour phenotypes in this transect.

In transect 1 the PUR phenotype was detected in four soil samples, ranging in distance from 20 m to 60 m into the dune system and the YEL phenotype was

detected in three soil samples, ranging in distance from 80 m to 120 m into the dune system (Figure 2.8A). In transect 2 the PUR phenotype was detected in only one soil sample, at 50 m (Figure 2.8B), and in transect 3 the PUR phenotype was detected at 40 m, 60 m, 70 m, 80 m and 90 m (Figure 2.8C). The number of bait insects killed per sample ranged from 1 (1/30, 3%) to 3 (3/30, 10%) (Figures 2.8A-C).

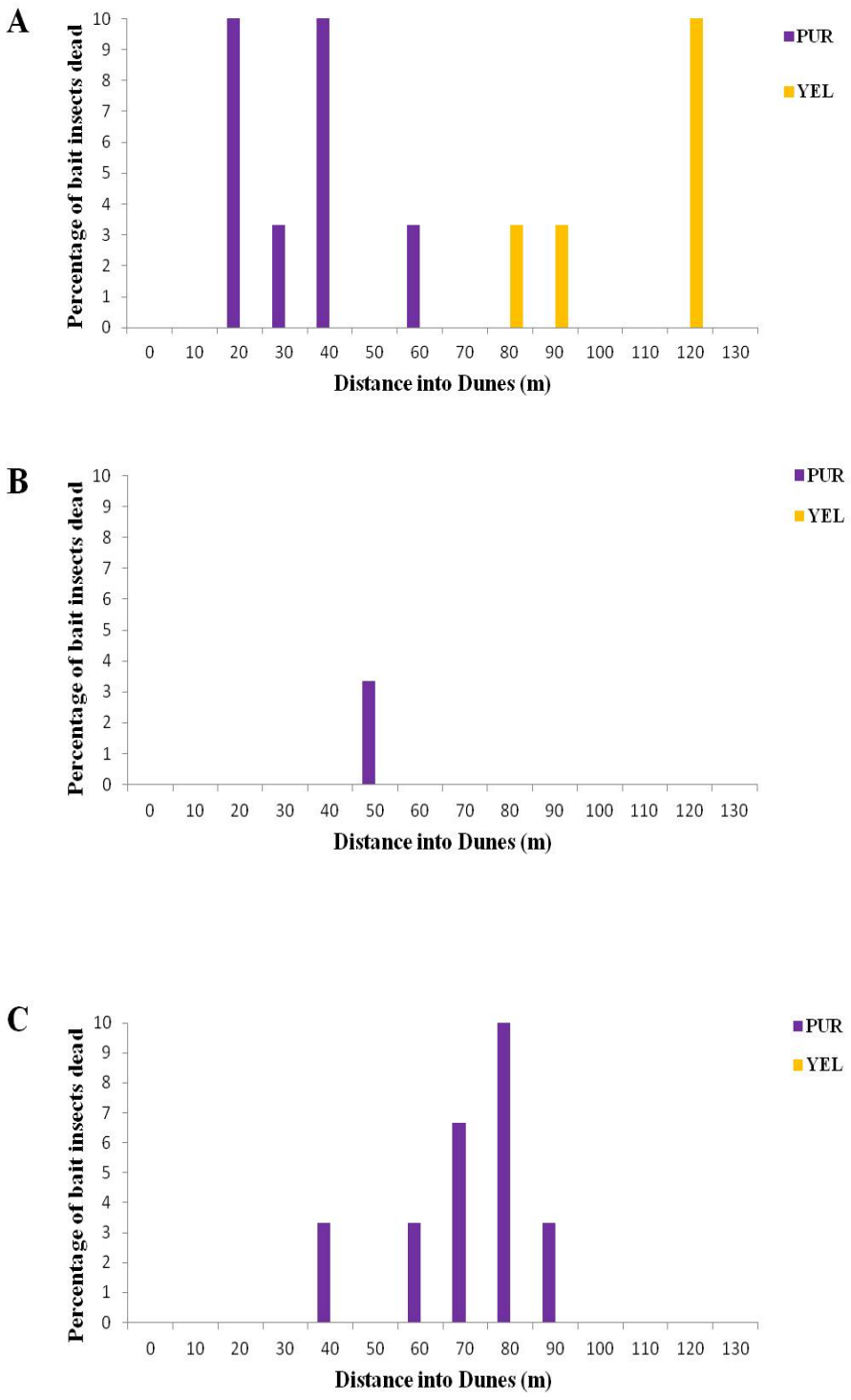

Figure 2.8 Percentage mortality of bait insects due to *H. downesi* PUR and YEL colour variants in A - transect 1, B - transect 2 and C - transect 3, North Bull Island. Each soil sample consisted of 30 pooled subsamples and was baited twice with 15 *G. mellonella* in each baiting. (n = 30). The first sampling location in each transect (0 m) was 2 metres above the strand line.

Bait insects from transect 1 were chosen to establish stock cultures of *H. downesi* as described in Section 2.2.1.3. Where multiple insects were infected in a particular soil sample one cadaver was chosen at random. Table 2.15 lists the isolates used to establish stock nematode cultures, along with the distance into the sand dunes of the soil sample from which they were recovered, and the symbiotic bacteria associated with each nematode isolate.

Table 2.15: *H. downesi* and *P. temperata* isolates from North Bull Island used in this study. The colour phenotype is indicated by the isolate name: PUR and *pur* for purple phenotype nematode and symbiotic bacteria respectively and YEL and *yel* for the yellow phenotype nematode and symbiotic bacteria respectively. Distance* indicates the distance into the sand dunes of the soil sample from which the nematode was recovered. All isolates listed in the table were isolated from transect 1.

	isolates	
distance (m)*	nematode	bacteria
20	PUR1	*pur1*
30	PUR2	*pur2*
60	PUR3	*pur3*
80	YEL1	*yel1*
90	YEL2	*yel2*
120	YEL3	*yel3*

* The first sampling location in each transect (0 m) was 2 metres above the strand line.

2.3.1.3 Repeat sampling 2012

In 2012 transect 1 was re-sampled (Table 2.17). Both *H. downesi* and *S. feltiae* were detected. On this occasion both *H. downesi* colour phenotypes were found to be co-occurring in the front of the dunes. A total of 26/48 (54.2%) of the soil samples were positive for nematodes when baited with *G. mellonella*. Overall, *H. downesi* was detected in 9/48 (18.8%) and *S. feltiae* was detected in 17/48 (35.4%) of the soil samples, and more nematodes were detected in the first baiting than in the second

(27/48 and 2/48, respectively). The PUR phenotype was more abundant than the YEL in the front section of the dunes, with the PUR phenotype being detected in five of the individual soil samples, ranging in distance from 20 to 70 m into the dune system and the YEL phenotype being detected in four of the individual soil samples, ranging in distance from 20 to 130 m into the dune system. Overall there was no difference in the number of bait insects positive for either phenotype, as one soil sample was positive for the YEL phenotype in both baitings.

2.3.1.4 Distribution of *H. downesi* colour phenotypes

Overall, 13 sampling locations were positive for *H. downesi* in 2008 and 7 sampling locations were positive in 2012. Although there was some overlap in their occurrence the Pur phenotype tended to occur in the front section of the dunes while the Yel phenotype was found mainly in the rear section of the dunes and the distribution of the two phenotypes was significantly different when positive sampling locations (distance into dunes) of each colour phenotype were compared were compared (Mann Whitney U test, W = 101.5, P = 0.006). The first positive sample in 2012 was at 20 m above the strand line, in embryonic dunes, forward of the first dune ridge. In 2008 the first soil sample was taken at 2 m above the strandline. In 2012 the first soil sample was again taken at 2 m above the strandline, however, in 2012 the dunes had advanced such that starting from a point 2 m above the strandline two extra soil samples were taken on the seaward side of the dunes in order to reach the same end point in the rear of the dunes (Table 2.17). Cadavers were scored positive for *H. downesi* on the basis of bioluminescence. It was difficult to discriminate cadaver colour in some instances as the YEL cadavers were pale and early in an infection PUR cadavers tend to be achromatic. The YEL colour phenotype was confirmed by carrying out individual bacterial extractions from 10 IJs

(Sections 2.2.3.1b above) for each isolate except for one of the positive cadavers at 20 m and the one at 90 m as there was no emergence from these two cadavers. These two cadavers were scored as YEL phenotypes on the basis of the bacterial extractions from the other potential YEL cadavers (data not shown) which yielded *yel* bacterial phenotype in all instances since all five cadavers had the same appearance.

Table 2.16: Number of dead bait insects recovered from soil samples from North Bull Island in 2012. Three individual soil samples were taken at each sampling location and each sample was baited twice with a single *G. mellonella* larva at each baiting (n = 6 bait insects per distance (10 m) into the dunes).

	Number of dead bait insects (/3)			
Distance (m)*	bait 1		bait 2	
	H. downesi	*S. feltiae*	*H. downesi*	*S. feltiae*
0	0	0	0	0
10	0	0	0	0
20	2	0	0	0
30	2	0	0	0
40	1	1	1	0
50	0	2	0	0
60	0	0	0	0
70	1	0	0	0
80	0	1	0	0
90	0	1	1	0
100	0	2	0	0
110	1	1	0	0
120	0	3	0	0
130	1	1	0	0
140	0	3	0	0
150	0	2	0	0
Total	8	17	2	0

* The first sampling location in each transect (0 m) was 2 metres above the strand line

Table 2.17: Detection of *H. downesi* PUR and YEL colour phenotype in sand dunes on North Bull Island in two surveys. Data shown is number of dead bait insects recovered from soil samples in transect 1. In 2008* each sample was baited twice and each baiting consisted of 15 *G. mellonella.* (n = 30 bait insects per distance). In 2012 each sample was baited twice and each baiting consisted of 1 *G. mellonella.* (n = 6 bait insects per distance).

		Transect 1			
distance (m)		2008		2012	
2008	2012	PUR	YEL	PUR	YEL
	0			0	0
	10			0	0
0 †	20	0	0	2	0
10	30	0	0	2	0
20	40	3	0	0	2 ‡
30	50	1	0	0	0
40	60	3	0	0	0
50	70	0	0	1	0
60	80	1	0	0	0
70	90	0	0	0	1 ‡
80	100	0	1	0	0
90	110	0	1	0	1 ‡
100	120	0	0	0	0
110	130	0	0	0	1
120	140	0	3	0	0
130	150	0	0	0	0
Total		8	7	5	5

* In 2008 30 subsamples per sampling location were pooled, mixed and then divided into three for baiting, with a total of 15 insects per distance per baiting. In 2012 three subsamples per sampling location were baited individually, with a total of 3 insects per distance per baiting.

† In both 2008 and 2012 the first soil sample was taken at 2 m above the strandline (= distance 0). In 2012 the dunes had advanced seaward such that two extra soil samples were taken to reach the same end point in the rear of the dune system.

‡ It was difficult to discriminate cadaver colour as the YEL cadavers were pale in some instances. The colour phenotype was confirmed by carrying out a bacterial extraction from 10 IJs for each isolate except for one of the positive cadavers at 20 m and the one at 90 m as there was no emergence from these two cadavers.

2.3.1.5 Habitat survey

The most common composite habitat recorded was Marram Dunes/Semi-Fixed Dunes/Fixed Dunes/Grassy Path, recorded at 10/42 sampling locations, followed by Marram Dunes/Semi-Fixed Dunes/Sandy Path, recorded at 8/ 42 sampling locations (Table 2.18). No *H. downesi* were detected in the categories that included Embryonic Dune habitat. Both colour phenotypes were detected in Marram and Semi-Fixed/Fixed Dune habitats that did or did not include paths. There was no clear difference between categories in the detection of the two colour phenotypes. The flora, soil temperature and pH records are given in Appendix Table A 5.

Table 2.18: Composite habitat types recorded at soil sampling locations on North Bull Island. The number of EPN infected bait insects is given. Detection of *H. downesi* PUR and YEL phenotypes is indicated by purple and yellow shading respectively. Absence of shading indicates detection of *S. feltiae* only. The first sampling location in eachtransect (0 m) was 2 metres above the strand line.

Distance (m)	Transect 1	Transect 2	Transect 3
0	Embryonic Dunes*	Embryonic Dunes	Embryonic Dunes
10	Embryonic Dunes, Marram Dunes, Sandy Path	Marram Dunes, Semi-Fixed Dunes, Sandy Path	Embryonic Dunes, Marram Dunes*
20	Marram Dunes, Semi-Fixed Dunes, Sandy Path*	Marram Dunes, Semi-Fixed Dunes, Fixed Dunes, Sandy Path*	Marram Dunes, Semi-Fixed Dunes, Sandy Path*
30	Marram Dunes, Semi-Fixed Dunes, Sandy Path*	Marram Dunes, Semi-Fixed Dunes, Fixed Dunes, Sandy Path*	Marram Dunes, Semi-Fixed Dunes, Sandy Path
40	Marram Dunes, Semi-Fixed Dunes, Sandy Path*	Marram Dunes, Semi-Fixed Dunes, Fixed Dunes*	Marram Dunes, Semi-Fixed Dunes, Fixed Dunes*
50	Marram Dunes, Semi-Fixed Dunes, Sandy Path*	Marram Dunes, Semi-Fixed Dunes, Fixed Dunes, Grassy Path	Marram Dunes, Semi-Fixed Dunes, Sandy Path*
60	Marram Dunes, Semi-Fixed Dunes, Fixed Dunes, Sandy Path, Blowout*	Marram Dunes, Semi-Fixed Dunes, Fixed Dunes, Grassy Path*	Marram Dunes, Semi-Fixed Dunes, Fixed Dunes, Grassy Path*
70	Marram Dunes, Semi-Fixed Dunes, Fixed Dunes, Sandy Path*	Marram Dunes, Semi-Fixed Dunes, Fixed Dunes, Grassy Path*	Marram Dunes, Semi-Fixed Dunes, Fixed Dunes, Grassy Path*
80	Marram Dunes, Semi-Fixed Dunes, Fixed Dunes, Sandy Path*	Marram Dunes, Semi-Fixed Dunes, Fixed Dunes, Grassy Path*	Marram Dunes, Semi-Fixed Dunes, Sandy Path, Grassy Path*
90	Marram Dunes, Semi-Fixed Dunes, Fixed Dunes*	Marram Dunes, Semi-Fixed Dunes, Fixed Dunes, Sandy Path, Grassy Path*	Marram Dunes, Semi-Fixed Dunes, Fixed Dunes, Sandy Path, Grassy Path*
100	Semi-Fixed Dunes, Fixed Dunes*	Marram Dunes, Semi-Fixed Dunes, Fixed Dunes, Grassy Path*	Marram Dunes, Semi-Fixed Dunes, Sandy Path, Grassy Path*
110	Semi-Fixed Dunes, Fixed Dunes, Grassy Path*	Marram Dunes, Semi-Fixed Dunes, Fixed Dunes, Grassy Path	Marram Dunes, Semi-Fixed Dunes, Grassy Path*
120	Semi-Fixed Dunes, Fixed Dunes, Grassy Path*	Marram Dunes, Semi-Fixed Dunes, Fixed Dunes, Sandy Path*	Marram Dunes, Semi-Fixed Dunes, Fixed Dunes, Grassy Path*
130	Semi-Fixed Dunes, Fixed Dunes*	Marram Dunes, Semi-Fixed Dunes, Fixed Dunes, Grassy Path*	Marram Dunes, Semi-Fixed Dunes, Grassy Path*

***** Sampling location positive for *S. feltiae*.

2.3.2 Phylogenetic analysis of the nematode ITS1 region

An initial alignment in ClustalW of the ITS1 sequences showed very little variation between the six isolates from North Bull Island with only 7 single nucleotide polymorphism in a total of 1031 shared positions (Appendix Figure A. 3). The BLAST search in the GenBank nucleotide database using the blastn algorithm showed that the ITS1 sequences from the *PUR* and *YEL* isolates were homologous with sequences within the ITS region from a range of *Heterorhabditis* species. Alignment of sequences in ClustalW is shown in Appendix Figure A. 4. There was no greater diversity within the North Bull Island isolates than there was between the two K122 isolates. All the North Bull Island nematode isolate shared a greater than 99% identity with each other and for one pair, PUR1 and YEL3 there was 100% identity over 705 bases in the final alignment. Similarly, there was a greater than 99% identity to two the sequences from *H. downesi* K122 produced by two different laboratories (see Section 2.2.2.6 Table 2.5). There was also a greater than 98% identity with two *H. downesi* isolates from Hungary, H3107 and H3173, the symbionts of which have been identified as *P. temperata* subsp. *cinerea* and *P. temperata* subsp. *temperata*, respectively.

The phylogenetic analysis showed that all six North Bull Island isolates belonged to the same cluster within the set of 29 sequences analysed (Figure 2.9). There appeared to be no separation of the PUR and YEL colour variants. The ITS1 sequences formed two main clusters. Cluster I contained all the North Bull Island isolates, the two K122 *H. downesi* isolates, the two Hungarian *H. downesi* isolates, H3107 and H3173, along with all *H. megidis* isolates, with 70% support for this cluster. Within cluster I the North Bull Island isolates, and the *H. downesi* isolates formed a separate clade from the *H. megidis* isolates with 76% support. Cluster II

contained all the *H. zealandica* isolates. The other species *H. marelatus, H. atacamensis* and *H. safricana*, formed a separate group that branched more deeply. Neighbour-joining and Minimum Evolution methods of phylogenetic inference showed the same clustering with practically identical bootstrap values for 1000 replications (data not shown).

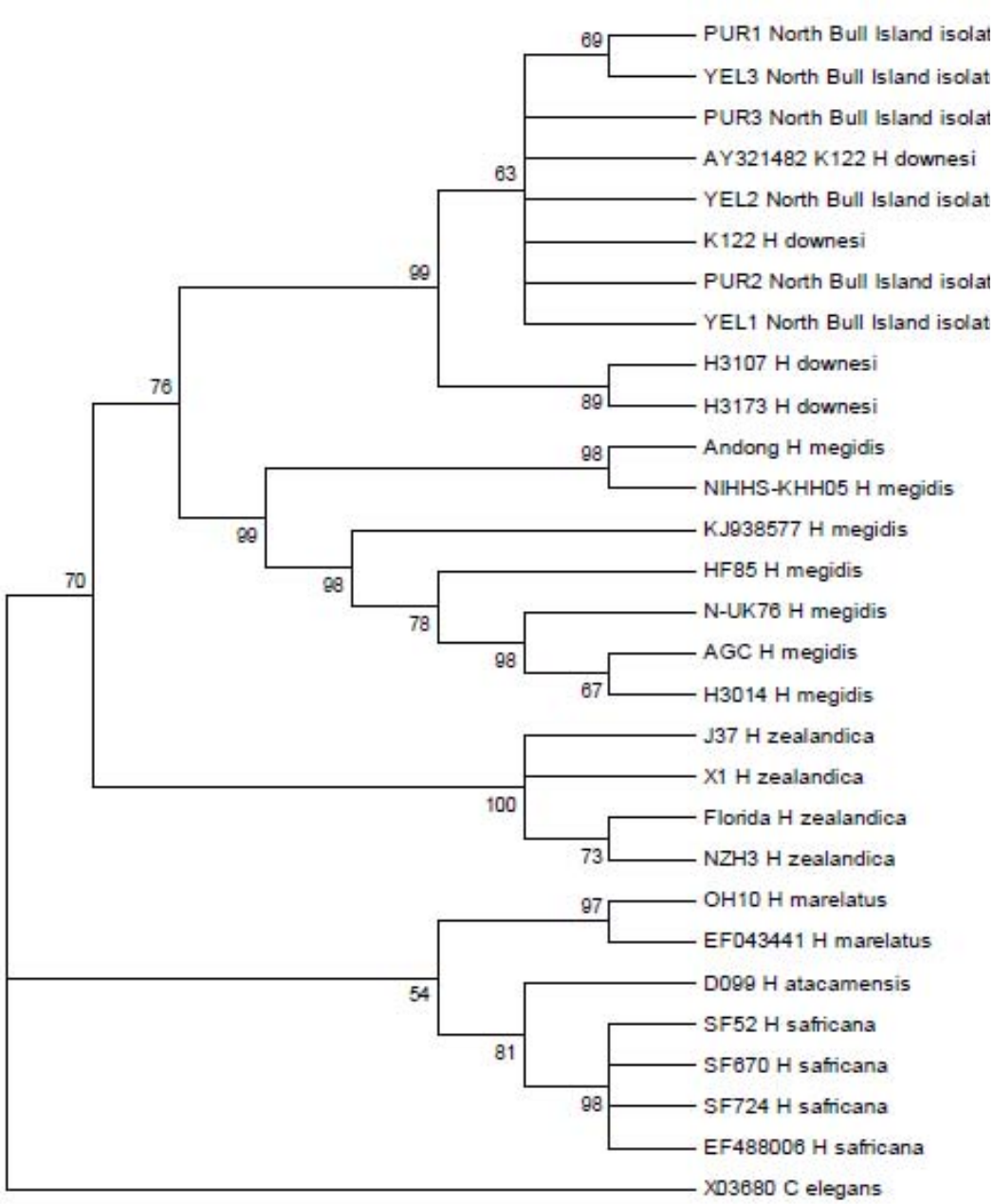

Figure 2.9: Maximum Likelihood phylogenetic tree based on a portion of the ITS1 sequence from six North Bull Island isolates and members of the *Heterorhabditis* species. *C. elegans* was used as an outgroup. Tree shown is the bootstrap consensus tree from 1000 replications.

2.3.3 Phenotypic characterisation of symbiotic bacteria

a) Morphological characterisation

The symbiotic bacteria were isolated from each of the nematode isolates listed in Table 2.15. All isolates were identified as *Photorhabdus* sp. on the basis of bioluminescence. All isolates adsorbed dye from MacConkey agar and NBTA plates. All isolates formed large, convex colonies with irregular margins. All isolates were gram-negative, motile, rod shaped bacteria.

b) Microbial growth - direct cell count

Direct cell counts for the isolates *pur1* and *yel3* were carried out in three separate experiments using liquid cultures set up as described in Section 2.2.3.3. Two of the experiments were carried out using cultures grown in nutrient broth and one used cultures grown in MacConkey broth. The elapsed time between sampling ranged from 6 hours to 24 hours. The doubling time was found to vary between experiments and growth media, ranging from 1.61 to 2.7 hrs/generation (*pur1*) and 1.62 to 2.59 hrs/generation (*yel3*) (Table 2.19). However, there was no significant difference in doubling time between the colour phenotypes (paired t-test, $T = 1.61$, $P > 0.05$). (see also Appendix Figure A. 5 for growth curves).

Table 2.19: Doubling times of *pur* and *yel* isolates. Cultures were sampled twice with at least 6 hours between time points. Doubling time was calculated from the average of two counts at each time point.

		Doubling time (hrs/generation)	
Experiment	Growth medium	*pur1*	*yel3*
1	Nutrient Broth	1.61	1.62
2	Nutrient Broth	2.60	2.53
3	MacConkey broth	2.55	2.59

c) Microbial growth – plate counts

In experiments 1 and 2 of the direct cell counts in Section 2.3.3b an aliquot of each cell culture was spread on replicate NA plates. In experiment 1 the colony counts showed both isolates grew on NA. Growth was similar for both isolates with a higher number of colony forming units (CFU) being recorded for the *yel* phenotype than for the *pur* (Figure 2.10). In experiment 2 at the second sampling point both cultures were spread on NA, NBTA and on MacConkey agar plates. It was noted that a greater number of colonies of the *pur* isolate grew on MacConkey agar than on NA or NBTA plates. This difference in number of colonies was not observed with the *yel* isolate.

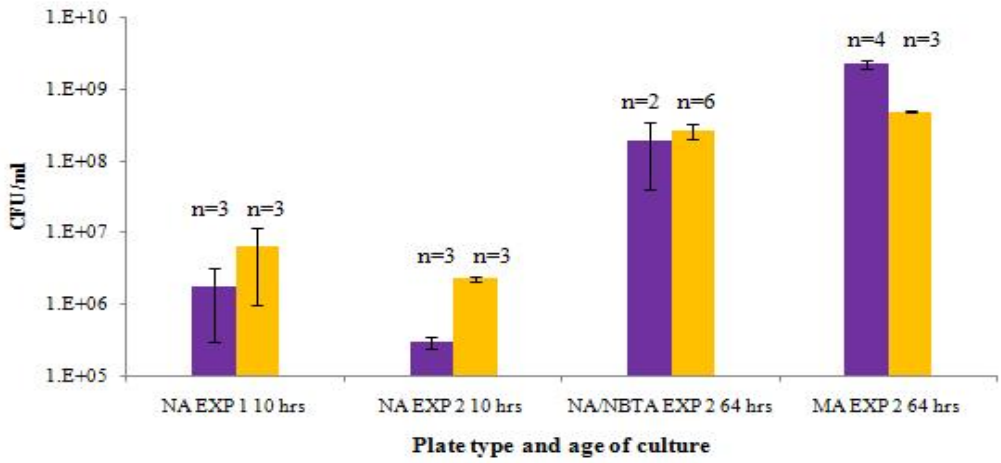

Figure 2.10: Growth of *Photorhabdus pur* and *yel* isolates on nutrient agar (NA), nutrient agar with Bromothymol Blue (NBTA) and MacConkey agar (MA) in two trials. All samples were drawn from cultures in nutrient broth. Values are mean (± SE) colony forming units (CFU) per ml of culture medium Number of plates (n) in each trial is indicated above the bar.

This result was further investigated in a separate experiment by carrying out replicate plating on all three media. More colonies of the *pur* isolate grew on MacConkey agar than on either the nutrient agar or nutrient agar supplemented with Bromothymol Blue (Figure 2.11). This result was significant at the $P < 0.05$ level. (2-sample t-test, 4 df, $T = 4.25$, $P < 0.05$). The nutrient agar (NA) and nutrient agar supplemented with Bromothymol Blue (NBTA) plates were treated as one type as there was no difference between the NA and NBTA plates when these were treated as separate media and a one-way ANOVA was carried out on NA, NBTA and MA (results not shown). There was no difference in the number of *yel* colonies between nutrient agar and MacConkey agar.

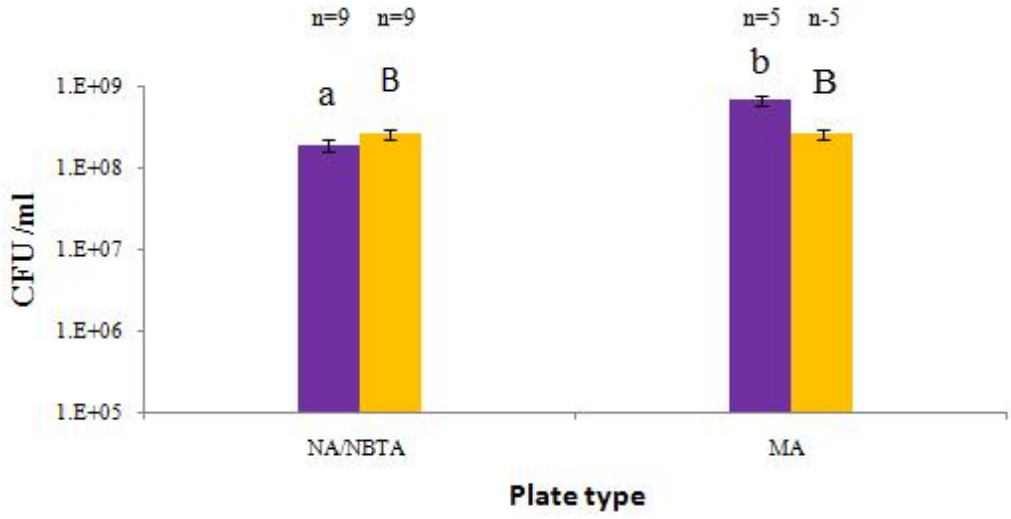

Figure 2.11: Growth of *Photorhabdus pur* and *yel* isolates on nutrient agar (NA), nutrient agar with Bromothymol Blue (NBTA) and MacConkey agar (MA) Number of plates (n) in each trial is indicated above each bar. Within colour (*pur, yel*) same letters indicate no significant difference between plate type.

As the *pur* isolate appeared to be disadvantaged in nutrient agar or broth versus MacConkey agar or broth and growth rate in MacConkey broth was the same for both isolates (Table 2.19) this medium was used for subsequent experiments requiring an inoculum of liquid culture.

d) Biochemical characterisation

In the API 20E tests all isolates were negative for oxidase activity, β-galactosidase activity on *o*-nitrophenyl-βD-galactopyranoside, lysine decarboxylase, ornithine decarboxylase, hydrogen sulphide production, urease activity, acetoin production and nitrate reduction to nitrite (Table 2.20). All isolates were positive for arginine dihydrolase, citrate utilisation and gelatinase. Only the *yel* isolates were positive for

tryptophan deaminase. The *pur* isolates and a single *yel* isolate (*yel1*) were positive for indole production.

Table 2.20: Characteristics of *Photorhabdus* isolates from North Bull Island (API 20 E test strip). Results are indicated as follows: +, positive; -, negative; w, weakly positive. Unusual results are highlighted**.**

Reaction	*pur1*	*pur2*	*pur3*	*yel1*	*yel2*	*yel3*
β-galactosidase	-	-	-	-	-	-
Arginine dihydrolase	+	+	+	+	+	+
Lysine decarboxylase	-	-	-	-	-	-
Ornithine decarboxylase	-	-	-	-	-	-
Citrate utilisation	+	+	+	+	+	+
H_2S production	-	-	-	-	-	-
Urease	-	-	-	-	-	-
Tryptophane deaminase	-	-	-	+	+	+
Indole production	w	w	w	+	-	-
Acetoin production	-	-	-	-	-	-
Gelatinase	+	+	+	+	+	+
Cytochrome-oxidase	-	-	-	-	-	-
NO_2 production	-	-	-	-	-	-
reduction to N_2 gas	-	-	-	-	-	-

In the API 50 CH tests acid was produced from 21 of the 49 carbohydrates in the test kit. Only substrates where at least one isolate released acid are shown (Table 2.21). All isolates produced acid from D-glucose, D-mannose, Esculin, D-fructose (weakly), D-ribose (weakly), inositol (weakly), N-acetylglucosamine, glycerol and D-maltose (weakly). Only *pur* isolates produced acid from salicin, D-sorbitol and methyl-αD-glucopyranoside and amygdalin. Only *yel* isolates produced acid from D-

trehalose, methyl-βD-xylopyranoside and L-lyxose. Some but not all isolates of each colour phenotype produced acid from D-turanose, L-fucose, gluconate, 2-ketogluconate and 3-ketogluconate.

Table 2.21: Carbohydrate fermentation by *Photorhabdus* isolates from North Bull Island (API 50 CH test strip). Results are indicated as follows: +, positive; -, negative; w, weakly positive. Substrates where all isolates were negative have been omitted. Substrates where only *pur* or *yel* were positive are outlined in purple or yellow outline respectively.

Acid produced from	*pur1*	*pur2*	*pur3*	*yel1*	*yel2*	*yel3*
D-Glucose	+	+	+	+	+	+
D-Mannose	+	+	+	+	+	+
Esculin	+	+	+	+	+	+
D-Fructose	w	w	w	w	w	w
D-Ribose	w	w	w	w	w	w
Inositol	w	w	w	w	w	w
D-Maltose	w	w	w	w	w	w
N-Acetylglucosamine	+	w	w	w	+	+
Glycerol	+	+	+	w	w	w
Salicin	w	w	w	-	-	-
D-Sorbitol	w	-	-	-	-	-
Methyl-αD-Glucopyranoside	+	-	-	-	-	-
Amygdalin	-	w	-	-	-	-
D-Trehalose	-	-	-	w	w	w
Methyl-βD-Xylopyranoside	-	-	-	w	-	-
D-Lyxose	-	-	-	-	-	w
D-Turanose	+	-	-	-	-	w
L-Fucose	w	w	-	w	+	+
Potassium Gluconate	w	-	w	w	w	w
Potassium 2-Ketogluconate	w	-	-	-	w	-
Potassium 3-Ketogluconate	w	w	-	-	w	w

2.3.3.1 Antibiosis

In antibacterial tests all *Photorhabdus* isolates inhibited the growth of Gram-negative (*E. coli*) and Gram-positive (*B. subtilis, M. luteus)* organisms (Figure 2.12). The mean size of the inhibition zone varied between target organisms. For both colour phenotypes the greatest inhibition was seen against *M. luteus* (Figure 2.12C),

followed by *E. coli (*Figure 2.12A). The least inhibition was seen against *B. subtilis* (Figure 2.12B).

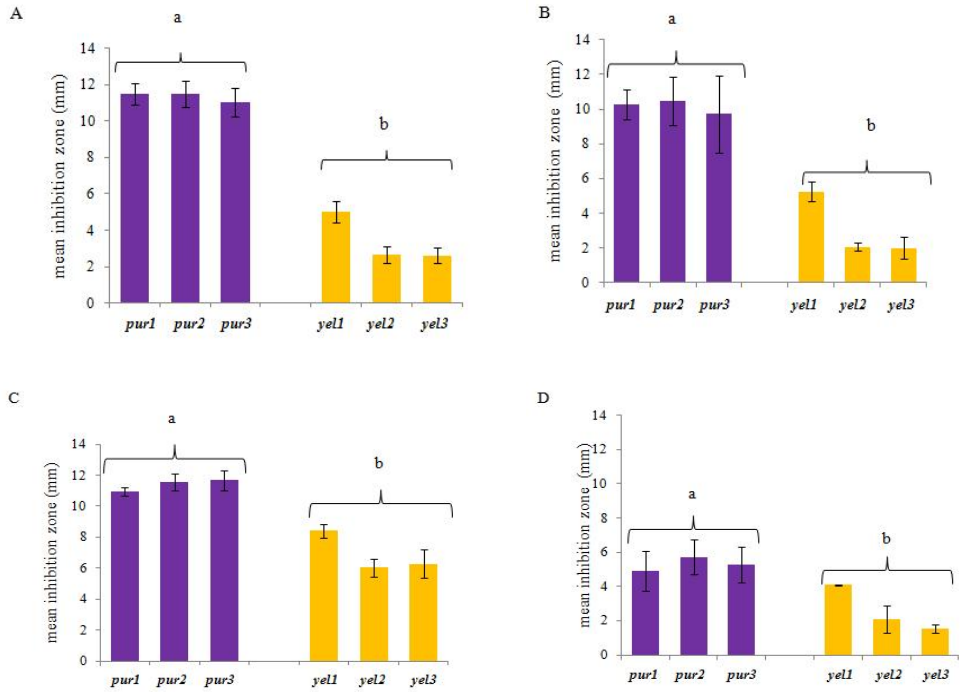

Figure 2.12: Mean size of inhibition zone around individual *Photorhabdus* isolate spot colonies. Plates were examined after 20 hr (A - *E. coli*, B - *B. subtilis*, D - *X. bovienii*) or 40 hr (C - *M. luteus*). Results are mean of three trials with three *pur* and three *yel* isolates in each trial. Treatments which do not share the same letter are significantly different. (nested ANOVA, See Table 2.22).

For all four target organisms larger inhibition zones were recorded for the *pur* phenotype than for the *yel* phenotype. The difference between phenotypes was significant ($P < 0.05$), while that between individual isolates when phenotype was included as a factor was not significant (nested ANOVA, see Table 2.22). Figure 2.13 shows typical inhibition of target organisms.

Table 2.22: Inhibition zone for two *Photorhabdus* colour phenotypes (n = 3) against four target organisms. Inhibition zone was calculated from average inhibition in two (*X. bovienii*) or three trials. Data were analysed using nested ANOVA. Treatments which do not share the same letter are significantly different.

	Mean inhibition zone (mm)		Test statistic (df)	
	pur	*yel*	F	P-value
E. coli	11.3 a	3.4 b	$94.336_{1,4}$	0.001
B. subtilis	10.1 a	3.1 b	$41.321_{1,4}$	0.003
M. luteus	11.4a	6.9b	$32.689_{1,4}$	0.005
X. bovienii	5.3a	2.6b	$11.542_{1,4}$	0.027

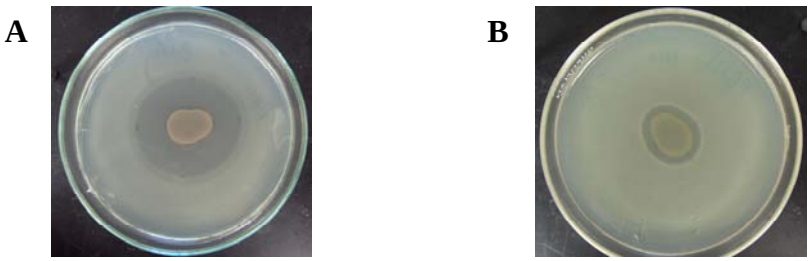

Figure 2.13: Inhibition of *E. coli* by *pur1* (A) and *yel3* (B).

In intraspecific tests inhibition was observed in only two combinations, *pur2/yel1* and *yel1/pur2* (Figure 2.14). The mean size of the inhibition zone was greater in the case of the *yel* isolate as target organism (Table 2.23).

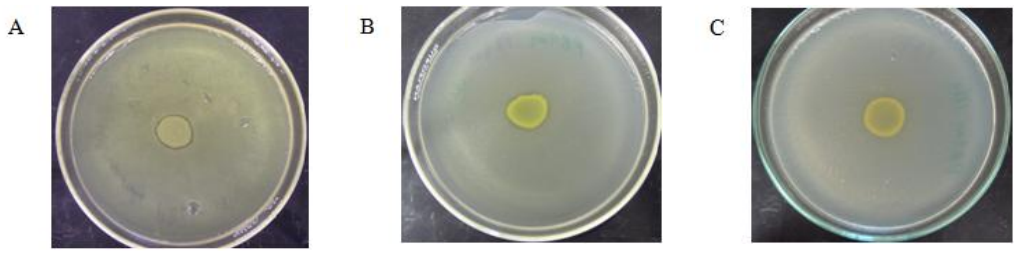

Figure 2.14: There was very slight intraspecific inhibition between *pur* and *yel Photorhabdus* isolates from North Bull Island. Interactions are A: *pur2* inhibits *yel1*. B: *yel1* inhibits *pur2*. C: *yel1* does not inhibit *pur1*.

Table 2.23: Intraspecific inhibition in *Photorhabdus* colour phenotypes (n = 2)

Spot colony	target	mean inhibition (mm)
pur1	*yel3*	0.00
pur2	*yel1*	0.50
pur3	*yel2*	0.00
yel1	*pur2*	0.20
yel2	*pur3*	0.00
yel3	*pur1*	0.00

In antimycotic tests all target organisms were inhibited by all *Photorhabdus* isolates (Figure 2.15A-C and Figure 2.16A-C). The *pur* isolates produced larger zones of inhibition than the *yel.* For each colour phenotype the greatest inhibition was seen against *B. bassiana (*Figure 2.15*A)* followed by *M. anisopliae* (Figure 2.15B) and *Mucor* (Figure 2.15C). For all three target organisms larger inhibition zones were recorded for the *pur* phenotype than for the *yel* (Table 2.24). The difference between phenotypes was significant for all three target organisms ($P < 0.05$) while the difference between individual isolates was not significant (nested ANOVA, see Table 2.24).

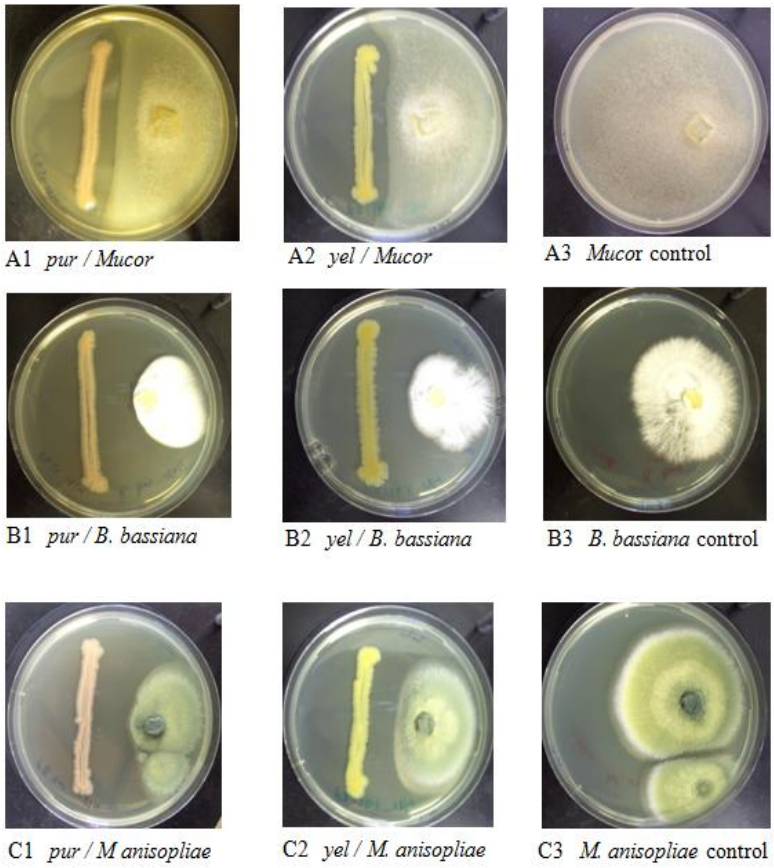

Figure 2.15: Inhibition of A - *Mucor*, B – *B. bassiana*, C – *M. anisopliae* by *pur* (A1, B1, C1) and *yel* (A2, B2, C2,) *Photorhabdus* isolates. Control plates (A3, B3, C3). All tests were run 3 times with 3 isolates of each colour phenotype per trial.

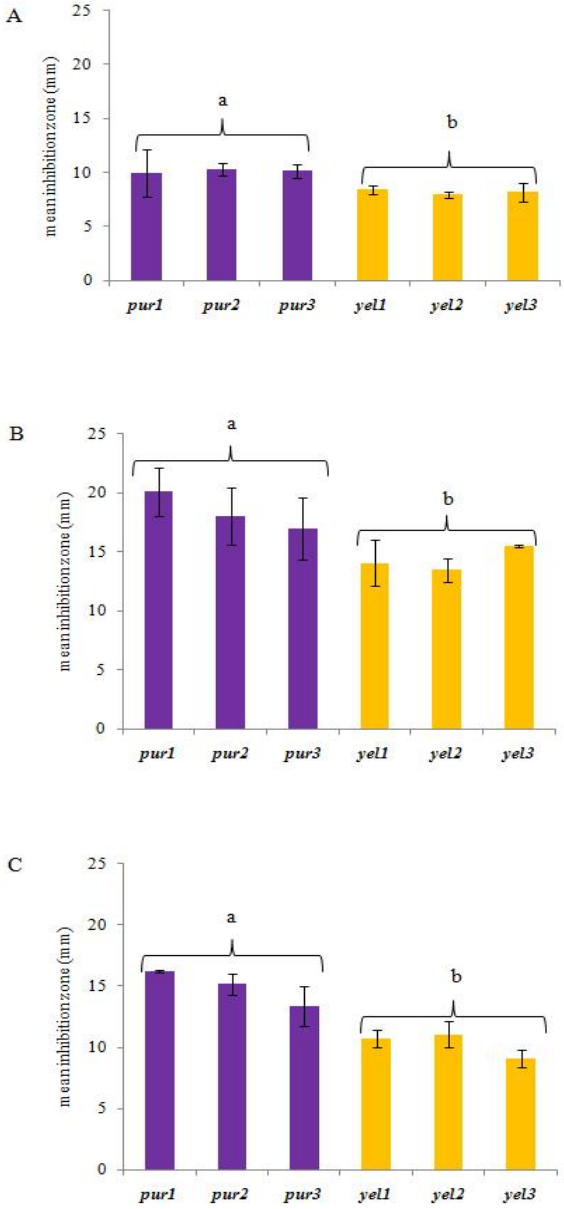

Figure 2.16: Inhibition of fungal growth by *Photorhabdus* isolates. Mean size of inhibition zone for individual *Photorhabdus* isolates. Data shown is for 7 days (A - *Mucor*) or 28 days (B - *B. bassiana*, C - *M. anisopliae*) growth. Results are mean of three trials with three *pur* and three *yel* isolates in each trial (2 *yel* in trial 1 *B. bassiana* only). Treatments which do not share the same letter are significantly different (nested ANOVA See Table 2.24).

Table 2.24: Inhibition zone for two *Photorhabdus* colour phenotypes (n = 3) against three fungal organisms. Inhibition zone was calculated from average inhibition in three trials. Data were analysed using nested ANOVA. Treatments which do not share the same letter are significantly different.

	Mean inhibition zone (mm)		Test statistic (df)	
	pur	*yel*	F	P-value
B. bassiana	18.4a	14.3b	$13.18_{1,4}$	0.020
M. anisopliae	14.9a	10.3b	$20.20_{1,4}$	0.011
Mucor sp.	10.1a	8.2b	$163.58_{1,4}$	<0.001

2.3.4 Phylogenetic analysis of symbiotic bacteria

Phylogenetic analysis of the *gyrB* gene sequences showed that the *pur* and *yel* bacterial isolates from North Bull Island belong to distinct subspecies of *Photorhabdus temperata*: *Photorhabdus temperata* subsp. *cinerea* and *Photorhabdus temperata* subsp. *temperata*, respectively. The initial alignment of the edited nucleotide sequence of the six isolates showed single nucleotide polymorphisms (SNPs) at a total of 78 sites (Appendix Figure A. 6). At each SNP site all three isolates of each colour variant shared the same nucleotide. Six of the SNP sites produced non-synonymous amino acids in the translated protein sequence identified by ORF Finder (Table 2.25).

Table 2.25: Position of non-synonymous amino acids in translated *gyrB* gene sequence from *Photorhabdus* isolates from North Bull Island (see also Appendix Figure A. 6)

amino acid position	*pur* isolates	*yel* isolates
39/460	Isoleucine (Ile)	Valine (Val)
83/460	Arginine (Arg)	Serine (Ser)
125/460	Glutamate (Glu)	Lysine (Lys)
155/460	Glycine (Gly)	Serine (Ser)
160/460	Alanine (Ala)	Glycine (Gly)
279/460	Serine (Ser)	Glycine (Gly)

The BLAST search in the GenBank nucleotide database using the blastn algorithm showed that the *pur* and *yel* isolates were homologous with *gyrB* gene sequences from a range of *Photorhabdus* species. Alignment of sequences in ClustalW is shown in Appendix Figure A. 7. The three purple isolates shared 100% identity over the 847 nucleotide sequence analysed with three *P. temperata* subsp. *cinerea* isolates and one other *P. temperata* sp. isolate and for one of these, H3014, there was 100% identity over the full 1402 base sequence. However, the *pur* isolates had an alignment score of only 93% against the three *yel* isolates and *P. temperata* subsp. *temperata* isolate K122, the only Irish isolate for which a *gyrB* sequence was available in GenBank. Conversely, the three *yel* isolates had an alignment score of 100% against K122, and two other Hungarian isolates, *P. temperata* subsp. *temperata* isolate H3179, isolated from *H. downesi* and isolate H111, described as *P. temperata* sp., isolated from *H. megidis*.

The phylogenetic analysis showed that the *pur* and *yel* isolates belonged to separate clusters within the set of sequences analysed (Figure 2.17). The *gyrB* sequences

formed four main clusters with three *P. temperata* subsp. *tasmaniensis* forming a separate group that branched more deeply. Cluster I contained the three *P. temperata* subsp. *khanii* American isolates, Meg, C1 and Habana, isolated from *H. megidis, H. bacteriophora* and an unidentified *Heterorhabditis* species respectively. There was 90% support for this cluster. Cluster II contained the three *pur* isolates along with four Hungarian isolates: three *P. temperata* subsp. *cinerea* isolates, H3240, H3107, from *H. downesi,* and H3014, isolated from *H. megidis,* one *Photorhabdus temperata* isolate, H267, isolated from *H. downesi* and one *Photorhabdus* sp. isolate, H3086, isolated from *H. downesi.* There was 98% support for this cluster. Cluster III contained three *P. temperata* subsp. *thracensis* isolates, H3210, CIP108426 isolated from *H. bacteriophora* from Hungary and Turkey respectively and FR32, isolated from an unidentified nematode from France. There was 98% support for this cluster. Cluster IV showed more variation with the three *yel* isolates grouped together with two *P. temperata* subsp. *temperata* isolates, K122 isolated from *H. downesi* from Ireland and H3179 isolated from *H. downesi* from Hungary and one isolate described as *P. temperata* sp., H111, isolated from *H. megidis* from Hungary, 80% support. The isolate H3182, isolated from *H. downesi* from Hungary was distinct from this group with 80% support. These seven isolates were separated from the other eight isolates in Cluster IV with 97% support for the two clades. This last group contained seven *P. temperata* subsp. *temperata* isolates along with one isolate described as *P. temperata* sp., isolated from *H. megidis* and *H. downesi* from The Netherlands, Lithuania, Russia and Hungary. Neighbour-joining and Minimum Evolution methods of phylogenetic inference showed the same clustering with practically identical bootstrap values for 1000 replications (data not shown).

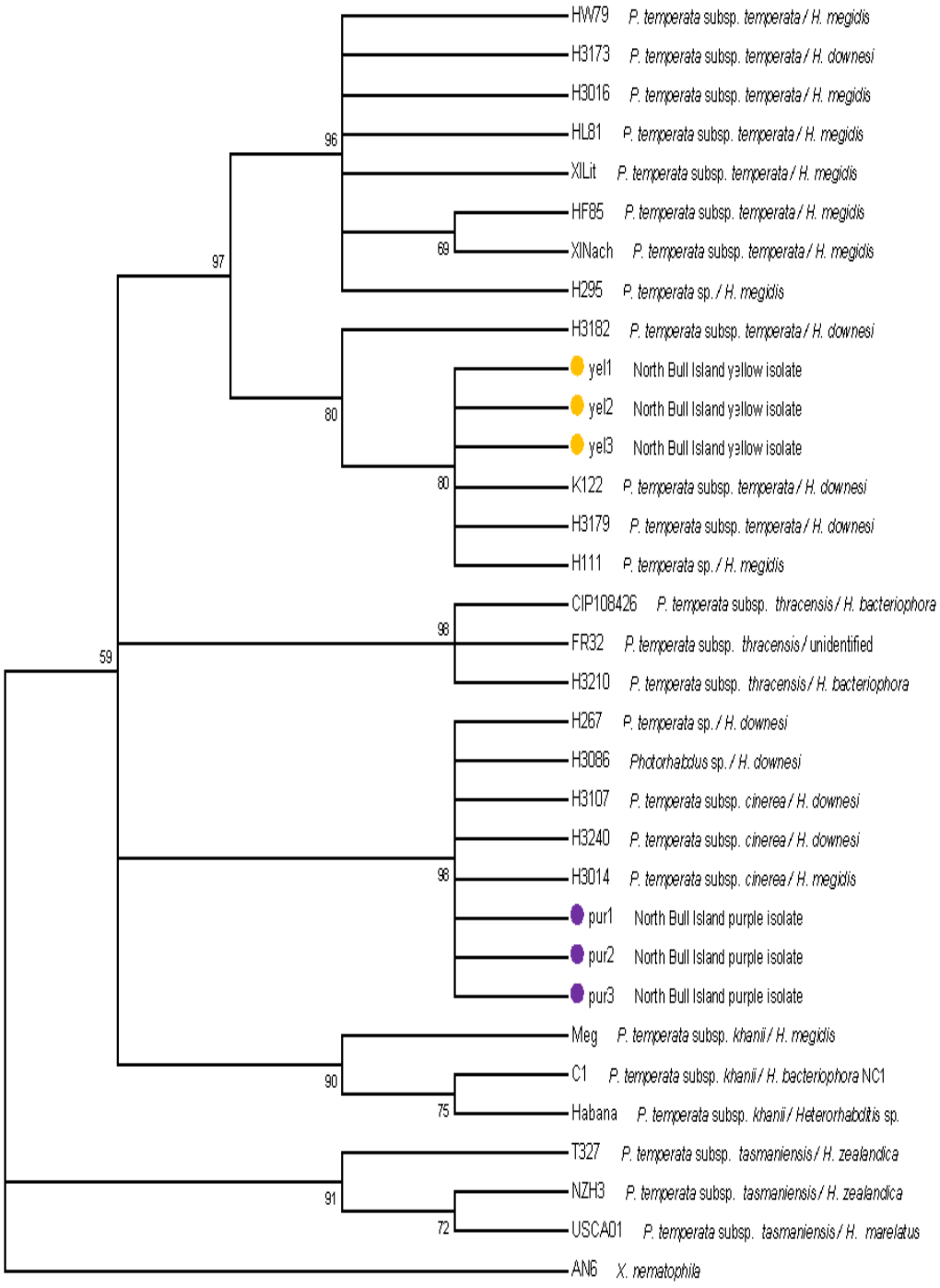

Figure 2.17: Maximum Likelihood phylogenetic tree, based on a portion of the *gyrB* gene from six North Bull Island isolates and members of the *Photorhabdus temperata* species. *X. nematophila* was used as an outgroup. Tree shown is the bootstrap consensus tree from 1000 replications**.**

2.4 **Discussion**

The detection of two species of entomopathogenic nematodes, *Heterorhabditis downesi* and *Steinernema feltiae*, in the sand dune complex on North Bull Island is consistent with a previous study (Rolston *et al.*, 2005) (see Appendix Figure A. 1 for overlap of sampling areas in this study and the Rolston *et al.* (2005) study). These two species along with *S. affine* have previously been recorded in Ireland (Griffin *et al.*, 1991). In the study reported here *H. downesi* was detected in 13/42 (31%) of soil samples and *S. feltiae* was detected in 34/42 (34%) of soil samples. This is much higher than the levels detected by Rolston *et al.* (2005) in surveys of the same site in 2001 and 2002 but comparable to those reported by Griffin *et al.* (1994; 1999) for sites in mainland Europe and Ireland and the UK respectively. In the current study in 2008 the detection of the two species, as percentage of bait insects killed, was 1.7% and 10.4% for *H. downesi* and *S. feltiae* respectively in 2008 (three transects) and 10.4% and 17.1% for *H. downesi* and *S. feltiae* respectively in 2012 (transect 1 only). The levels detected by Rolston *et al.* (2005) at the same site in two successive years, 2001 and 2002, as percentage of bait insects killed, was 0.48% and 1.88% respectively, while that of *S. feltiae* was 0.63% and 1.6% respectively. While the 2008 level of detection of *H. downesi,* as percentage of bait insects killed, was similar to that of the Rolston *et al.* (2005) study the level of detection of *S. feltiae* was higher. This was due in part to two high observations in transect 1 with detection levels of 36.7% and 56.7% as percentage of bait insects killed at the 40 m and 90 m sampling locations respectively. The level of detection for both nematode spp. was much higher in 2012.

The distribution of entomopathogenic nematodes is reported as patchy by several studies and can be expected to vary both spatially and temporally (Stuart & Gaugler,

1994; Lawrence *et al.*, 2006; Campos-Herrera *et al.*, 2007). This variation can be due to a number of factors such as host availability, presence of competitors and predators, as well as a range of abiotic factors such as soil type, moisture, temperature and land use (Mrácek *et al.*, 2005; Hoy *et al.*, 2008). Localised areas of high density of infective juveniles, such as that observed in transect 1 for *S. feltiae* (2008) may also be due to the death of infected insects and subsequent emergence and dispersal of infective juveniles (Timper *et al.*, 1988; Spiridonov *et al.*, 2007).

In this study and the Rolston *et al* (2005) work soil baiting with *G. mellonella* was used to detect entomopathogenic nematodes; however the Rolston *et al* (2005) study differed in that soil samples consisted of individual 10 x 10 x 150 mm cores and was conducted in July and August 2001 and 2002 whereas in 2008 this study used a bucket type corer (12 mm x 10 cm, Figure 2.2) and pooled soil samples and sampling was carried out in September and October 2008 and in 2012 a targeted sampling of transect 1 where both *H. downesi* colour phenotypes had previously been detected was carried out. Differences in detection levels of entomopathogenic nematodes between this study and the previous studies on this site may be due to differences in sampling and baiting method or to time of sampling. It is also the case that repeat sampling may fail to detect EPN in sites that were previously positive for them (Hominick & Briscoe, 1990; Rolston *et al.*, 2005).

In this study, bait insects infected by *H. downesi* showed two colour phenotypes, purple and yellow; designated PUR and YEL respectively. This is similar to Rolston *et al.* (2005). In 2008 the PUR phenotype was detected in all three transects sampled whereas the YEL phenotype was detected in transect 1 only, and the colour phenotypes were spatially segregated here. This is similar to Rolston *et al.* (2005) who, although they found no significant difference in the distribution of the colour

phenotypes into the dunes, found that the PUR was more dominant in the front 50 m of the dunes in the second year (2002) of that study. In a subsequent study in 2005 Rolston found that the purple phenotype was significantly more prevalent in terms of number of positive soil samples and in terms of number of bait insects invaded in the front section of the dune system (0 m - 40 m) than in the rear section (60 m -100 m) and that for the yellow phenotype significantly more bait insects were invaded in the rear section (Rolston *et al.*, 2006). In 2012 the PUR and YEL phenotypes co-occurred in the front of the dunes and the PUR were more abundant here but not significantly so. None of the PUR phenotype was detected in the rear portion of the dunes in 2012. In the 2012 both *H. downesi* phenotypes had migrated forward with the advancing dunes and the PUR phenotype was detected 20 m forward of the first location that was positive for PUR in 2008 (Table 2.17). The YEL phenotype was detected 60 m forward of the first location that was positive for YEL in 2008. This forward movement of the nematodes with the advancing sand dunes may be due to the movement of infected insects prior to death or may be due to infected insects being dispersed by other predators such as birds and small mammals. It also appears to be the case the humans may play a role with higher occurrence of EPN reported where disturbance has occurred (Mracek & Webster, 1993; Rolston *et al.*, 2005). These repeat surveys of the same site point to the variability of the population of entomopathogenic nematodes associated with a particular site and is consistent with other studies (Lawrence *et al.*, 2006).

While it is difficult to directly correlate habitat with colour phenotype in this study, because soil samples were pooled at each sampling distance, a number of observations are possible. The PUR phenotype occurred where the ground cover was fragmented and *A. arenaria* was the dominant species. The ground in this front

section of the dunes was transected with many paths, leading to stretches of bare sand. In all sampling locations where the PUR phenotype was detected sandy paths comprised 1/5 to 1/3 of the cores taken. *Ammophila arenaria* was present throughout the dune system but was not the dominant species beyond 60 m into the dune system. The YEL phenotype was more likely to be associated with fixed dune type habitat. This habitat is characterised by broadleaved herbs such as *Anthyllis vulneraria, Galium verum*, *Ononis repens*, *Plantago lanceolata* and grasses such as *Festuca rubra* and *Agrostis* spp. (Fossitt, 2000; Rodwell *et al.*, 2000) and was the dominant habitat from 60 m into the dune system along transect 1. Ground cover was more or less complete in the rear 70 m of the dunes where a dense mat of living and dead plant material tended to accumulate, leaving few bare patches. Where this occurred the soil was well shaded and tended to be more moist. This may have been an important factor in the survival of the YEL phenotype. This is discussed in more detail in Chapter 5.

Little is known about the natural host range of entomopathogenic nematodes although they have been isolated from a range of insects in the field with *Heterorhabditis* spp. being recovered from Lepidoptera and Coleoptera (Peters, 1996; Adams & Nguyen, 2002). *Heterorhabditis downesi*-infected larvae of the garden chafer, *Phyllopertha horticola* have been found in modified dune grassland in Co. Clare by R. Enright (C. Griffin, pers. comm.). The harsher environment at the front of the dune system tends to favour sand-dune specialists whilst grassland species, some but not all of which are coastal species, tend to occupy the dune grassland of the fixed dunes (Speight, 1997). The black Marram weevil, *Otiorhynchus atroapterus* was observed throughout the dune system both in the Rolston *et al.* (2005) surveys and in the course of the present work larvae of the

cinnabar moth *Tyria jacobaeae* and adult *O. atroapterus* were observed throughout the dune system and adult *Psilothrix* spp. (Coleoptera) were observed in the front section of the dune system (Maher, personal observation). Many of the insects which are potential hosts for the entomopathogenic nematodes on North Bull Island may be nocturnal, live in the litter layer at the base of the grass tussocks or are viable hosts only in the larval stage. While this study did not include a systematic faunistic survey of North Bull Island adults of Lepidoptera, Diptera, Coleoptera and Hymenoptera were frequently observed and the larvae of these may be hosts for entomopathogenic nematodes. In a survey of the Czech Republic entomopathogenic nematodes occurred more frequently in habitats that had a high abundance of suitable hosts than those that had a low insect abundance (Mrácek *et al.*, 2005). Emelianoff *et al*. (2008b) suggested that there was an association between nematode strain and habitat type in a survey of soils in Southern France. Factors such as host availability and soil type as well as physiological adaptations all play a role in nematode distribution (Adams *et al.*, 2006). Differences in range of potential hosts between the front and the back of the dune system may be part of the explanation for the spatial segregation of the colour phenotypes.

An alignment of the *gyrB* sequence from the bacterial symbionts associated with the *H. downesi* nematode from North Bull Island showed that the *pur* and *yel* bacterial isolates could be separated on the basis of nucleotide sequence. Phylogenetic analysis of the six North Bull Island bacterial symbionts of *H. downesi* identified the three *pur* isolates as *P. temperata* subsp. *cinerea*, first described by Toth & Lakatos (2008), and identified the three *yel* isolates as *P. temperata* subsp. *temperata*. Thus the *H. downesi* nematodes on North Bull Island are symbiotically associated with two different *Photorhabdus temperata* subspecies. This is the first finding of two

different subspecies of symbiotic bacteria associated with a single entomopathogenic nematode in Ireland. While the recovery of two different subspecies of bacterial symbiont from a single species of *Heterorhabditis* is unusual it is not unknown. It has been shown that a single *Photorhabdus* species can be associated with more than one *Heterorhabditis* species (Fischer-Le Saux *et al.*, 1999; Akhurst *et al.*, 2004; Tailliez *et al.*, 2010). Conversely, a single *Heterorhabditis* species can be associated with more than one *Photorhabdus* species or subspecies (Hazir *et al.*, 2004; Adams *et al.*, 2006; Emelianoff *et al.*, 2008b; Maneesakorn *et al.*, 2011). The Emelianoff *et al.* (2008b) survey in Southern France found two strains of *H. bacteriophora,* at different sampling locations, one of which was associated with *P. luminescens* subsp. *kayaii* and was recovered from four different beach sites and one meadow while the other strain was associated with *P. luminescens* subsp. *kayaii* in a vineyard site and associated with *P. luminescens* subsp. *laumondiii* in a separate vineyard site and a non-agricultural field site. While the surveys by Emelianoff *et al*. (2008b) and Hazir *et al*. (2004) and others (Noujeim *et al.*, 2011) reported the association of a single species of nematode with more than one species or subspecies of bacterial symbiont their findings showed that the different combinations occurred at separate sites. However, in Hungary Toth & Lakatos (2008), isolated *P. temperata* subsp. *cinerea* from both *H. downesi* and *H. megidis.* In a further study they reported the isolation of both *P. temperata* subsp. *cinerea* and *P. temperata* subsp. *temperata* from both *H. downesi* and *H. megidis* isolated from the two sites in central Hungary, an oak forest and a clearing 1 km distant from the forest site (Tóth & Lakatos, 2009). The Hungarian studies do not give any details of soil types associated with the occurrence of the nematode/bacterial combinations, or the spatial distribution of the bacterial subspecies within the sampling sites, however sampling areas were chosen

on the basis of being the preferred habitat of the common cockchafer, *Melolontha melolontha,* a major horticultural pest in Hungary. The results from North Bull Island are a further confirmation of the association of a single *Heterorhabditis* species, *H. downesi,* with two different bacterial subspecies, *P. temperata* subsp. *temperata* and *P. temperata* subsp. *cinerea* in a single site.

The clustering obtained in the *gyrB* phylogenetic tree is broadly consistent with other studies (Akhurst *et al.*, 2004; Kuwata *et al.*, 2008; Tóth & Lakatos, 2008; Tóth *et al.*, 2008; An & Grewal, 2010a; Tailliez *et al.*, 2010; An & Grewal, 2011a). The grouping at subspecies level matches that obtained in these studies. The three *P. temperata* subsp. *tasmaniensis* isolates USCA01, NZH3 and T327 form a separate monophyletic group as do the American isolates C1, Meg and Habana, which are classified as *P. temperata* subsp. *khanii* by Tailliez *et al,* (2010) and as *P. temperata* subsp. *stackebrandtii* subsp. nov. by An & Grewal (2010b). Within the European species and subspecies the position of K122 and XINach in separate clades within the *P. temperata* subsp. *temperata* group and the position of the three Hungarian *P. temperata* subsp. *cinerea* isolates, H3107, H3240 and H3107 as a separate monophyletic group is well supported in the literature. Thus the identification of the *pur* and *yel* isolates as belonging to separate subspecies is based on a phylogeny that matches previous work.

To date only a single *Heterorhabditis* species, *H. downesi,* has been recorded from Ireland. The isolate K122, the type specimen for the species, has been sequenced by two other groups (Nguyen *et al.*, 2004; Regeai & Burnell, 2006). The analysis of the ITS1 region of the ribosomal genes identifies the nematode isolates in this study as *Heterorhabditis downesi (*Figure 2.9*).* The six North Bull Island isolates (PUR1, PUR2, PUR3, YEL1, YEL2 and YEL3) along with the two K122 isolate and two

Hungarian *H. downesi* isolates, H3017 and H3173, associated with *P. temperata* subsp. *cinerea* and *P. temperata* subsp. *temperata* respectively (Tóth & Lakatos, 2008; Tóth *et al.*, 2008), were placed in a single clade, although the Hungarian isolates were placed on a separate branch of the clade. Based on an analysis of the ITS1 region of the *H. downesi* isolates recovered in this study there is no evidence of different nematode types being associated with the two different bacterial subspecies discussed above. This is in agreement with the findings of Toth *et al.* (2008), and Toth & Lakatos (2008; 2009), who first identified *P. temperata* subsp. *cinerea* and found both *P. temperata* subspecies associated with *H. downesi* at a single site. This is in contrast to Emelianoff *et al*. (2008b) where two *H. bacteriophora* strains which differed by four nucleotide sites in their ITS1 sequence were found to have a different bacterial subspecies associated with each nematode strain. In that study the two *H. bacteriophora* strains were isolated from sites with different habitats. Given the similarity of the nematode isolates in this study and their close association in a single site it might be expected that their symbiotic bacteria would be interchangeable. This is investigated in Chapter 3.

Biochemical characterisation of bacteria is useful in describing a species and can provide useful data for identifying new isolates (Akhurst *et al.*, 1996; Fischer-Le Saux *et al.*, 1999). In the case of the symbiotic bacteria associated with entomopathogenic nematodes they are also useful in differentiating between phase I and phase II variants (Boemare & Akhurst, 1988). Biochemical tests are widely used and are particularly useful when investigating new isolates. The use of this type of test adds to the profile of the strain and can be useful in identifying further isolates of a particular strain. The API 20 test strips were used to investigate the enzymatic profile of the North Bull Island isolates. The results showed that there was a great

deal of similarity between all the isolates, but that certain tests were useful for distinguishing between the colour phenotypes. The most striking result was that all isolates were positive for arginine dihydrolase. This is in contrast to the type specimen for *P. temperata* subsp. *temperata*, XINach (Fischer-Le Saux *et al.*, 1999). However, this character has been observed in other *P. temperata* subspecies, for example *P. temperata* subsp. *stackebrandtii* strain GPS11 (An & Grewal, 2010b). Another enzymatic character which may be used to distinguish *P. temperata* subspecies is tryptophan deaminase activity (Tóth & Lakatos, 2008). The three *pur* isolates were negative for this, while the three *yel* isolates were positive. In the Toth & Lakatos (2008) study the majority of *P. temperata* subsp. *cinerea* isolates were positive for tryptophan deaminase activity although the *P. temperata* subsp. *cinerea* type specimen was negative. The ability to carry out fermentative metabolism in the anaerobic conditions which persist in host cadavers is an important adaptation for organisms that exploit this niche. Entomopathogenic bacteria display an ability to ferment a number of carbohydrates under anaerobic conditions (Rosner *et al.*, 1996). The use of test kits such as the API 50 strips allows a rapid analysis of the fermentative profile of bacteria. Only the *yel* isolates produced acid from trehalose, this is similar to the type strain for *P. temperata* subsp. *temperata* (Fischer-Le Saux *et al.*, 1999), as well as other *Photorhabdus* species but is not universal in the genus (Akhurst *et al.*, 1996). Trehalose is a major component of insect haemolymph (Wyatt & Kalf, 1957) and the ability to utilise this energy source could be an important ecological advantage, enabling a particular nematode-bacteria partnership to fully exploit the insect host. The production of acid from salicin and amygdalin was noted for only a few of the strains in the Fischer-Le Saux *et al.* (1999) study and none produced acid from D-sorbitol, methyl-αD-glucopyranoside. In contrast, in the case

of the North Bull Island symbionts at least one *pur* isolate produced acid from each of these substrates and acid was produced by all three *pur* isolates in the case of salicin. None of the North Bull Island *yel* isolates from produced acid from these four substrates. The characters which are exhibited by only one colour phenotype may prove useful in distinguishing bacterial isolates from *H. downesi* in the future. While acid production from a number of substrates was noted by only one or other of the bacterial colour phenotypes the results also showed within-type variation and between-type similarity. A number of studies have shown that biochemical characters can be variable between isolates of the same strain (Fischer-Le Saux *et al.*, 1999; Kuwata *et al.*, 2008; Tóth & Lakatos, 2008; Tóth *et al.*, 2008) as well as between subspecies (Hazir *et al.*, 2004; An & Grewal, 2011a) within the genus *Photorhabdus,* so this variation is not unexpected. In order for biochemical characterisation to be a useful tool for determining species and subspecies it should be used as part of a range of diagnostic methods and multiple isolates of each strain should be included. The results presented here are a useful step in building a profile of the bacterial symbiont associated with *Heterorhabditis downesi* in North Bull Island and the island of Ireland as a whole. The ability of the *yel* isolates but not the *pur* to ferment trehalose would appear to give the former a competitive advantage in utilising host resources and presents some interesting questions for future investigation.

The difference in the number of colonies of the *pur* isolate which grew on nutrient agar versus MacConkey agar which was noticed while determining the growth rate of the isolates in liquid culture was unexpected. There was no significant difference in growth rate of *pur* or *yel* isolates in the liquid cultures of these media (Table 2.19). One possible explanation for the differential growth on solid media was a greater

requirement for salt for optimal growth of the *pur* isolate, as a notable difference between media is the presence of bile salts (5 g/l) and NaCl (5 g/l) in MacConkey agar while nutrient agar contains 5 g/l NaCl only (Appendix Table A 3). Hazir *et al.* (2004) found that two Turkish species of *Photorhabdus* had heavier growth and a faster growth rate in the presence of 5 g/l NaCl in nutrient broth. Hodgson *et al.* (2003) found that three *P. temperata* strains grew more quickly in tryptone or peptone based media containing 5 g/l or 10 g/l NaCl. Some work was carried out by undergraduate students in the Behavioural Ecology laboratory under my co-supervision to test the hypothesis that the *pur* phenotype had a greater requirement for salt in the growth medium (Daly, 2010; Kennedy, 2011). Both bacterial colour variants were cultured in Luria broth with varying salt concentrations. This work was inconclusive in demonstrating a greater requirement for salt by the *pur* phenotype. Another major difference between nutrient agar and MacConkey agar is the make-up of nutrients available for bacterial growth and in particular the higher concentration of peptone in MacConkey agar (20 g/l) compared to nutrient agar (5 g/l) (Appendix Table A 3). MacConkey agar also contains lactose (10 g/l). Although nutrient agar contains yeast extract (2 g/l) and Lab-Lemco powder (1 g/l) the total concentration of complex nutrients is lower compared to MacConkey agar. Complex media such as peptone and yeast extract contain compatible solutes such as betaine and choline (Schmitz & Galinski, 1996). Schmitz & Galinski (1996) found that *P. luminescens* grown in nutrient broth supplemented with an additional 30 g/l NaCl accumulated compatible solutes, notably betaine and glutamine as well as choline, a precursor of betaine, presumably from the growth medium. Schmitz & Galinski (1996) also showed that when nutrient broth was supplemented with additional compatible solutes (1mM betaine or 1mM ecotine) the bacteria could tolerate 50 g/l NaCl and

accumulated compatible solutes including betaine, ecotine, glutamine and alanine. Crawford *et al.* (2010) found that *P. luminescens* and *X. nematophila* grown on agar containing 2 g/l tryptone and 5 g/l yeast extract could tolerate salt (NaCl) concentrations of 10 g/l when the agar was supplemented with L-proline (100mM), which is known to serve as an osmoregulator in *E. coli.* The salt concentration of insect haemolymph is high (Wyatt, 1961), and so the bacteria need to be able to tolerate conditions of high osmolarity. It may be the case that the *pur* phenotype requires nutrient conditions high in compatible solutes which can be used for osmoregulation to achieve optimum growth in the presence of salt. As the growth rate for both *pur* and *yel* isolates in MacConkey broth was the same and as nutrient agar appeared to be suboptimal for *pur* growth MacConkey broth was used for subsequent experiments requiring an inoculum of liquid culture.

The *yel* bacterial symbionts were expected to demonstrate higher antimicrobial effects than the *pur* isolates as the rear of the dune system where they mostly occur would be expected to harbour a greater range of antagonistic and/or competitor organisms. In fact the opposite result was observed. Antimicrobial compounds which are produced by entomopathogenic bacteria in the course of nematode infection of an insect are effective against both bacterial and fungal antagonists. These types of compounds are believed to be involved in out-competing other microorganisms for resources in the cadaver (Hu & Webster, 2000; Derzelle *et al.*, 2002). It has been shown that the midgut microbiota of certain Lepidoteran and Dipteran species can include between five and 22 different phyla, with *Enterococcus, Pseudomonas,* and *Serratia* occurring as common isolates (Walsh & Webster, 2003; Gouge & Snyder, 2006; van der Hoeven *et al.*, 2008; Erkosar *et al.*, 2013). The compounds produced by the North Bull Island *Photorhabdus* isolates have not been identified but it is

known that entomopathogenic bacteria produce a range of compounds with antifungal, antibacterial, insecticidal and nematicidal activity (Webster *et al.*, 2002; Hinchliffe *et al.*, 2010). The range of compounds produced by species of *Xenorhabdus* includes, for example, xenorhabdins, xenorxides, xenocoumacins and nematophins while species of *Photorhabdus* produce indole, and a range of stilbenes. In addition, members of both genera produce bacteriocins. Some of these compounds are believed to play a role in maintaining the specificity of the nematode-bacterium symbiosis as they are effective against other entomopathogenic microorganisms including closely related species and even different strains of the same species (Boemare *et al.*, 1997; Hawlena *et al.*, 2010b; Morales-Soto & Forst, 2011). This type of activity was evident in both the *pur* and *yel* phenotypes as both inhibited *X. bovienii*, the symbiont of *S. feltiae*, with the *pur* isolates having a greater inhibitory effect. There was limited inhibition in some *pur/yel* combinations. In contrast, Sharma *et al* (2002) found that all four strains inhibited each other in an *in vitro* study of the bacteriocin activity of *P. luminescens subsp. akhurstii* W14, *P. luminescens* subsp. *laumondii* TT01, *P. temperata* subsp. *temperata* K122 and *P. asymbiotica* ATCC43949. In the current study both phenotypes also inhibited two strains of entomopathogenic fungi, *B. bassiana* and *M. anisopliae.* This was similar to the findings of Ansari *et al.* (2005), who found that both *P. luminescens* and *X. poinarii* inhibited the growth of both of these fungi.

The enhanced antimicrobial properties of the *pur* isolates could also have a role in competition between *H. downesi* and *S. feltiae* and their respective bacterial symbionts *Photorhabdus* and *Xenorhabdus* for scarce resources as indole and stilbenes have nematicidal activity (Hu & Webster, 2000; Webster *et al.*, 2002). It is also the case that antimicrobial compounds produced *in vitro* and *in vivo* differ both

in terms of quantity and the range of compounds produced (Hu *et al.*, 1998). Studies by Crawford *et al.* (2010; 2012) showed that the production of some antimicrobial compounds was induced in response to molecular signals in insect haemolymph. Thus the *in vitro* investigation discussed here gives an indication of the range of antimicrobial activity of the *pur* and *yel* phenotypes but does not necessarily represent the *in vivo* activity. In chapter three the *in vivo* activity of a single strain of each bacterial subspecies towards the other host and bacterial subspecies will be investigated as part of a cross-compatibility study which will include co-infection of insects with both *P. temperata* subspecies.

Chapter 3
Cross compatibility of *Heterorhabditis downesi – Photorhabdus temperata*

3.1 **Introduction**

Many multicellular organisms have over the course of their evolution formed close natural associations with microorganisms, to the extent that both partners now depend on the other for survival, with the larger organism providing a stable habitat with access to nutrients for the microbe while the microbe provides metabolic functions (Moran, 2006). (See Chapter 1 for a more general discussion of symbiosis). Many natural associations of entomopathogenic nematode-bacterium have been described, Lewis and Clarke (2012) for example, list 20 *Steinernema-Xenorhabdus* associations and seven *Heterorhabditis-Photorhabdus* associations. In some cases the nematode is associated with more than a single bacterial species and as has been seen in Chapter 2 this can occur at a single site, although this is unusual. The specificity of a symbiotic relationship is modulated by a number of recognition factors including tolerance or suppression of host defences, chemical or metabolic interactions and behavioural interactions. In the case of the entomopathogenic nematode-bacterium complex the specificity of the symbiotic relationship operates on a number of different levels: the food signals produced by the bacteria which influence IJ recovery in the insect (Strauch & Ehlers, 1998), the requirement by the nematode for the bacteria as a food source to complete its life cycle (Grewal *et al.*, 1997) and the retention of the bacteria by the nematode IJ (Han & Ehlers, 1999). The level of specificity of the symbiotic relationship and indeed the requirement of the nematode for the bacteria appears to be variable.

In general, *Steinernema* spp. appear to be less dependent on their symbiont than *Heterorhabditis* spp., for example, Grewal *et al* (1997) found that *S. scapterisci* could develop on and retain *Xenorhabdus* spp. from *S. carpocapsae* and *S. riobravis*, whereas Han & Ehlers, (1998, 2000), found that while axenic *H. bacteriophora* H06 could reproduce on the symbiont of *H. megidis* HNA the next generation of infective juveniles did not retain the bacteria. The ability of a heterologous symbiont (non-natural) to support the development of a particular nematode species and be transmitted to the next generation of infective juveniles appears to depend on the degree of relatedness of the homologous and heterologous bacteria (Sicard *et al.*, 2004). At the time of the Han & Ehlers (1998) study all the symbionts of the different *Heterorhabditis* species were categorised as *P. luminescens* species. The authors suggested that the inability of the different symbionts to support the growth of each other's nematode supported the subdivision of the group into different species. Taxonomic studies showed that the genus *Photorhabdus* contained three species, each with a number of subspecies (Fischer-Le Saux *et al.*, 1999; Akhurst *et al.*, 2004; Tailliez *et al.*, 2010). The two *Heterorhabditis* spp. investigated by Han & Ehlers (1998) were found to carry different species of symbiotic bacteria; the symbiont of *H. megidis* HNA is *P. temperata* (Fischer-Le Saux *et al.*, 1999) while that of *H. bacteriophora* H06 is *P. luminescens* (Qiu *et al.*, 2012). Evidence from coevolutionary studies of *Heterorhabditis* spp. – *Photorhabdus* spp. partnerships (Maneesakorn *et al.*, 2011) and *Steinernema* spp. - *Xenorhabdus* spp. partnerships (Lee & Stock, 2010) suggest that there is a tighter host-symbiont specificity in *Heterorhabditis* spp. – *Photorhabdus* spp. partnerships as there was evidence of a greater degree of cospeciation in the Maneesakorn study.

On the other hand, Gerritsen & Smits (1993) found that in some novel combinations of *Heterorhabditis – Photorhabdus* the IJs retained the heterologous bacteria. In the case of *H. megidis* strain HE, the IJs retained Xbac, the symbiont of *H. bacteriophora* strain Hbac. The *H. bacteriophora* IJs did not retain the HE symbiont although in a further study the same authors found that a very small percentage of *H. bacteriophora* IJ strains Hbac and Hmol retained the symbiont of *H. megidis* strain Pjun (Gerritsen *et al.*, 1998). The same authors found that two strains of *H. megidis,* HE and HSH, could develop on and retain each other's symbionts (Gerritsen & Smits, 1997). Susurluk *et al* (2001) found that *H. bacteriophora* (TUR-H2), were able to grow on and retain the symbionts of both *H. megidis* HSH2 and *H. bacteriophora* HYB. The varying results in these studies demonstrate that the ability of both *Heterorhabditis* and *Steinernema* species and strains to form novel combinations is very variable and seems to be strain specific. In all of these cases (Gerritsen & Smits, 1993, 1997; Grewal *et al.*, 1997; Gerritsen *et al.*, 1998; Han & Ehlers, 1998, 2000; Susurluk *et al.*, 2001) the recombination was carried out *in vitro*. Novel combinations have also been produced *in vivo*, for example (Chapuis *et al.*, 2009) recombined aposymbiotic *S. carpocapsae* strain SK27 with a number of different *X. nematophila* strains and aposymbiotic *S. feltiae* strain VIN with a number of different *X. bovienii* strains from different geographical regions to that of the wild type combinations of both nematodes.

In nature it appears that each *Steinernema* species has a specific association with a single *Xenorhabdus* species although a single *Xenorhabdus* species can form an association with more than one nematode species (Adams *et al.*, 2006; Lee & Stock, 2010) (See Section 1.2.5 Table 1.2). In the case of *Heterorhabditis – Photorhabdus* associations the nematodes and the symbiotic bacterium will combine with multiple

partners rather than forming strict single species pairings (Adams *et al.*, 2006; Emelianoff *et al.*, 2008b; Tóth & Lakatos, 2008; Tóth & Lakatos, 2009), although the combinations appear to be strain-specific (Chaston & Goodrich-Blair, 2009). In a survey of forest sites in Hungary Toth & Lakatos (2009) found both *H. megidis* and *H. downesi* associated with both *P. temperata* subsp. *temperata* and *P. temperata* subsp. *cinerea*, indicating that both the nematode and the bacteria can form associations with more than one partner in the same area. Similarly, the *H. downesi* isolates from North Bull Island were found to be associated with two different subspecies of *P. temperata* (Chapter 2).

The symbiosis between the nematode and the bacteria has been shown to have a cost for both the nematode and the bacteria in terms of survival (Mitani *et al.*, 2004; Emelianoff *et al.*, 2007; An & Grewal, 2010a). However, this cost is not universal, as Emelianoff *et al.,* (2007) found that while there was a difference in mortality between symbiotic and aposymbiotic IJs of *S. carpocapsae* strain SK27 there was no difference between symbiotic and aposymbiotic IJs of *S. scapterisci* strain SE61. In the Chapuis (2009) study the survival rate of the wild type *S. carpocapsae-X. nematophila* combination was lower than that of the wild type *S. feltiae-X. bovienii* combination. Furthermore, the wild type combination may not be the most pathogenic as a novel combination of *H. megidis* strain HE with the symbiont from *H. megidis* strain PE was found to be significantly more pathogenic against the dipteran *Tipula oleracea* than the wild type combination (Gerritsen *et al.*, 1998). The specificity of the symbiosis between the North Bull Island nematode and the bacteria isolates will be investigated in this chapter using an *in vivo* approach. One bacterial subspecies will be injected into a host which will then be infected with IJs carrying the other subspecies. The ability of the nematode to recombine with and

retain the heterologous bacteria will be investigated using IJs emerging from the cadavers. The pathogenicity of any novel combinations of nematode-bacterium obtained in the experiment will be investigated. The effect of the different subspecies of bacteria on the survival of the nematode will be investigated for the isolates from North Bull Island.

The objectives of this chapter are:

i) To determine the ability of the PUR and YEL nematodes identified in Chapter 2 to carry each other's symbiont by means of a cross-compatibility experiment.
ii) To look for differences in the infectivity of wild type and hybrid strains of the nematode-bacterium complex
iii) To look for differences between the *pur* and *yel* bacterial subspecies in terms of infective juvenile survival and number of bacteria carried.

3.2 **Materials and Methods**

3.2.1 Cross-compatibility of *Heterorhabditis downesi – Photorhabdus temperata* strains

A no-choice experiment was carried out to investigate the preference of either the PUR or YEL nematode for its own colour bacteria variant within a host. The experiment consisted of four treatments (*pur* bacteria, *yel* bacteria, a mixture of both bacteria or no bacteria). Final instar larvae of *G. mellonella* were injected with the bacterial treatments and three days later the cadavers were infected with nematodes (Figure 3.). A subset of the injected insects was used to quantify the bacterial load in

the cadavers at 72 hours post injection. A subset of the injected and infected insects was used to estimate the invasion rate of the nematodes. The final subset of cadavers was used to quantify the progeny from each of the bacterial treatments. A single experiment was carried out separately for a single nematode/bacteria isolate of each colour phenotype (PUR1/*pur1* and YEL/*yel3*) (see Chapter 2, Table 2.15).

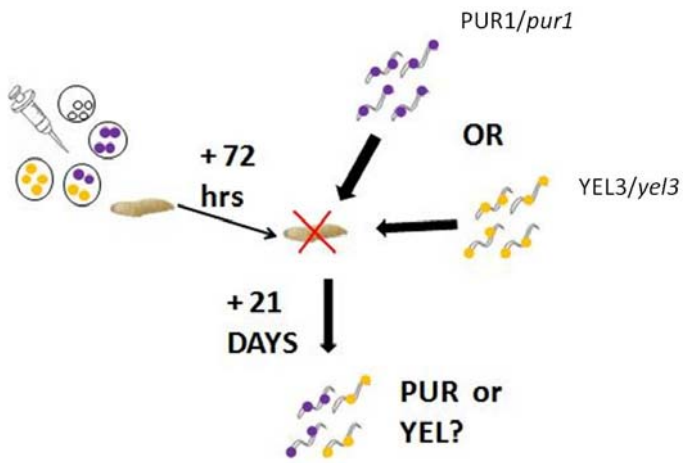

Figure 3.1: Experimental design for cross-compatibility experiment. Final instar *G. mellonella* larvae were injected with one of four treatments (*pur* bacteria, *yel* bacteria, a mixture of both bacteria or no bacteria) and subsequently infected with nematodes carrying either *pur* or *yel* bacteria. The bacterial phenotype carried by the emerging IJs was determined in subsequent experiments.

3.2.1.1 Preparation of bacterial cell suspension

Liquid cultures of *P. temperata* isolates *pur1* and *yel3* were set up in MacConkey broth as described in Section 2.2.3.2 and grown for 48 hours. Cells were collected by centrifugation at 3,850 x *g* for 10 min. The supernatant was discarded and the cells were resuspended in ¼ strength sterile Ringer's solution. The optical density was

measured at 660 nm (OD_{660}) and the concentration was adjusted to 4 x 10^7 cells/ml, calculated from the standard curve (see Appendix Figure A. 2A and Figure A. 2B). For each experiment the bacteria were freshly cultured.

3.2.1.2 Injection of *G. mellonella*

Final instar *G. mellonella* larvae were injected with 2.5 µl bacterial cell suspension (4 x 10^7 cells/ml, equivalent to 1 x 10^5 cells), prepared as outlined in Section 3.2.1.1. Larvae were injected behind the second proleg using a 5 µl microsyringe with fitted with a 70 mm plunger-in-needle (0.63 mm OD, 0.37 mm ID), (SGE Europe Ltd., Milton Keynes, UK). The syringe was rinsed five times with ethanol (70%) and five times with sterile distilled water between insects. Thirty insects per treatment received *P. temperata* isolates *pur1, yel3,* or *pur1/yel3* (1:1) bacteria. For each bacterial treatment ten insects were randomly allocated to one of three subsets for subsequent treatments:

i) bacterial quantification
ii) dissection to assess number of IJs invading
iii) White traps to assess progeny

Twenty insects received a no bacteria treatment, consisting of an injection of 2.5 µl sterile ¼ strength Ringer's solution and were randomly assigned to subsets ii) and iii) above. Each insect was placed in a well of 24-well plates lined with two 1.5 cm filter papers (Whatman no. 1) (Corning Costar 3527, Fischer Scientific, Ireland). The plates were sealed with Parafilm and stored at 20 ^oC. Insects were checked for mortality after 3 days. All insects exhibiting evidence of death due to *P. temperata* infection (see Section 2.2.1.3) were used in the subsequent stages of the experiment. The colour of each insect cadaver was recorded as purple (P), yellow (Y) or

purple/yellow (P/Y), indicating mainly purple, mainly yellow or neither colour predominating. For each bacterial treatment the ten cadavers allocated for dissection were set aside. The remaining cadavers were used in the infection stage of the experiment.

3.2.1.3 Infection of injected cadavers

Three days after injection of the respective bacteria (Section 3.2.1.2), 50 µl of 2000 IJ/ml nematode suspension (= 100 IJs/insect) was added to each of 20 cadavers per bacterial treatment in the 24-well plates. Nematode suspension (50 µl of 2000 IJ/ml) was also added to all insects in the no bacteria treatment, which were still alive at this stage. The 24-well plates were resealed with parafilm which was punctured along each side for ventilation and stored at 20 ^{o}C. All insects were removed from the 24-well plates 24 hours after application of the nematodes, rinsed 3 times in sterile distilled water to remove any IJs on the exterior, placed individually on moist tissue paper in 3.5 cm Petri dishes and stored at 20 ^{o}C. The colour of each cadaver in the *pur1/yel3* (1:1) treatment was recorded as purple (P) when the cadaver was achromatic/purple, yellow (Y) when the cadaver had a distinctive yellow colour or purple/yellow (P/Y) when the cadaver colour was indeterminate (see also Chapter 2, Section 2.2.1.3 and Figure 2.3A-B).

3.2.1.4 Bacterial quantification from injected *G. mellonella*

Three days after injection of the respective bacteria the insects previously allocated to the bacterial quantification subset (Section 3.2.1.2i) were surfaced sterilised by rinsing in 70% ethanol, igniting and extinguishing by plunging into sterile ¼ strength Ringer's solution and placed in a 3.5 cm Petri dish. Each cadaver was partially macerated with forceps and then transferred to a sterile 15 ml plastic vial (Sarstedt,

Numbrecht, Germany). The Petri dish was rinsed with 2 x 1 ml aliquots of ¼ strength Ringer's solution which was also transferred to the plastic vial. All work was carried out beside a flame on the bench. The body contents were allowed to soften at room temperature for 4 hr with gentle agitation on a Grant Bio PS-3d platform rotator at 30 rpm. The contents were then vortexed for 2 min and allowed to stand at room temperature for 20 min. A 20 µl aliquot of the homogenate was added to 0.98 ml sterile ¼ strength Ringer's solution in a 1.5 ml Eppendorf tube (Fisher Scientific, Ireland) and a 10-fold serial dilution was made. Three replicate 100 µl aliquots of each of 3 dilutions were spread on NA, NBTA and MA plates and incubated at 27 ^{o}C for 48 - 72 hours to assess the bacterial load in the insects at day 3 post injection.

3.2.1.5 Invasion rate

At day 4 following nematode infection the insects previously allocated to the dissection subset (Section 3.2.1.2ii) were examined to assess the number of nematodes invading in each treatment. Dissections were carried out in sterile ¼ with the aid of a dissecting microscope. The insect cadaver was placed in a 9 cm Petri dish to which approximately 25 ml of ¼ strength Ringer's solution was added. The cadaver was oriented with the ventral side uppermost and held steady with a coarse forceps. The body was opened along the line of the spiracles from the anus to the head using a fine forceps and the body contents were removed and dispersed in the ¼ strength Ringer's solution and the number of nematodes per cadaver was recorded.

3.2.1.6 Infective juvenile emergence and harvest

Fourteen days after nematode infection (Section 3.2.1.3) the remaining ten cadavers for each treatment were placed on individual White traps (Section 3.2.1.2iii) (Figure 2.4). The individual White traps were harvested by carefully pouring the IJ

suspension into a 50 ml tube. The White trap was rinsed three times with tap water to transfer any remaining IJs to the 50 ml tube. Each White trap was harvested at day 4 following first emergence of IJs. A further harvest was carried out 24 hours later. This was repeated at day 11 and 12 following first emergence and at day 18 and 19 following first emergence. A final harvest was carried out on day 26 following first emergence. All IJs were stored at 9 ^{o}C prior to counting or use in subsequent experiments. Nematode counts were carried out as follows:

a) Sample count (1 ml): The volume of nematode suspension from an individual White trap was noted and the 50 ml tube was inverted three times. A 1 ml sample was drawn and pipetted in a line of drops into a Petri dish. Live IJs in each drop were counted with the aid of a dissecting microscope. Three 1 ml samples were counted and the number of IJs in the total volume was calculated based on the mean of the three counts.
b) Total count: The entire volume in the 50 ml tube was transferred to a 9 cm Petri dish marked with a grid and the total number of IJs in the sample was counted.
c) Sample count (100 µl): The volume in the 50 ml tube was adjusted to 50 ml with tap water and three 100 µl samples were counted and the total number of IJs calculated as in a). If the initial suspension was too concentrated it was diluted 10-fold by drawing a 1 ml sample, transferring to a 10 ml graduated cylinder which was topped up to 10 ml with tap water, sealed and inverted three times prior to counting as outlined above.
d) Semi quantitative estimation: A series of nematode suspensions was made up in 50 ml tubes to the concentrations listed in Table 3.. The total number of IJs present in a pooled harvest was estimated by eye by comparing the density of

the nematode suspension against the prepared concentrations and scored accordingly.

Table 3.1: Scoring used for semi-quantitative estimation of nematode harvest from White traps.

score	Nematode concentration (IJs/ml)
1	100
2	250
3	500
4	1000
5	2500
6	5000

Methods a) or b) were used where the IJ concentration was low and method c) was used where the IJ concentration was high. Method d) was used where individual counts were not carried out.

3.2.2 Bacteria carried by emerging infective juveniles

The bacterial symbiont carried by the emerging IJs harvested as described in Section 3.2.1.6 was assessed using *in vitro* and *in vivo* assays.

3.2.2.1 *In vitro* assay

The IJs were stored for 1 week at 9 ^{o}C. A 600µl aliquot of IJ suspension was desheathed as described in Section 2.2.3.1a. The IJs were collected by centrifugation at 1000 x *g* for 5 min and the supernatant was removed. The IJs were rinsed three times with ¼ strength sterile Ringer's solution, collecting by centrifugation after each rinse. The IJs were crushed with a sterile micro pestle for 1 min, the volume was adjusted to 600µl with ¼ strength sterile Ringer's solution and two 1 in 10 serial

dilutions with ¼ strength sterile Ringer's solution were made. A 100µl aliquot of each dilution was plated in triplicate on MacConkey agar plates and incubated in the dark at 27 ^{o}C for 48 hr.

The assay was carried out with IJs emerging from cadavers in all the bacterial treatments infected with PUR1 IJs. Several problems occurred with the method, for example it was noted that some IJs were lost at each step in the collection and rinsing process, also some IJs remained uncrushed after the final step in the method. Both of these issues gave rise to very low colony counts. As one of the objectives in carrying out the assay was to determine the bacterial partner of the IJs an *in vivo* assay was used instead. A separate experiment to assess the level of bacterial colonisation of individual IJs was carried out using the method outlined in Section 2.2.3.1b). Briefly, IJs were surface sterilised using Hyamine 1622 solution (0.16 mM), rinsed three times with sterile PBS and single IJs were crushed in 100 µl sterile PBS and 50 µl of the homogenate was plated onto Luria-Bertani (LB) agar (Difco) supplemented with 0.1% sodium pyruvate (Sigma-Aldrich) (LB pyruvate agar) and incubated in the dark at 27 ^{o}C for 48 hr.

3.2.2.2 *In vivo* assay

A series of single nematode infections based on the one-on-one bioassay described by Grewal (2005) were set up as follows: with the aid of a dissecting microscope one IJ, picked up by pipette in a 10 µl volume from a dilute suspension, was transferred to a well of 24-well plates lined with two 1.5 cm filter papers (Whatman no. 1) (Corning Costar 3527, Fischer Scientific, Ireland). The pipette was rinsed with 2 x 10 µl tap water which was also transferred to the well to ensure the IJ did not remain in the pipette tip. A single final instar *G. mellonella* larva was added to each well, the lid was replaced and the plate was sealed with parafilm which was punctured along

each side for ventilation. Plates were randomly stacked in blocks consisting of one plate of each of the wild type and hybrid combinations of nematode/bacterial treatment and one negative control plate consisting of a 24-well plate with two filter papers, 30 µl of tap water and one *G. mellonella* larva per well. One empty 24-well plate with filter papers and 30 µl tap water only was placed on the top and bottom of each stack as a buffer. Each stack was wrapped in tinfoil to ensure constant light conditions and stored at 20 ^{o}C. Plates were checked daily for eight days and insect mortality was recorded. Tap water (30 µl) was added to the wells as required to avoid the filter paper drying out. Presence of *P. temperata* infection was confirmed as by bioluminescence by observing the plate in a darkened room for ten min (see also Section 2.2.1.3). The colour of the insect cadaver was recorded. Twenty four infections were set up with progeny from each of four separate cadavers for each of the wild type and hybrid combinations of nematode/bacterial treatment (see Table 3.2) and for four cadavers in the *pur1/yel3* 1:1 bacterial mixture treatment (= 96 insects per nematode/bacterial treatment). The experiment carried out once with IJs harvested at day 4 and repeated with IJs harvested at day 19 (Section 3.2.1.6). In addition, the experiment was also repeated with progeny harvested at day 4 and at day 19 from each of the remaining cadavers in the *pur1/yel3* 1:1 bacterial mixture treatment in each run of the cross-compatibility experiment (= 144 insects using progeny from PUR IJs and 120 insects using progeny from YEL IJs).

3.2.3 Tests of hybrid nematode/bacteria strains against *G. mellonella*

Throughout the following text capitals are used to describe the nematode colour phenotypes PUR and YEL for purple and yellow respectively, and italics are used to describe the bacteria colour phenotypes *pur* and *yel* for purple and yellow respectively. The nematode/bacteria designations of the wild type and hybrid strains

along with details of the North Bull Island isolates from which they originated are given in Table 3.2.

Table 3.2: Designations of wild type and hybrid combinations of nematode/bacteria cultures established from North Bull Island isolates (see also Table 2-14).

Nematode/bacteria combination		Parent	
		nematode	bacteria
Wild type	PUR/*pur* (Pp)	PUR1	*pur1*
	YEL/*yel* (Yy)	YEL3	*yel3*
Hybrid	PUR/*yel* (Py)	PUR1	*yel3*
	YEL/*pur* (Yp)	YEL3	*pur1*

Four separate infections of each type (Pp, Py, Yy, Yp) were set up to assess the infectivity of progeny from each of the wild type and hybrid nematode/bacteria combinations produced in Section 3.2.1.6 (see also Table 3.2). The lid and base of a 9 cm Petri dish were each lined with a 90 mm filter paper. Ten final instar *G. mellonella* larvae were placed in each dish (= 40 insects per hybrid) and 1 ml of IJ suspension (1000 IJs/ml) was evenly pipetted onto the lid and base, equivalent to a dose of 100 IJs per insect. The dishes were incubated at 20 ^{o}C. Insect mortality was recorded every four hours between the periods from 40 hours to 48 hours following infection. Mortality due to P. *temperata* infection was assessed previously described (Section 3.2.2.2). The experiment was carried out once with IJs harvested at day 4 (Section 3.2.1.6).

3.2.3.1 Cultivation of wild type and hybrid nematode/bacteria strains

Laboratory lines of wild type and hybrid nematode/bacteria combinations were established from the IJs which developed from the infections set up in Section 3.2.3. All ten cadavers from each infection were placed on White traps (see Section 2.2.1.7), harvested as described in Section 2.2.1.4 and counted as described in Section 2.2.1.6. Equal volumes of nematode suspension (1000 IJs/ml) from the four White traps for each of the wild type or hybrid nematode/bacteria combinations were pooled to give a single nematode suspension for each wild type or hybrid nematode/bacteria combination.

3.2.4 Survival of IJs in water

The survival of IJs of the *H. downesi* nematode isolates from North Bull Island was investigated. A 200 µl aliquot of nematode suspension (1000 IJ/ml) was added to 8 ml of tap water in a 5 cm Petri dish. The experiment was set up in randomised block design with 50 blocks. A block consisted of one dish of each of the six nematode isolates listed in Table 2.14 randomly arranged in a stack with one dish of tap water only on top and bottom of each stack as a buffer. Nine blocks were randomly arranged in each of 5 plastic food boxes. Each box was covered with a close fitting lid, placed in a plastic tray, covered with tinfoil to give constant light conditions and stored at 20 ^{o}C. The remaining five blocks were assessed immediately. A different block was removed from each food box every two weeks for 14 weeks and the number of surviving IJs in each dish was counted with the aid of a dissecting microscope. The block was replaced in the box so that there nine blocks in each box at all times.

3.2.5 Bacterial quantification from individual infective juveniles

IJs were surface sterilised by adding Hyamine 1622 solution (0.16 mM) (Sigma-Aldrich) to nine ml of nematode suspension (1000 IJs/ml), inverting 3 times and allowing to settle. The supernatant was discarded and the IJs were rinsed by adding 9 ml sterile phosphate buffered saline (PBS), inverting 3 times and allowing to settle. The rinse was repeated twice. Individual IJs were pipetted in 10 µl aliquots with the aid of a dissecting microscope and added to 90 µl PBS in a 1.5 ml microcentrifuge tube. Each IJ was crushed by grinding with a sterile micro pestle for 1 min. A 50 µl aliquot of the product was plated onto Luria-Bertani (LB) agar (Difco) supplemented with 0.1% sodium pyruvate (Sigma-Aldrich) (LB pyruvate agar) and incubated in the dark at 27 ^{o}C for 48 hr. The experiment was carried out twice for each of the six isolates from North Bull Island and once for each of the hybrid nematode/bacteria combinations listed in Table 3.2, with 20 IJs of each isolate or hybrid in each experiment.

3.2.6 Statistical analysis

Routine statistical analysis, including tests for normality, was carried out using Minitab statistical software, version 16.1.1 (Minitab Inc., 2010). Normality of data was tested using the Anderson-darling method. Analysis of variance was carried out using a one-way ANOVA or when groups had an uneven number of observations the general linear model with an unbalanced nested design was used to compare isolate and colour phenotype. Analysis of variance was followed by pair-wise multiple comparisons using Tukey's test. In experiments where nematode type and bacterial type were factors results were analysed using a two-way ANOVA. Where no significant interactions occurred treatment means for each factor were compared using a one-way ANOVA and post-hoc Tukey's test for three or more treatments or

2-sample T-test for two treatments. When data could not be normalised treatment medians were compared using a Kruskal-Wallis test, followed by multiple pairwise comparisons using a Mann-Whitney U-test test with Bonferroni 95% confidence intervals (CI), obtained by calculating $(1 - \alpha / g)$ where α is the level of significance and g is the number of pairwise comparisons carried out. Spearman's Rank Correlation was carried out online (http://vassarstats.net/corr_rank.html). In all tests a P value of < 0.05 was taken as significant unless indicated otherwise.

3.3 **Results**

3.3.1 Cross-compatibility of Heterorhabditis downesi – Photorhabdus temperata strains

In the cross-compatibility experiments both the PUR (experiment 1) and YEL (experiment 2) nematode colour phenotypes invaded and reproduced in *G. mellonella* cadavers that had been injected with any of the three *P. temperata* treatments and the no bacteria treatment described in Section 3.2.1.2. Invasion is described in Section 3.3.1.2, reproduction is described in Sections 3.3.1.3 and 3.3.1.4. At the outset, the colonisation of the *G. mellonella* haemocoel by the *P. temperata* isolates following injection is described.

3.3.1.1 Bacterial quantification from injected *G. mellonella*

In each experiment a number of the *G. mellonella* cadavers in the injection only subset (Section 3.2.1.2i) were homogenised 72 hours post injection and an aliquot of the homogenate was spread on agar plates. Initial trials using NBTA and MacConkey agar plates showed that it was not possible to accurately quantify the individual colour phenotypes of the *P. temperata* colonies in the *pur1/yel3* 1:1 treatment on the selective agar plates as the colonies absorbed dye from the NBTA

and MacConkey agar, which masked the pigmentation of the *pur* and *yel* isolates. Replicate plating from the selective plates onto NA plates was also unsuccessful for enumerating colour phenotypes. Aliquots of the homogenate were also spread directly on NA plates. In the case of the NA plates, contamination from the endogenous flora present in the *G. mellonella* cadavers prevented accurate enumeration of the separate colour phenotypes as young *pur* colonies are achromatic and can be mistaken for the *yel* phenotype. Colonies need to grow for 48 – 96 hr before the distinctive purple pigmentation of the *P. temperata pur* phenotype is apparent. This extra incubation period allowed the endogenous flora to overgrow the *P. temperata* colonies in some cases. A number of observations were possible however.

In the first experiment, set up for the PUR1 nematodes, colonies of both colour phenotypes were isolated from insects which received an injection of the *pur1/yel3* 1:1 bacterial mixture (Figure 3.2A). More of the *pur* phenotype than the *yel* phenotype appeared to be present in cadavers from the *pur1/yel3* 1:1 treatment, based on colony counts (data not shown). In the individual treatments more *P. temperata* colonies grew on plates from the insects that received the *pur1* bacteria compared to plates from insects that received the *yel3* bacteria, based on colony counts. There did not appear to be a difference in luminescence between the *pur* and *yel* colonies (plates were checked in the dark as outlined in Section 2.2.3.3a). Overall, more colonies grew on plates from the insects that received the *pur1/yel3* 1:1 bacterial mixture than either of the individual bacterial types. The data were analysed using a one-way ANOVA. There was no difference between the bacterial treatments ($P > 0.05$).

In the second experiment, set up for the YEL3 nematodes, the same trend in the relative number of colonies of the *pur* and *yel* phenotypes was observed (Figure 3.1B), with more *pur* colonies than *yel* in the *pur1/yel3* 1:1 treatment, overall more colonies in the *pur1/yel3* 1:1 treatment than the individual treatments and more colonies in the *pur1* individual treatment than the *yel3* individual treatment. The data were not normally distributed but were normalised using a log to the base 10 transformation. The log to the base 10 of the data were analysed using a one-way ANOVA. There was a significant difference in the number of colonies between the *pur1/yel3* 1:1 treatment and the individual *yel3* treatment (one-way ANOVA, $F_{2,14} = 5.04$, $P = 0.022$).

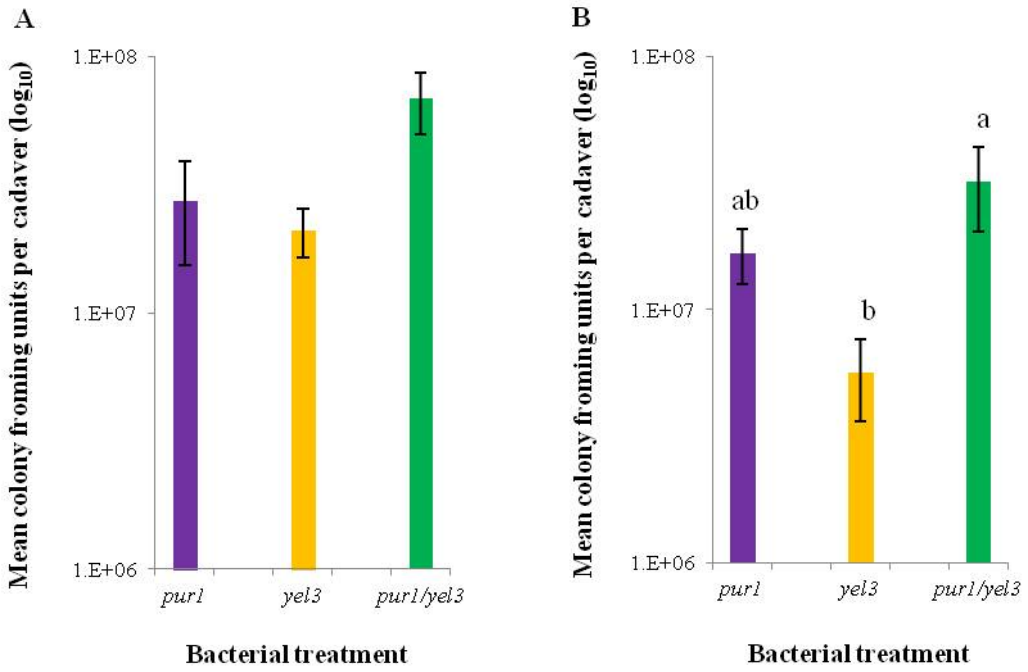

Figure 3.2: Mean number (± SE) of colony forming units of *P. temperata* colour phenotypes *pur* and *yel* in *G. mellonella* cadavers 72 hours post injection. Each insect had been injected with 1 x 10^5 cells *P. temperata* isolates *pur1*, *yel3* or *pur1/yel3* 1:1 mixture. Data shown is mean number of colony forming units on NA plates. In experiment 1 (A) n = 6 for *pur1/yel3* 1:1 treatment, n = 4 for *pur1* and *yel3* treatments. In experiment 2 (B) n = 9 for *pur1/yel3* 1:1 treatment, n = 3 for *pur1* treatment and n = 5 for *yel3* treatment. Within each graph treatments which do not share the same letter were significantly different (One-way ANOVA, $P < 0.05$). Data from experiment 2 were log to the base 10 transformed for analysis.

As a similar trend in the number of colony forming units was observed in each experiment, the log to the base 10 data for both experiments were combined and analysed with bacterial treatment and experiment as fixed factors, using a general linear model as n varied between treatments and experiments. This showed that both bacterial treatment and experiment had an effect on the number of colony forming units observed, with more colonies in the *pur1/yel3* 1:1 treatment compared to the *yel3* treatment and this difference was highly significant (GLM, $F_{2,27}$ =6.02, P = 0.007) (Table 3.3).

Table 3.3: Mean number (± SE) of colony forming units (cfu) of *P. temperata* colour phenotypes *pur* and *yel* in *G. mellonella* cadavers 72 hours post injection. Each insect had been injected with 1 x 10^5 cells *P. temperata* isolates *pur1, yel3* or *pur1/yel3* 1:1 mixture. Data shown is mean number of colony forming units on NA plates. n = 7 for *pur1* treatment, n = 9 for *yel3* treatment, and n = 15 for *pur1/yel3* 1:1 treatment for two experiments. Results of GLM analysis are also given. Data were log to the base 10 transformed for analysis. Treatments which do not share the same letter were significantly different.

Bacterial treatment	mean number cfu per cadaver	
pur1	2.27E+07^{ab} (7.03E+06)	
yel3	1.25E+07^{b} (3.89E+06)	
pur1/yel3	4.66E+07^{a} (1.09E+07)	
Factor	Test statistic	P value
Bacterial treatment	$F_{2,27} = 6.02$	0.007
Experiment	$F_{1,27} = 7.09$	0.013

Because there was an effect of experiment when the data for both experiments were combined by bacterial treatment the log to the base 10 transformed data for all three bacterial treatments were pooled for each experiment and analysed using a one-way ANOVA. The overall level of colonisation of the insects by the *P. temperata* isolates appeared be to significantly greater in the first experiment than in the second (one-way ANOVA, $F_{1,29} = 4.48$, P = 0.043) (Figure 3.3).

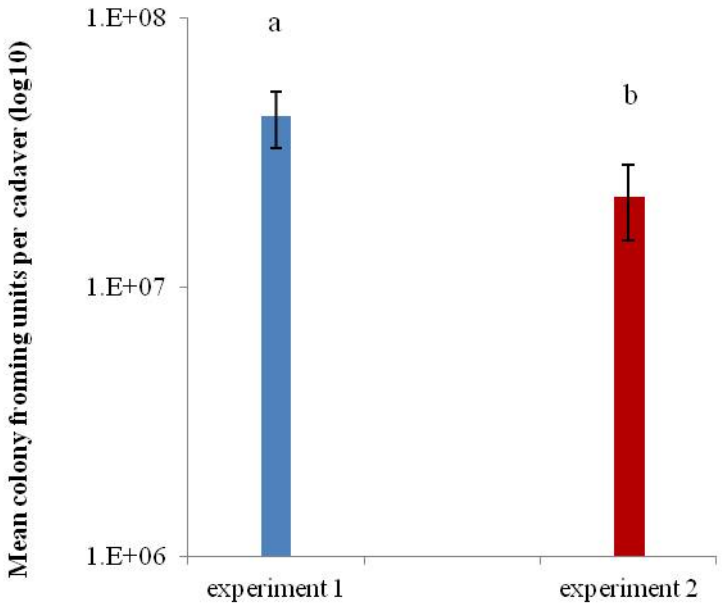

Figure 3.3: Mean number (± SE) of colony forming units of *P. temperata* isolates per insect in *G. mellonella* cadavers 72 hours post injection in two experiments. Each insect had been injected with one of three treatments of 1 x 10^5 cells *P. temperata*. Data shown is pooled for all three bacterial treatments in each experiment with n = 14 in experiment 1 and n = 17 in experiment 2. Treatments which do not share the same letter were significantly different (One-way ANOVA, $P < 0.05$). Data were log to the base 10 transformed for analysis.

3.3.1.2 Invasion rate

In the first experiment, using PUR1 IJs, on average more nematodes invaded insects which had previously been injected with one of the three *P. temperata* treatments compared to the insects in the no bacteria treatment (Figure 3.4A). The PUR1 nematodes did not seem to have a preference for their own bacterial type or the other bacterial type as the mean number of nematodes invading in the *pur1/yel3* 1:1 bacterial treatment was approximately 38 compared to means of approximately 25 and 22 nematodes in the *pur1* and *yel3* treatments respectively and the mean number of nematodes invading in the no bacteria treatment was approximately 11. The

difference between treatments was highly significant (one-way ANOVA, $F_{3,36} = 6.8$, $P = 0.001$) for the number of nematodes invading insects in the no bacteria treatment compared to the number of nematodes invading insects in the *pur1/yel3* 1:1 treatment but not the other two treatments.

In the second experiment, using YEL3 IJs, on average more nematodes invaded insects in the no bacteria treatment compared to insects in the three *P. temperata* treatments (Figure 3.4B). Similarly to the PUR1 nematodes, the YEL3 nematodes did not seem to have a preference for their own bacterial type or the other bacterial type as the mean number of nematodes invading in the *pur1/yel3* 1:1 bacterial treatment was approximately 15 compared to means of approximately 10 and 5 nematodes in the *pur1* and *yel3* treatments respectively. However, the mean number of nematodes invading the no bacteria treatment was approximately 17. There was a highly significant difference in mean number of nematodes invading (one-way ANOVA, $F_{3,36} = 9.24$, $P < 0.001$) comparing the *yel3* treatment to the *pur1/yel3* 1:1 treatment and the no bacteria treatment but not to the *pur1* individual treatment.

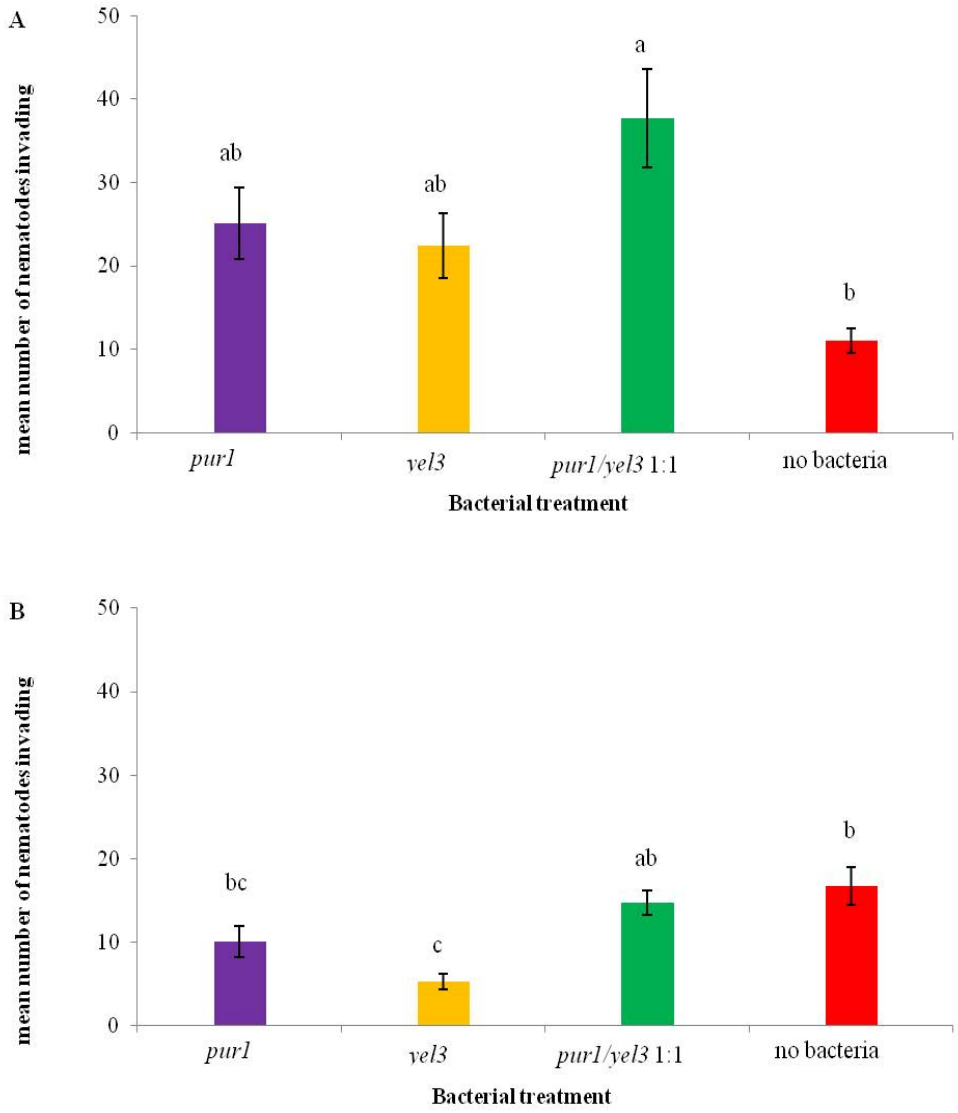

Figure 3.4: Mean number (± SE) of adult nematodes per insect in *G. mellonella* at day 4 following infection with 100 IJs per insect of (A) PUR1 and (B) YEL3 isolates from North Bull Island with n = 10 for all treatments. Insects had previously been injected with 1 x 10^5 cells of *P. temperata* isolates *pur1*, *yel3*, or a *pur1/yel3* 1:1 mixture. Insects in the no bacteria treatment were injected with an equal volume of ¼ sterile Ringer's solution only. For each nematode type, PUR1 or YEL3, bacterial treatments which do not share the same letter are significantly different. (One-way ANOVA, $P < 0.05$)

Both nematode types appeared to prefer the bacterial mixture rather than their own or the other bacterial type. As the trend in invasion rate in the three *P. temperata* treatments only, (*pur1/yel3* > *pur1* > *yel3*), was similar for both nematode types, the

data were analysed excluding the no bacteria treatment to see the effect of bacterial treatment on nematode invasion. For the YEL3 nematodes the difference in the mean number of nematodes invading was still found to be highly significant comparing the *yel3* treatment to the *pur1/yel3* 1:1 treatment but not the *pur1* treatment (one-way ANOVA, $F_{2,27} = 10.51$, $P < 0.001$). For the PUR1 nematodes there was a difference at the 10% level with fewer nematodes invading in the *yel3* treatment compared to the *pur1/yel3* 1:1 treatment but not to the *pur1* individual treatment (on-way ANOVA, $F_{2,27} = 2.92$, $P = 0.071$) .

The data from both experiments were combined and the number of nematodes invading were analysed using a two-way ANOVA with bacterial treatment and nematode type as factors. This showed that both bacterial treatment and nematode type had a highly significant effect on the number of nematodes invading the cadavers (Table 3.4).

Table 3.4: Results of two-way ANOVA comparing the effect of both bacterial treatment and nematode type on the number of IJs invading *G. mellonella* in three bacterial treatments.

Factor	Test statistic	P
Bacterial treatment	$F_{5,54} = 6.47$	0.003
Nematode type	$F_{1,54} = 40.76$	< 0.001
Interaction term	$F_{2,54} = 0.67$	0.516

Since there was no interaction the combined data were analysed by bacterial type and by nematode type. When the numbers of nematodes invading the injected insects from both experiments were pooled by bacterial treatment more nematodes invaded

insects in the *pur1/yel3* 1:1 treatment than either of the individual bacterial treatments and the difference compared to the *yel3* treatment was significant (one-way ANOVA, $F_{2,57} = 5.16$, P = 0.009) (Figure 3.5). The data were log to the base 10 transformed for analysis. The mean number of nematodes invading the insects in the *pur1/yel3* 1:1 treatment was almost twice that in the *yel3* treatment and more nematodes also invaded insects in the *pur1* treatment compared to the *yel3* treatment, but not significantly so. This preference for the *pur1/yel3* 1:1 treatment when comparing the pooled nematode data was in line with the results for the individual nematode types.

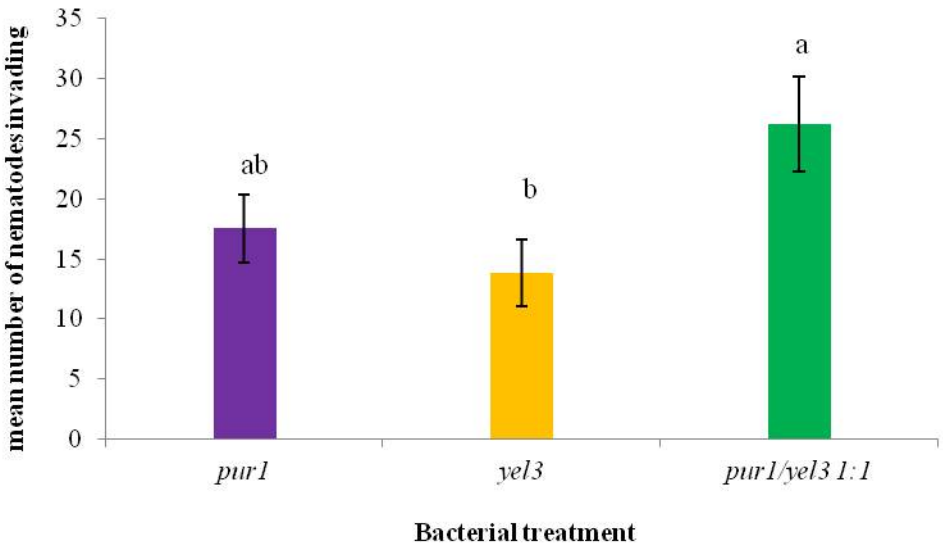

Figure 3.5: Mean number (± SE) of adult nematodes per insect in *G. mellonella* at day 4 following infection with 100 IJs per insect. Insects had previously been injected with 1 x 10^5 cells of *P. temperata* isolates *pur1*, *yel3*, or a *pur1/yel3* 1:1 mixture. Data for both nematode types are pooled by bacterial treatment and are a mean of two experiments with n = 20 for all treatments. Treatments which do not share the same letter were significantly different (one-way ANOVA, $P < 0.05$).

When the numbers of nematodes invading the injected insects from both experiments were pooled by nematode type more PUR1 nematodes than YEL3 nematodes invaded and the difference was highly significant (one-way ANOVA, $F_{1,58} = 34.62$, $P < 0.001$) (Figure 3.6).

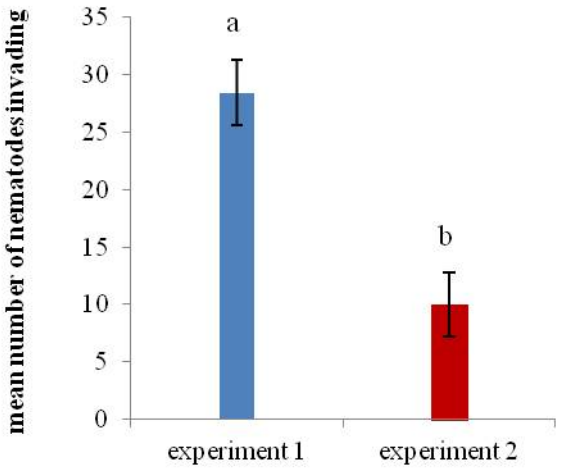

Figure 3.6: Mean number (± SE) of adult nematodes per insect in *G. mellonella* at day 4 following infection with 100 IJs per insect. Insects had previously been injected with 1 x 10^5 cells of *P. temperata* isolates *pur1, yel3,* or a *pur1/yel3* 1:1 mixture. Nematode used were PUR1 (experiment 1) and YEL3 (experiment 2). Data for all three bacterial treatments are pooled by nematode type and are a mean of two experiments with n = 30 in each experiment. Treatments which do not share the same letter were significantly different (one-way ANOVA, $P < 0.001$).

As a similar trend was observed for number of colony forming units from injected insects (Figure 3.3) and number of nematodes invading injected insects (Figure 3.6) in both experiments, (experiment 1 > experiment 2 for each parameter), the relationship between the level of bacterial colonisation of the insects and nematode

invasion rate was assessed using a Spearman's correlation (Figure 3.7). The test showed that there was a positive correlation between the mean number of colony forming units of bacteria in an insect and the invasion rate of the nematodes (ρ = 0.6406, n = 60, P < 0.001).

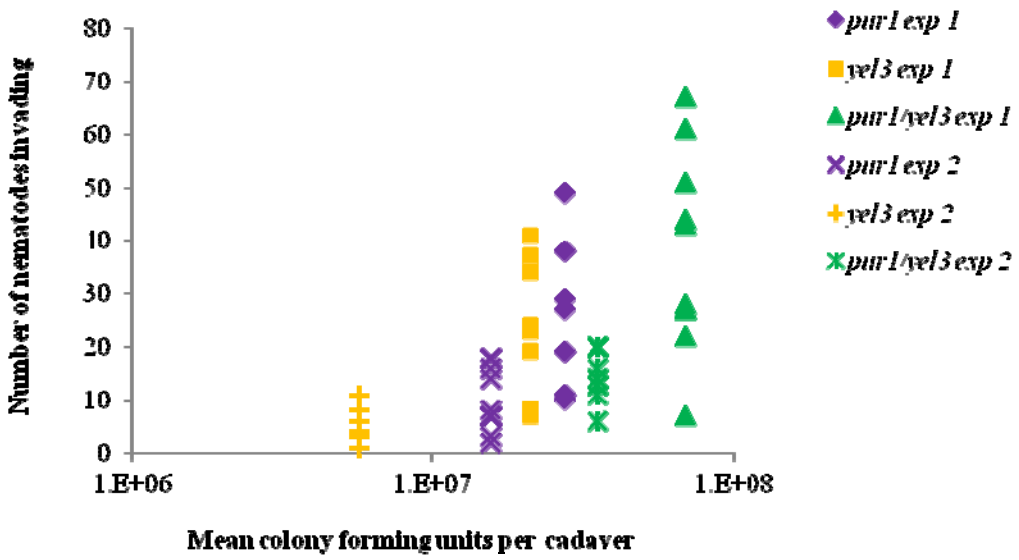

Figure 3.7: Correlation between invasion rate of nematodes in *G. mellonella* injected with one of three *P. temperata* treatments and subsequently infected with 100 IJs per insect and mean colony forming units in insects injected with one of three *P. temperata* treatments in two experiments. Nematode used were PUR1 (experiment 1) and YEL3 (experiment 2) with n = 10 in each experiment and each bacterial treatment.

3.3.1.3 Time of infective juvenile first emergence

Both of the *H. downesi* isolates PUR1 and YEL3 successfully reproduced and emerged from insects in all treatments, with first emergence of IJs occurring in some insects in all four treatments at 16 days post infection with nematodes (Appendix Table A 6). However, there was some variation in time to first emergence for all insects in each treatment with first emergence occurring at 31 days for one insect in

the *pur1/yel3* 1:1 treatment. The data were not normally distributed and could not be transformed to fit the normal distribution therefore a series of non parametric tests were used to analyse the data. The median time to first emergence was compared for each nematode type separately using a Kruskal-Wallis test. This showed that in each case there was a highly significant difference in the time to first emergence of infective juveniles between bacterial treatments (P = 0.006 and P = 0.004 for PUR1 and YEL3 IJs respectively). To determine which treatments differed a series of multiple pairwise comparisons using a two-tailed Mann-Whitney U-test with a Bonferroni 95% CI were carried out (Appendix Table A 7A and Table A 7B). In the case of the PUR1 nematodes the median time to first emergence was longer in the no bacteria treatment insects (19 days) than any of the *P. temperata* treatments (16 to 18 days) and this difference was significant compared to the *yel3* treatment (P = 0.0041) (Table 3.5). The opposite trend was observed for the YEL3 nematodes where the shortest median times to first emergence occurred in the no bacteria treatment insects and the *yel3* treatment (16.5 and 17 days respectively) compared to the other two bacterial treatments (18 and 19.5 days for the *pur1/yel3* 1:1 and *pur1* treatments, respectively) and the difference was highly significant for the no bacteria treatment compared to the *pur1* treatment (P = 0.0003).

Table 3.5: Time to first emergence for PUR1 and YEL3 IJs from *G. mellonella* infected with 100 IJs. Insects had previously been injected with 1 x 10^5 cells of *P. temperata* isolates *pur1, yel3* or a *pur1/yel3* 1:1 mixture. Insects in the no bacteria treatment were injected with an equal volume of ¼ sterile Ringer's solution only.

Bacterial treatment	Median time to first emergence (days)	
	Nematode type	
	PUR1	YEL3
pur1	18.5 ab*	19.5 a
yel3	16.0 b	17.0 ab
pur1/yel3 1:1	17.5 ab	18.0 ab
no bacteria	19.0 a	16.5 b

* Treatments which do not share the same letter were significantly different (Mann-Whitney U-test, 95% CI Bonferroni-adjusted significance level of $\alpha = 0.008$).

However, when comparing the time to first emergence in the *P. temperata* treatments only the trend for both nematode types was *pur1* > *pur1/yel3* 1:1 > *yel3.* Since the median time to first emergence for both the PUR1 IJs and the YEL3 IJs showed a similar trend the no bacteria treatment was omitted and the data were pooled and analysed by bacterial treatment and by nematode type. When time to first emergence for all nematodes was pooled by bacterial treatment the median time ranged from 17 days in the *yel3* treatment to 18 days in the *pur1/yel3* 1:1 treatment to 19 days in the *pur1* treatment (Figure 3.8A) suggesting that the nematodes were more likely to emerge sooner from insects infected with *yel3* bacteria. A Kruskal-Wallis test showed that there was a highly significant difference between the three bacterial treatments ($H = 10.16$, $df = 2$, $P = 0.006$). Subsequent pairwise comparisons using a Mann-Whitney U-test with Bonferroni 95% CI showed that the difference in the median time to first emergence was significant for the *pur1* compared to the *yel3* treatment ($P = 0.0031$) (Appendix Table A 8). When the time to first emergence was

pooled by nematode type the median time for the PUR1 IJs was 17 days while the median time for the YEL3 IJs was 18 days (Figure 3.8B) however this difference was not significant (Mann-Whitney U-test, $P > 0.05$).

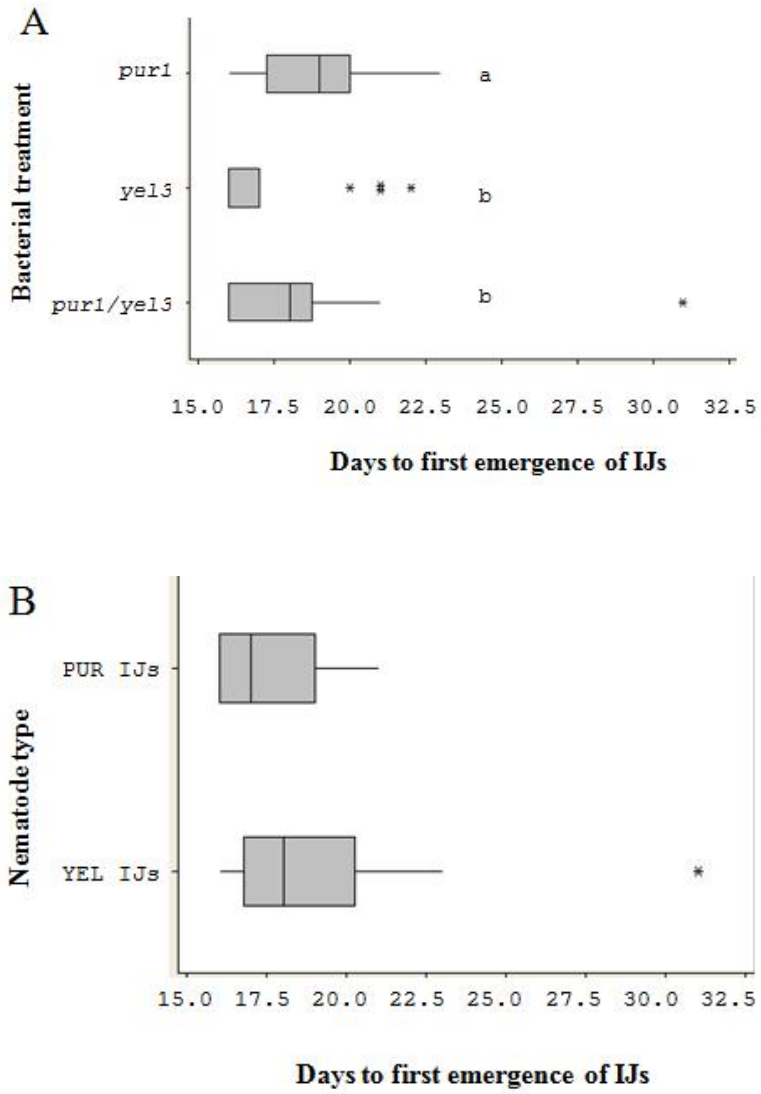

Figure 3.8: Median time to first emergence of IJs from insects following infection with 100 IJs per insect of PUR1 or YEL3 isolates from North Bull Island. Insects had previously been injected with 1 x 10^5 cells of *P. temperata* isolates *pur1*, *yel3*, or a *pur1/yel3* 1:1 mixture. Data shown is (A) both nematode types pooled by bacterial treatment with n = 20 for each treatment and (B) all three bacterial treatments pooled by nematode type with n = 30 for each nematode type. (A) Treatments which do not share the same letter are significantly different (Two-tailed Mann-Whitney U-test, $P < 0.05$, with 95% CI Bonferroni-adjusted significance level of $\alpha = 0.017$).

3.3.1.4 Numbers of IJs emerging

For each of the nematode and bacterial treatments the White traps were harvested at a number of time points. Numbers of IJs emerging are dealt with as follows:

i) Estimates of total harvests from all four treatments at week 1, week 2 and week 3.
ii) Day 4 and Day 19 counts of IJs from *pur1* and *yel3* treatments only.
iii) Counts of pooled total emergence for 26 days from *pur1* and *yel3* treatments only.

i) Estimates of total harvests from all four treatments at week 1, week 2 and week 3.

Emergence was estimated as described in Section 3.2.1.6d) for each cadaver each week for three weeks in all four treatments (Table 3.6). In both the PUR and YEL nematodes the emergence in week 1 was highest in the no bacteria treatment and the emergence in this treatment declined in subsequent weeks. For both nematode types more IJs emerged from insects in the *pur1* treatment in week 1 compared to the *yel3* treatment or the *pur1/yel3* 1:1 treatment. Unlike the no bacteria treatment, emergence for all IJs except the YEL3 nematode/*pur1* bacteria combination was highest in week 2. By week 3 IJ emergence had declined in all three bacterial treatments and at this time point more IJs emerged from both the *yel3* treatment and the *pur1/yel3* 1:1 compared to the *pur1* treatment for both nematode types.

Table 3.6: Estimated IJ emergence over three weeks from *G. mellonella* infected with 100 nematodes per insect. Insects had previously been injected with 1 x 10^5 cells of *P. temperata* isolates *pur1, yel3,* or a *pur1/yel3* 1:1 mixture. Insects in the no bacteria treatment were injected with an equal volume of ¼ sterile Ringer's solution only. N = 10 for each treatment for each nematode type except YEL3/*pur1/yel3* where n = 9.

Nematode type	Bacterial treatment	Week 1	Week 2	Week 3	total (± SE)
PUR1	*pur1*	486467	896250	142027	1524744_{31603}
	yel3	226341	1492150	294753	2013244_{19823}
	pur1/yel3 1:1	119638	705140	246802	1071580_{21356}
	no bacteria	780804	752500	198540	1731845_{27920}
YEL3	*pur1*	599945	261325	219528	1080798_{34133}
	yel3	229115	446800	309875	985790_{19556}
	pur1/yel3 1:1	522750	1118450	327627	1968827_{72544}
	no bacteria	791050	584350	252100	1627500_{16908}

There appeared to be a trend for greater numbers of IJs from both the PUR1 and the YEL3 nematode infections to emerge earlier in the *pur1* treatment compared to the *yel3* or the *pur1/yel3* treatments, therefore the estimated emergence in week 1 as a proportion of the total estimated emergence for three weeks in all four treatments was analysed to investigate the trend for higher numbers of IJs to emerge early from cadavers in the *pur1* treatment. As n varied between treatments the data were analysed using a general linear model with nematode type and bacterial treatment as factors. The analysis showed that bacterial treatment had a highly significant effect on the proportion of IJs emerging early while nematode type did not (Table 3.7). All proportion data were arcsine square root transformed for analysis

Table 3.7: Results of analysis of variance using a general linear model comparing the effect of both bacterial treatment and nematode type on estimated numbers of IJs emerging early. All proportion data were arcsine square root transformed for analysis

Factor	Test statistic	P
Bacterial treatment	$F_{3,74} = 5.25$	0.002
Nematode type	$F_{1,74} = 0.50$	0.481

When the data were pooled and analysed by bacterial treatment the overall mean estimated proportion of IJs emerging early (week 1) in the *pur1* and no bacteria treatments was more than double that of the *pur1/yel3* 1:1 mixture and individual *yel3* treatments and this difference was highly significant (one-way ANOVA, $F_{3,75} = 5.31$, P = 0.002) (Table 3.8). All proportion data were arcsine square root transformed for analysis.

Table 3.8: Estimated proportion of IJs emerging early from *G. mellonella* infected with 100 IJs per insect. Insects had previously been injected with 1 x 105 cells of *P. temperata* isolates *pur1, yel3* or *pur1*/*yel3* 1:1 mixture. Insects in the no bacteria treatment were injected with an equal volume of ¼ sterile Ringer's solution only. Data for both nematode types are pooled by bacterial treatment and are number of IJs emerging in week 1 as a percentage of total emergence over three weeks with n = 20 for all treatments except *pur1*/*yel3* (n = 19). All proportion data were arcsine square root transformed for analysis. Treatments which do not share the same letter are significantly different (one-way ANOVA, $F_{3,75} = 5.31$, P = 0.002).

Bacterial treatment	Mean estimated proportion of IJs emerging early (% week 1/total) (± SE)
pur1	42.0(7.32) a
yel3	18.7(3.33) b
pur1/yel3	19.7(4.40) b
no bacteria	48.6(7.35) a

ii) Day 4 and Day 19 counts of IJs from *pur1* and *yel3* treatments only.

Individual counts were carried out for harvests from each of four White traps for the wild type and hybrid combinations listed in Table 3.2 for early (day 4) and late (day 19) harvests (Figure 3.9**Error! Reference source not found.**A and Figure 3.9B). The day 4 harvest contained IJs emerging over the first four days for each White trap while the day 19 harvest contained IJs emerging over a 24 hour period from day 18 to day 19. While there was a large variation in the emergence there appeared to be a trend for greater numbers of IJs from both the PUR1 and the YEL3 nematode infections to emerge earlier in the *pur1* bacterial treatment (Table 3.9).

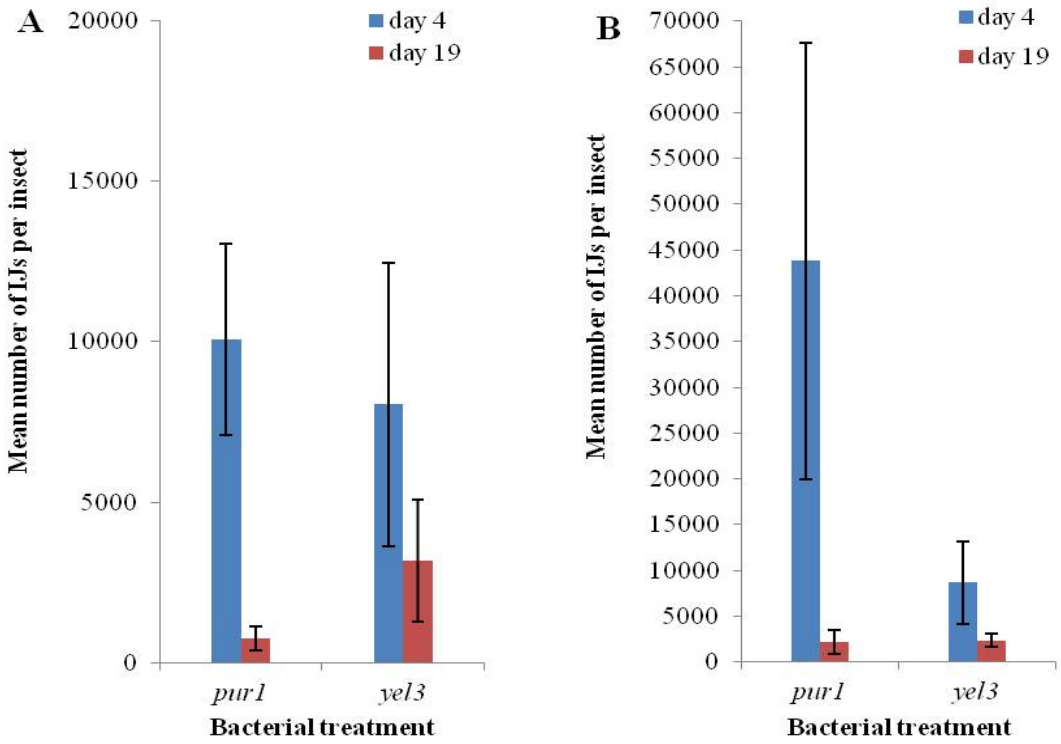

Figure 3.9: Mean (± S.E.) emergence per insect of (A) PUR1 and (B) YEL3 IJs from insects following infection with 100 nematodes per insect. Insects had previously been injected with 1 x 10^5 cells of *P. temperata* isolates *pur1* or *yel3*. Data shown are for harvests at day 4 and day 19, n = 4 for two bacterial treatments at each time point.

Table 3.9: Mean (± SE) percentage of IJs emerging early from insects following infection with 100 nematodes per insect. Insects had previously been injected with 1 x 10^5 cells of *P. temperata* isolates *pur1* or *yel3*. Data shown are for harvests at day 4 as a percentage of the total harvest at day 4 and day 19, n = 4 for each bacterial treatments.

	Bacterial treatment	
Nematode type	*pur1*	*yel3*
PUR1	92.5(3.08)	72.5(16.38)
YEL3	81.7(17.19)	61.4(18.34)
Overall	87.1(8.34)	66.9(11.57)

The data were combined and the number of PUR1 and YEL3 IJs emerged by day 4 as a proportion of the total harvest for each IJ in each bacterial treatment at both time points was compared using a two-way ANOVA with bacterial treatment and nematode type as factors (Appendix Table A 9). The analysis showed neither bacterial type nor nematode type had a significant effect on the number of IJs emerging early and there was no interaction between bacterial treatment and nematode type ($P > 0.05$ in all cases).

iii) Counts of pooled total emergence for 26 days from *pur1* and *yel3* treatments only.

The data from experiment 1 and experiment 2 were combined and the total emergence for 26 days for each White trap was pooled and counted (Table 3.10). A Two-way ANOVA comparing number of IJs emerging with bacterial treatment and nematode type as factors showed neither bacterial type nor nematode type had a significant effect on the number of IJs over the 26 day period and there was no

interaction between bacterial treatment and nematode type (P > 0.05 in all cases) (Appendix Table A 10).

Table 3.10: Mean number of IJs (± SE) emerging per insect from *G. mellonella* over 26 days following infection with 100 IJs per insect. Nematodes used were PUR1 (experiment 1) or YEL3 (experiment 2) isolates from North Bull Island. Insects had previously been injected with 1 x 10^5 cells of *P. temperata* isolates *pur1*or *yel3*. Data is from two experiments with n = 5 for all treatments.

	Bacterial treatment	
Nematode type	*pur1*	*yel3*
PUR1	103095(21356)	104016(14456)
YEL3	85062(12769)	65863(22152)
Overall	94078(14813)	84940(11095)

3.3.2 Bacteria carried by emerging infective juveniles

A series of continuous exposure single nematode infections were set up as described in Section 3.2.2.2 and insect mortality due to entomopathogenic nematode infection was recorded for a period of eight days for IJs harvested at each time point (day 4 and day 19). Mean mortality ranged from approximately 19 to 44 % (Table 3.11). The overall mean mortality for both nematode types and both harvest time points ranged from 31% for progeny from the *pur1* treatment to 32.6% for progeny from the *yel3* treatment to 36.5% for progeny from the *pur1/yel3* 1:1 treatment (Figure 3.10). The data for both harvest time points were combined for analysis. As n varied between treatments the data were analysed using a general linear model with nematode type, bacterial treatment and harvest time point as factors. The analysis

showed there was no effect of any of these factors on the percentage mortality of the insects and no interaction between any factors ($P > 0.05$ in all cases).

Table 3.11: Mean insect mortality (± SE) in single nematode infections using IJs from insects that had been injected with 1 x 10^5 cells of *P. temperata* isolates *pur1, yel3* or *pur1/yel3* 1:1 mixture and infected three days later with 100 IJs per insect. Nematodes used were PUR1 or YEL3 isolates from North Bull Island. Data shown is pooled by bacterial treatment for IJs from PUR1 and YEL3 infection types for each harvest time point, with n = 4 for each nematode type in the individual bacterial treatments at each time point and n = 10 and 9 for the PUR1 and YEL3 nematodes respectively in the pur1/yel3 1:1 bacterial treatment at each harvest time point.

		Bacterial treatment		
Nematode type	Harvest time point	*pur1*	*yel3*	*pur1/yel3*
PUR1	Day 4	$39.58_{(4.96)}$	$32.29_{(6.88)}$	$34.58_{(4.12)}$
	Day 19	$18.75_{(7.12)}$	$29.17_{(4.50)}$	$31.67_{(1.55)}$
YEL3	Day 4	$30.21_{(5.48)}$	$33.33_{(2.41)}$	$37.04_{(5.49)}$
	Day 19	$30.56_{(3.67)}$	$35.40_{(4.72)}$	$43.52_{(4.72)}$

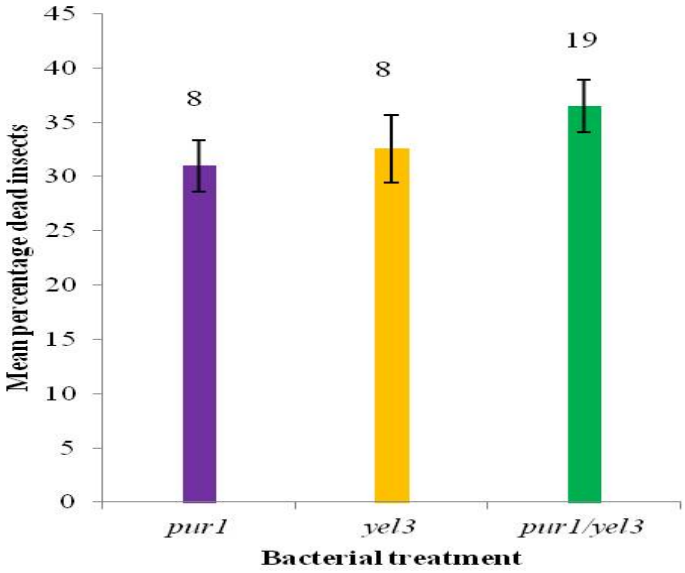

Figure 3.10: Mean *G. mellonella* mortality (± SE) in single nematode infections using IJs from insects that had been injected with 1 x 105 cells of *P. temperata* isolates *pur1*, *yel3* or *pur1*/*yel3* 1:1 mixture and infected three days later with 100 IJs per insect. Nematodes used were PUR1 or YEL3 isolates from North Bull Island. Data shown is pooled by bacterial treatment for PUR1 and YEL3 IJs from two harvest time points, n for each bacterial treatment is displayed above the bars.

In this experiment the identity of the bacteria predominating in the IJ is ascertained based on the colour of the insect cadaver. In each of the single bacterial treatments the IJs always carried the bacterial phenotype which had been injected into the insect three days prior to nematode infection, irrespective of the colour phenotype of the nematode used in the subsequent infection. In the case of the *pur1/yel3* 1:1 bacterial treatment the IJs from any given source insect all tended to carry a single bacterial colour phenotype (Figure 3.11 and Table 3.12), although both bacterial colour phenotypes were detected in infections using IJs from 3/10 cadavers in the case of PUR1 IJs and from 2/9 cadavers in the case YEL3 IJs.

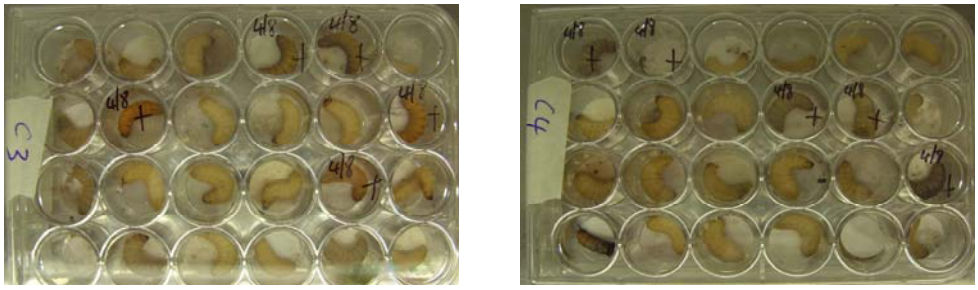

Figure 3.11: Cadaver colour in single nematode infections using *G. mellonella*. The IJs used for the infection were harvested on day 4 following emergence from cadavers which had been infected with 1 x 10^5 cells of *P. temperata* isolates *pur1*/*yel3* 1:1 mixture and infected three days later with 100 PUR1 IJs per insect. Images shown are for IJs from two separate cadavers: (C3) all infections resulted in yellow cadavers and (C4) all infections resulted in purple cadavers. Cells marked with a cross (+) indicate insects killed by nematode infection on day 4 of the experiment.

Table 3.12: Colour phenotype of *G. mellonella* in single nematode infections using emerging IJs from insects in the cross-compatibility experiment that had been injected with 1 x 10^5 cells of *P. temperata* isolates *pur1/yel3* 1:1 bacterial mixture and infected three days later with 100 IJs per insect with PUR1 or YEL3 isolates from North Bull Island. N = 24 insects at each time point per harvest from 10 (PUR IJs) or 9 (YEL3) cadavers.

		Cadaver colour in single nematode infection (/24 for each infection at each time point)							
		Harvest							
		day 4		day 19		Total			
Nematode type	Cadaver	*p*	*y*	*p*	*y*	*p*	*y*	source cadaver colour†	Cadaver colour in single nematode infections ‡
PUR1 IJs	1	4	0	0	8	4	8	P[1]	*p*→*y*[4]
	2	5	0	7	0	12	0	P	*p*
	3	0	7	0	8	0	15	P/Y[2]	*y*
	4	0	8	0	7	0	15	P/Y	*y*
	5	12	0	0	6	12	6	P	*p*→ *y*
	6	11	0	9	0	20	0	P	*p*
	7	2	6	0	6	2	12	P	*p* + *y* → *y*[5]
	8	0	14	0	7	0	21	P/Y	*y*
	9	0	7	0	9	0	16	P	*y*
	10	0	7	0	9	0	16	P/Y	*y*
YEL3 IJs	1	0	4	0	9	0	13	P/Y	*y*
	2	2	4	0	9	2	13	Y[3]	*p* + *y* → y
	3	0	6	0	12	0	18	Y	*y*
	4	11	0	13	0	24	0	P	*p*
	5	0	15	0	10	0	25	P	*y*
	6	0	8	0	8	0	16	P/Y	*y*
	7	0	15	0	17	0	32	Y	*y*
	8	7	0	0	11	7	11	P	*p*→ *y*
	9	8	0	5	0	13	0	P	*p*
Overall		62	101	34	136	96	237		

† The colour of the cadavers was assessed at day 4 of the cross-compatibility experiment:

1. P = achromatic cadaver colouration typically seen early in infection with *H. downesi* PUR isolates.
2. Cadaver colour was recorded as P/Y when the colouration was intermediate between typical PUR or YEL infection phenotypes.
3. Y (yellow) = yellow cadaver colouration typically seen early in infection with *H. downesi* YEL isolates.

‡ The cadaver colour in the one-on-one bioassay was recorded as *p* or *y* for PUR or YEL nematode infection respectively.

4. An arrow in the final column indicates the colour of the cadavers was different in the infection with IJs from the second harvest.
5. A plus (+) symbol indicates cadavers of both colour phenotypes.

Overall, ten source cadavers produced yellow IJs only (based on the cadaver colour resulting from the single nematode infections), four produced purple IJs only and five produced IJs of both colours, as noted already (Table 3.12). The proportion of cadavers with the *y* colour phenotype was higher for each nematode type from each harvest time point. The proportion of each cadaver colour phenotype, (*p, y*), was tested at each harvest time point for each nematode type (Table 3.13). The difference between the proportion of *y* and *p* colour phenotypes was significant at the 10% level for PUR1 IJs at the day 4 harvest time point. The difference between the proportion of *y* and *p* colour phenotypes was highly significant for both nematode types at all other harvest time points (χ^2, $P < 0.01$).

Table 3.13: Number of *G. mellonella* cadavers of each colour phenotype in single nematodes infections using emerging IJs from insects in the cross-compatibility experiment that had been injected with 1 x 10^5 cells of *P. temperata* isolates *pur1/yel3* 1:1 bacterial mixture and infected three days later with 100 IJs per insect with PUR1 or YEL3 isolates from North Bull Island. n = 240 (PUR IJs) or 216 (YEL3) cadavers at each time point.

	Cadaver colour in single nematode infections			
	Harvest			
	day 4		day 19	
Nematode type	*p*	*y*	*p*	*y*
PUR1 IJs	34	49	16	60
Test result	$\chi^2 = 3.278$, df = 1, P = 0.070		$\chi^2 = 30.266$, df = 1, P = 0.000	
YEL IJs	28	52	18	76
	$\chi^2 = 8.836$, df = 1, P = 0.003		$\chi^2 = 45.740$, df = 1, P = 0.000	

The data for IJs from the *pur1*/*yel3* 1:1 treatment were pooled and the colour of source cadaver (Section 3.2.1.3) was compared to the colour carried by the IJs (Table 3.14). All of the nine source cadavers classified as yellow (Y) or purple/yellow (P/Y)

produced IJs carrying *yel3* bacteria, whereas only eight of the sixteen source cadavers classified as purple (P) or P/Y produced IJs carrying *pur1* bacteria.

Table 3.14: Comparison of source cadaver colour and cadaver colour resulting from a single nematode infection using emerging IJs from the cross-compatibility experiment. Data shown is for IJs emerging from *pur1/yel3* 1:1 bacterial treatment in the cross-compatibility experiment and is pooled for PUR1 and YEL3 nematodes in that experiment. (Insect used were *G. mellonella* larvae)

Source cadaver colour	cadaver colour in single nematode infection		
	p	*p + y*	*y*
P	4	4	2
P/Y	0	0	6
Y	0	1	2

A number of two-way comparisons were carried out to test whether cadaver colour tends to predict the IJ colour (Table 3.15, see also Appendix Table A 11). The analysis showed that the cadaver colour tended to predict IJ colour as where the source cadaver appeared yellow (Y) or purple/yellow (P/Y) it is likely that all of the progeny from a cadaver will be carrying the *yel3* (*y*) bacteria, although in one case a yellow source cadaver produced some progeny with *pur1* (*p*) bacteria. Conversely, when there is no sign of yellow in the source cadaver it is likely that at least some of the progeny will be carrying the *pur1* bacteria, although in two cases for purple source cadavers no *pur1* progeny were detected using single nematode infections. These results suggest that the *yel3* bacteria are better at colonising the nematodes within the co-infected cadavers despite the apparent dominance of the *pur1* bacteria in colonising the insect (Section 3.3.1.1 above).

Table 3.15: Results of two-way comparisons of source cadaver colour and single nematode infection cadaver colour (Fisher's exact test). Data shown is for IJs emerging from *pur1/yel3* 1:1 bacterial treatment in the cross-compatibility experiment and is pooled for PUR1 and YEL3 nematodes in that experiment. (Insect used were *G. mellonella* larvae in all cases).

source cadaver colour	cadaver colour in single nematode infection	P-value
yellow or not	purple or not	0.005
yellow or not	yellow or not	0.087
purple or not	purple or not	1
purple or not	yellow or not	1

3.3.3 Tests of wild type and hybrid nematode/bacteria strains against *G. mellonella*

For each of the wild type and hybrid combinations listed in Table 3.2 four infections using *G. mellonella* were set up as described in Section 3.2.3. The mortality of insects in the various treatments was compared at a number of time points. The data at 40 hours and 48 hours was not normal and could not be normalised. The mean or median percentage of insects dead varied between the individual combinations, ranging from 0% at 40 hours to 100% at 48 hours (Table 3.16).

Table 3.16: Median† or mean‡ (± SE) percentage of *G. mellonella* dead at three time points for wild type and hybrid combinations of nematode and bacteria. All insects were infected with 100 IJs per insect using IJs emerging from cadavers in the *pur1* and *yel3* bacterial treatments in the cross-compatibility experiment. There were four replications of each combination with ten insects per replication. Results of Kruskal-Wallis test or one-way ANOVA comparison between treatment medians or means are shown for proportion dead at each time point.

	Time point (hrs)		
Nematode/ bacteria combination	40†	44‡	48†
Pp	30	75 (11.9)	100
Yp	15	60 (1.08)	85
Yy	0	47.5 (11.1)	75
Py	0	75 (6.45)	95
P value	0.121	0.226	0.051
Test statistic (3 df)	H = 5.81	$F_{3,12} = 1.67$	H = 7.77

In all four treatments all insects had died within 64 hours of infection and the PUR nematodes tended to produce higher mortality rate, irrespective of bacterial colour. As the data at 44 hours were normally distributed the percentage of insects dead at this time point were analysed using a two-way ANOVA with nematode type and bacteria type as factors. The analysis showed there was no effect of bacteria type and no evidence of an interaction between bacteria type and nematode type. Nematode type was significant at the 10% level but not at the 5% level (P = 0.061) (Appendix Table A 12). There was no significant difference in the median number of insects dead per treatment at 40 hours and 48 hours or in the mean number of insects dead at 44 hours at the 5% level, although there was a significant difference between treatments at the 10% level for the number of insects dead by 48 hours. However, pairwise post-hoc comparisons (Two-tailed Mann-Whitney U-test, with Bonferroni 95% CI) showed no difference between combinations (Appendix Table A 13).

The data were pooled by nematode type (Table 3.17) and by bacterial type (Table 3.18) and median or mean percentage mortality compared using either a Mann-Whitney U-test or a one-way ANOVA. When the data were pooled by nematode type a higher percentage of insects were dead at 40 hours in the YEL3 treatment compared to the PUR1 treatment but the difference was not significant. However, at 44 hours and 48 hours a significantly higher percentage of insects were dead in the PUR1 treatment compared to the YEL 3 treatment.

Table 3.17: Median† or mean‡ (± SE) percentage of *G. mellonella* dead at 3 time points for wild type and hybrid infections. Data is pooled by nematode type. Results of Mann-Whitney U-test or one-way ANOVA comparison between treatment medians or means are shown for number dead at each time point. N = 8 at each time point.

	Time point (hrs)			
Nematode type	40†	44‡		48†
PUR1	5	75.00	(6.27)	100
YEL3	10	53.75	(7.54)	80
P value	0.740	0.049		0.0116
Test statistic (df = 1)	W = 64.5	T = 2.17		W = 91.5

When the data were pooled by bacterial type a greater percent of insects were dead at 40 hours in the *pur1* treatment compared to the *yel3* treatment and this difference was highly significant. These results suggest that both nematode type and bacteria play a role in insect mortality and that the *pur1* bacteria kill the insects quicker than the *yel3* bacteria do.

Table 3.18: Median† or mean‡ (± SE) percentage of *G. mellonella* dead at 3 time points for wild type and hybrid infections. Data is pooled by bacterial type. Results of Mann-Whitney U-test or one-way ANOVA comparison between treatment medians or means are shown for percentage dead at each time point. N = 8 at each time point.

	Time point (hrs)		
Bacteria type	40†	44‡	48†
pur1	15	67.5 (7.96)	95
yel3	0	61.2 (7.89)	90
P value	0.0274	0.587	0.3720
Test statistic (1 df)	W = 89.5	T = 0.56	W = 77.0

3.3.4 Survival of IJs in water

The *in vitro* survival in water of the two *H. downesi* colour phenotypes from North Bull Island was investigated as described in Section 0. There was a gradual decline in the numbers of IJs surviving, with most isolates showing a reduction in survival by the first time point (week two) (Figure 3.12). Infective juveniles of both colour phenotypes, PUR and YEL, survived in water at 20 ^{o}C for up to 14 weeks, at which point the experiment was terminated. No IJs of the PUR3 isolate remained alive at week 14 and the mean percentage survival for the PUR1 and PUR2 isolates was 0.2% and 0.6% respectively at week 14 while the mean percentage survival for the YEL isolates was 0.2 % (YEL2), 0.3 % (YEL3) and 0.4 % (YEL1). There was no overall trend for either colour phenotype to survive better over the fourteen week duration of the experiment.

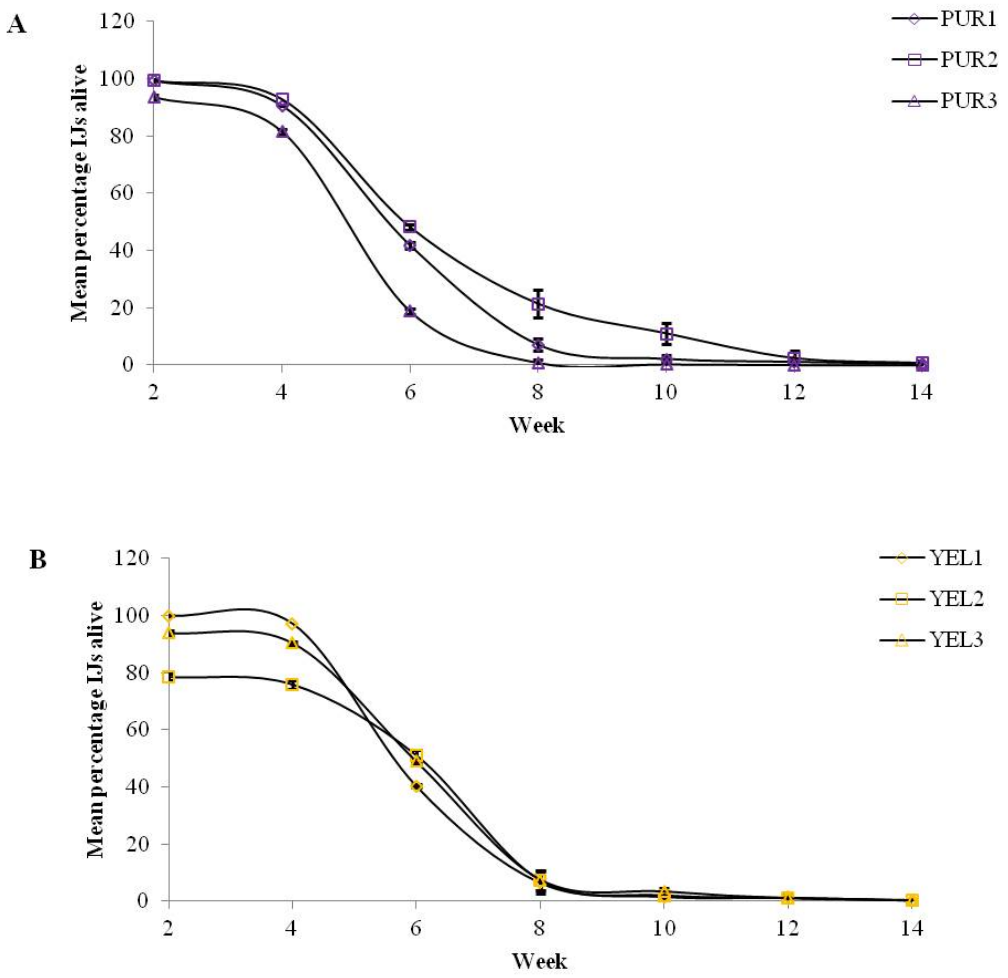

Figure 3.12: Mean percentage (± SE) survival in water of (A) PUR and (B) YEL isolates from North Bull Island over a 14 week period at 20 ^{o}C. N = 5 at each time point.

In week 2 and week 4 of the experiment both the highest percentage survival and the lowest percentage survival occurred in the yellow phenotype (YEL1 and YEL2 respectively), whereas for week 8 to week 14 the highest percentage survival and the lowest percentage survival occurred in the purple phenotype (PUR2 and PUR3 respectively). In week 6 the mean percentage survival for the PUR IJs was 41.8%, 48.1% and 18.8% for PUR1, PUR2 and PUR3 respectively while mean percentage survival for the YEL IJs was 40.2%, 51.0% and 48.9% for YEL1, YEL2 and YEL3 respectively. Thus the trend in percentage survival was YEL2 > YEL3 > PUR2 >

PUR1 > YEL1 > PUR3. A Regression with Life Data analysis of the survival data in weeks 2 to 8 was carried out using week, colour phenotype and isolate as model terms. This showed no effect of colour phenotype or isolate on survival.

3.3.5 Bacterial quantification from individual infective juveniles

The experiment described in Section b) was carried out twice to determine the number of *P. temperata* colonising individual IJs of each isolate. The first experiment was carried out using the six isolates from North Bull Island only. The two hybrid combinations were included in the second experiment along with the six North Bull Island isolates. In each experiment the IJs were found to have variable numbers of bacteria, with 5 to 10% of IJs having no detectable bacteria, (PUR2 in experiment 1 and YEL2, YEL3 and YEL/*pur* in experiment 2 (Appendix Table A 14)). Overall, bacteria were detected in 48/50 PUR IJs in experiment 1 and 56/60 PUR IJs in experiment 2 whereas the opposite trend was observed for the YEL IJs with bacteria being detected in 56/60 YEL IJs in experiment 1 and 59/60 YEL IJs in experiment 2. Bacteria were also detected in 19/20 PUR1/*yel3* hybrids and 20/20 YEL3/*pur1* hybrids in experiment 2. As there was no evidence of a colour bias no statistical tests were carried out on differences in the number of IJs having no detectable bacteria between the colour phenotypes.

In experiment 1 colonization levels ranged from 0 colony forming units (cfu) per IJ to 1746 cfu per IJ (Figure 3.13A). In experiment 2 colonization levels ranged from 0 cfu per IJ to 1154 cfu per IJ (Figure 3.13B). In order to compare the colonization level in the IJs which carried bacteria zeros and outliers were removed (Figure 3.14A-B). The data for both experiments were pooled and analysed using a fully nested general linear model with experiment, nematode type and isolate as factors. There was no effect of nematode type or isolate on the number of bacteria carried by

the IJs ($P > 0.005$ for both factors), however there was a highly significant effect of experiment (GLM, $F_{1,195} = 11.71$, $P = 0.001$). Since there was no evidence for either IJ phenotype, PUR or YEL to carry more bacteria in either experiment and there was a difference in the number of bacteria carried in each experiment no comparisons were carried out on the data pooled by nematode type.

For the wild type and hybrid strains in experiment 2 there was no significant difference between treatments when the number of cfu per IJ was compared within nematode phenotype (PUR or YEL) or within bacterial phenotype (*pur* or *yel*) (one-way ANOVA, $P > 0.05$).

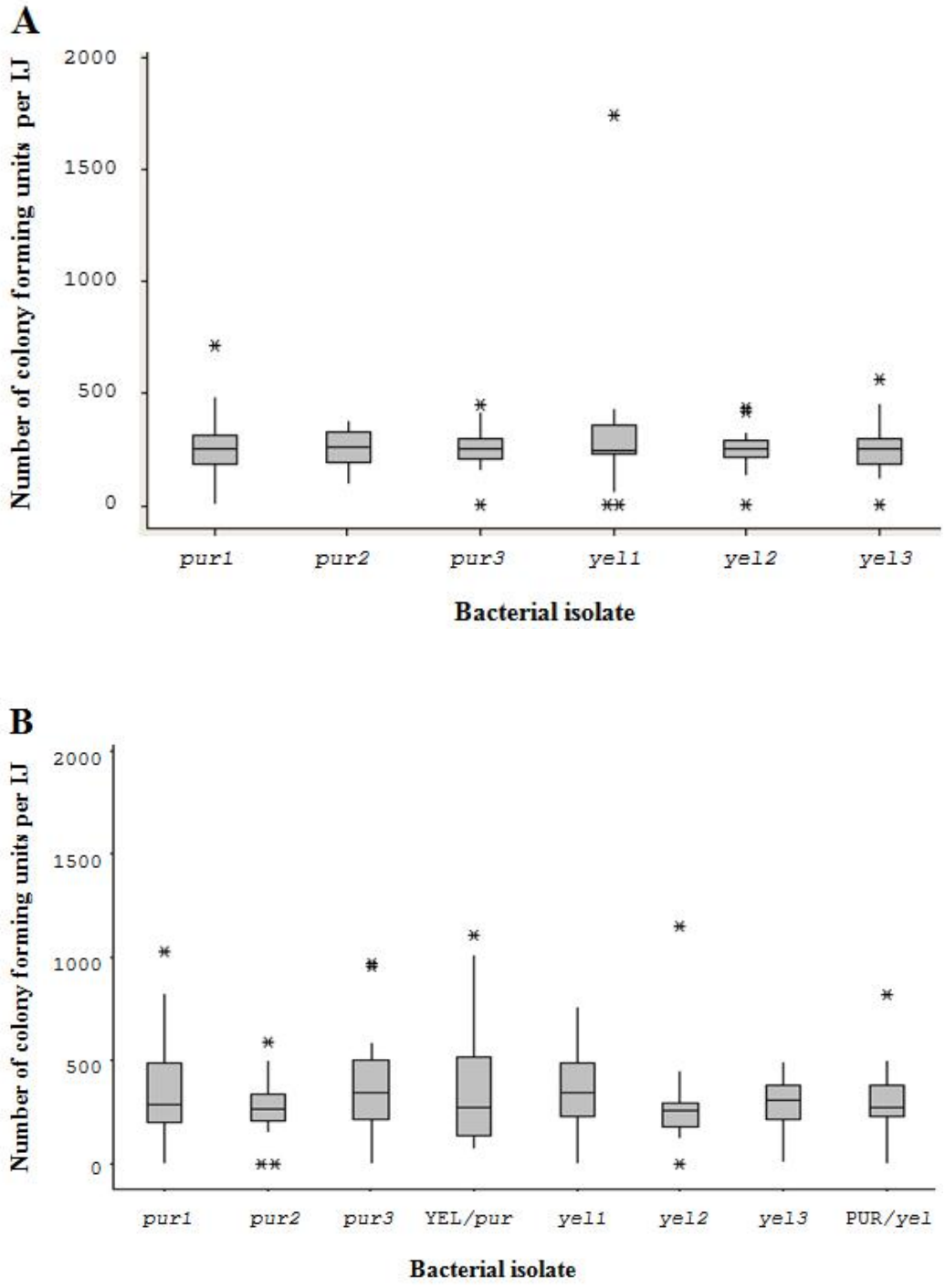

Figure 3.13: Box and whisker plot of median number of colony forming units (cfu) per IJ in two experiments using (A) six *H. downesi*[1] isolates from North Bull Island and (B) six *H. downesi*[1] isolates from North Bull Island plus two hybrids, YEL/*pur*[2] and PUR/*yel*[3].

[1] Natural associations are PUR1/*pur1*, PUR2/*pur2*, PUR3/*pur3*, YEL1/*yel1*, YEL2/*yel2*, YEL3/*yel3.*

[2] YEL3 nematode colonised with *pur1* bacteria.

[3] PUR1 nematode colonised with *yel3* bacteria.

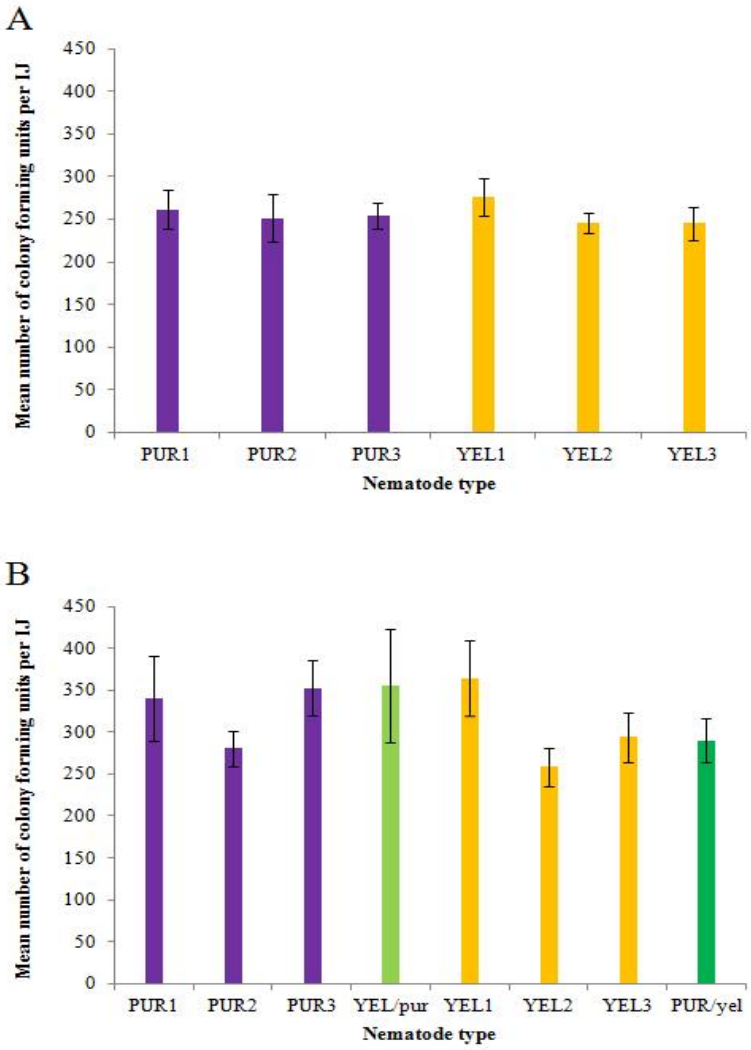

Figure 3.14: Mean (± SE) number of bacterial colonies of *P. temperata* from (A) six *H. downesi* isolates from North Bull Island and (B) six *H. downesi* isolates from North Bull Island plus two hybrid strains. IJs containing no bacteria and outliers were removed prior to analysis and the data were square root transformed for analysis.

3.4 **Discussion**

The cross-compatibility experiment showed that both strains of *H. downesi*, PUR1 and YEL3, were able to invade cadavers previously killed by two different subspecies of *Photorhabdus temperata, P. temperata* subsp. *temperata* (*yel3*) and *P temperata* subsp. *cinerea* (*pur1*). The nematodes were able to develop and reproduce on both the homologous and novel bacteria and infective juveniles of each nematode strain retained the novel bacterial subspecies. This study showed that nematodes would form new combinations when they invaded insects already infected with a novel *Photorhabdus* subspecies. Hybrid combinations subsequently were stable over many cycles of laboratory culturing and were used in experiments in Chapter 4 and Chapter 5.

Other studies have shown that nematode/bacteria recombination was possible. For example, Gerritsen & Smits (1993) found that *H. megidis* strains NLH-E87.3 and NLH-F85 were able to recombine with each other's symbionts, *Photorhabdus* strains PE (XE) and PF (XF) respectively, when axenic IJs of each strain were placed on lawns of the other bacteria, which were serologically different (Gerritsen *et al.*, 1995) and had distinct restriction fragment length polymorphism (RFLP) patterns (Smits *et al.*, 1991). (The symbionts of *Heterorhabditis* spp. were initially classified as *Xenorhabdus luminescens* and subsequently renamed *Photorhabdus luminescens* (Boemare *et al.*, 1993b)). Separate species and subspecies within the genus were recognised later (see Section 1.2.4). The two *Photorhabdus* strains PE and PF were later identified as *P. temperata* subsp. *temperata* (Susurluk *et al.*, 2001; Maneesakorn *et al.*, 2011) using phylogenetic techniques. The novel symbionts supported the growth and reproduction of the host nematode and there was no difference in mortality of *G. mellonella* larvae exposed to natural or recombined

nematode/bacteria partnerships (Gerritsen & Smits, 1993). Gerritsen and Smits (1997) found that there was no difference in the number of IJs produced by nematodes cultured on their own or other bacteria for two strains of *H. megidis,* DH-SH1 and NLH-E87.3. On the other hand, the ability of the symbiont of one nematode species to successfully recombine with another nematode species is more limited. Gerritsen & Smits (1993) found that the two *H. megidis* symbionts PE and PF supported the growth and reproduction of axenic IJs of *H. bacteriophora* strains Hbac and Hmol *in vitro* but the novel combinations tended to be non-pathogenic to *G. mellonella*, suggesting that the symbiont was not carried by the IJs, confirmed by Gerritsen *et al.* (1998). The *H. bacteriophora* strain H06 was able to reproduce on the symbiont of *H. megidis*, HNA, both *in vitro* and *in vivo*, but not on the symbiont of *H. indica* (Han & Ehlers, 2001). It has been shown that heterologous bacteria may provide better growth conditions than the homologous bacteria (Han *et al.*, 1991) where cultures of *Heterorhabditis* sp. H3 and HNZ both had higher yields of IJs when combined with non-homologous strains of *P. luminescens*. In an *in vitro* choice test using both homologous and heterologous bacterial lawns two *H. megidis* strains, HE and HSH IJs showed no preference for their own or the other bacteria (Gerritsen & Smits, 1997). Both strains were able to develop and reproduce on a combination of the two bacterial strains and both IJ strains cultured in this way were found to have both strains of bacteria in their gut. In an *in vitro* choice study of two *H. bacteriophora* strains from Italy Ith-CE1 and Ith-C6, which carried two different colour phenotypes of *Photorhabdus*, red and green respectively, IJs of both strains appeared to prefer the red bacterium (Tarasco *et al.*, 2003). A 4th year under graduate project, which I co-supervised, was designed to test the preference of the Bull Island nematodes for their own or the other colour phenotype (Spring, 2009).

This showed that both nematode types PUR1 and YEL3 had a slight preference for the *yel3* bacteria in choice experiments carried out an agar plates over 2.5 hour duration. These studies were looking at bacterial preference by IJs on artificial media. In the cross-compatibility experiment the bacteria are consumed by and colonise non-infective juveniles that develop into the next generation of IJs within the insect cadaver. The specificity of the nematode-bacterium complex is mediated by a number of factors including nutrition, surface structure recognition and gene regulation (reviewed in Chaston & Goodrich-Blair (2009) and Lewis & Clarke (2012)). In *H. bacteriophora* strain TT01 successful colonisation of the nematode by its bacterial symbiont, *P. luminescens* subsp. *laumondii* strain TT01 appears to involve the expression of specific cell surface characters such as lipopolysaccharide (LPS) (Easom *et al.*, 2010) and filaments known as fimbriae (Somvanshi *et al.*, 2010) by the bacteria. Other *Heterorhabditis/Photorhabdus* have not been studied in such detail and in *Steinernema carpocapsae/Xenorhabdus nematophila* complexes lectin-binding residues inside the specialised receptacle where the symbionts are carried in the nematode are involved (Martens & Goodrich-Blair, 2005). Until more detailed studies have been conducted on other EPN complexes it is uncertain how specific the symbiotic relationship is in each combination. Within the insect host the nematode is essentially a bacteria feeder and is probably not actively choosing which bacteria to eat. In the case of the North Bull Island *pur1* and *yel3* isolates both were able to colonise the *H. downesi* nematodes and it is probably the case that the relative proportion of each bacterial type present at a particular stage in the infection is determining the colonisation of the IJs in the *pur1/yel3* 1:1 treatment.

In Hungary both *H. downesi* and *H. megidis* have been found naturally associated with both *P. temperata* subsp. *temperata* and *P. temperata* subsp. *cinerea* at two

sites (Tóth & Lakatos, 2008; Tóth *et al.*, 2008; Tóth & Lakatos, 2009) and *H. bacteriophora* was found naturally associated with both *P. luminescens* subsp. *laumondii* at one site and with *P. luminescens* subsp. *thracensis* at another site (Tóth & Lakatos, 2009). In Turkey *H. bacteriophora* was found naturally associated with both *P. luminescens* subsp. *kayiii* at one site and with *P. luminescens* subsp. *thracensis* at another site (Hazir *et al.*, 2004). This is not unusual as *H. bacteriophora* is found associated with several species and even subspecies of *Photorhabdus* (Adams *et al.*, 2006; Lewis & Clarke, 2012), although there is some evidence that there may be different variants of *H. bacteriophora* as not all strains are reproductively compatible (Stack *et al.*, 2000).

I have already shown in Chapter 2 that the two isolates *pur1* and *yel3* did not inhibit each other *in vitro* (Section 2.3.3.1) and so they would not be expected to inhibit each other *in vivo.* The results of the cross-compatibility experiment support this (Section 3.3.1.1). From the NA plates (Figure 3.2) inoculated from the *G. mellonella* homogenate it was clear that colonies of both bacterial phenotypes were present in similar numbers at 72 hours post injection and this was true for both experiment 1 and experiment 2. A number of studies have investigated interactions between different genera (Massey *et al.*, 2004), species (Hawlena *et al.*, 2010a; Bashey *et al.*, 2012a), and isolates (Hawlena *et al.*, 2010b), of entomopathogenic bacteria from a range of nematode hosts. Interactions between species can be mutualistic, selfish, altruistic or spiteful depending on the cost or benefit to the actor and the recipient (Gardner & West, 2004). In bacteria the interaction is to the detriment of one or even both bacterial species if they both produce toxins that can kill the other. These interactions are mediated in part by bacteriocins (xenorhabdicins (Thaler *et al.*, 1995) and lumicins (Sharma *et al.*, 2002) in *Xenorhabdus* spp. and *Photorhabdus*

spp., respectively). In a study of *X. bovienii* and *X. koppenhoeferi* (Bashey *et al.*, 2011) found that where one bacterial species produced bacteriocins which inhibited the other this could give the host nematode a competitive advantage in mixed infections in *G. mellonella*. However, bacteriocins are costly to produce and may not always confer an advantage on the producer (Massey *et al.*, 2004). Variation among bacteria in sensitivity to bacteriocins has been shown to influence the outcome of such interactions both in terms of growth rate of the bacteria and virulence against an insect host. The interactions between closely related bacterial species is termed spiteful when they result in lower virulence and lower within-host growth rates of both bacteria in co-infections (Gardner *et al.*, 2004; Bashey *et al.*, 2012a; Bashey *et al.*, 2012b) or lower growth rates in co-cultures (Inglis *et al.*, 2009; Inglis *et al.*, 2011). In a study of *P. asymbiotica, P. luminescens* subsp. *luminescens* and *X. nematophilus* isolates Massey *et al* (2004) found that bacterial species which inhibited each other *in vitro* resulted in lower insect mortality when both were injected into *G. mellonella* compared to the mortality when each was used singly. The degree of relatedness may influence the bacteriocin-mediated interaction between the bacteria, with less inhibition occurring between genetically similar isolates of the same species compared to isolates of the same species that were genetically different (Vigneux *et al.*, 2008; Hawlena *et al.*, 2010b; Bashey *et al.*, 2012b). Other antimicrobial compounds such as xenocoumacins, water-soluble peptides, are also involved in both interspecific and intraspecific competition (Park *et al.*, 2009). In contrast to Massey *et al.* (2004), in the cross-compatibility experiment not only did neither subspecies inhibit the other, in both experiments there was a higher level of bacterial colonisation of the insects where both bacterial subspecies were injected than for either single subspecies, suggesting a possible

synergy between the two subspecies. It is also possible that differences in the metabolism of each isolate (Section 2.3.3.d) enabled both isolates to exploit the host to a greater extent than either isolate in single infections, contributing to an overall greater growth rate of the bacteria in the mixed infection.

In the cross compatibility experiments both the PUR1 and the YEL3 IJs successfully invaded cadavers which were already colonised by bacteria in all three bacterial treatments, *pur1* alone, *yel3* alone, and the *pur1/yel3* 1:1 mixture, as well as the control (no bacteria) insects which were alive at the time of nematode infection. In the case of the PUR1 IJs the invasion rate was higher in all three bacterial treatments compared to the control insects whereas the converse was true for the YEL3 IJs (Figure 3.4). The nematodes were applied to the insects 72 hours after they had been injected with bacteria. In the field it would be usual for entomopathogenic nematodes to encounter dead as well as live hosts. In any event there is only one first invader and subsequent invading nematodes are faced with assessing the status of an already infected host. In studies with *S. feltiae, S. affine* and *H. megidis* it was found that species from both genera would invade both live and dead (freeze-killed) *G.* mellonella larvae (San-Blas & Gowen, 2008; San-Blas *et al.*, 2008), although in the case of *H. megidis* the invasion rate in the dead insects was lower compared to live ones. Han & Ehlers (2001) found that *H. bacteriophora* strain HO6 infected *G. mellonella* larvae that had been injected with the homologous (*P. luminescens* HO6) or a heterologous (*P. luminescens* HNA, from *H. megidis* strain HNA) symbiont. Scavenging behaviour could be seen as an advantage for an entomopathogenic nematode as it might allow it to exploit hosts or life stages that would otherwise be unavailable. The chance of survival of the nematode and its symbiotic bacteria would also be increased if the nematode-bacterium complex didn't have to overcome

the host immune system in order to successfully colonise the host. The infection status of the cadaver would also be a factor; in the case of a cadaver already heavily infected with saprotrophic bacteria the symbiotic bacteria might not be able suppress them. This would be a disadvantage for EPN species that are dependent on their symbiont. However, invading cadavers already colonised by the symbiont does not have this disadvantage for the nematode. There is conflicting evidence as to the effect infection status of a host plays in subsequent invasion by conspecific and heterospecific entomopathogenic nematodes. Some studies have reported that hosts previously infected with *Steinernema* spp. were unattractive to conspecifics at 6-9 hours after infection (Glazer, 1997) or 48 hours post infection (Wang & Ishibashi, 1999). In contrast, Ramos-Rodriguez *et al* (2007) found that for *S. glaseri* there was no difference in invasion rate between infected and uninfected hosts. It should be remembered that in a successful entomopathogenic nematode infection there are in fact two colonisations occurring. There was a significant difference in the number of IJs invading between the first and second experiments (Figure 3.6). There was also a difference in the number of IJs invading the different treatments, with significantly more IJs invading the 1:1 *pur1/yel3* treatment compared to the *yel3* treatment in experiment two (Figure 3.4). This difference in invasion rate may be due to the level of bacterial colonisation as the number of colony forming units of bacteria recovered from *G. mellonella* was also significantly greater in the first experiment compared to the second (Figure 3.3), and the number of colony forming units was significantly greater in the *pur1/yel3* treatment compared to the *yel3* treatment in experiment two (Figure 3.2). It may be that the greater rate of nematode invasion can be explained in part by increased bacteria activity in the cadaver and that the bacterial feeding nematodes recognise the symbiont-colonised cadaver as a food source.

Bacterial type appeared to influence time of first emergence, the proportion of IJs emerging early on in the emergence period, and the number of IJs emerging. In both experiments the median time to first emergence of infective juveniles was longest in the *pur1* treatment and shortest in the *yel3* treatment. Nematode emergence was monitored over a period of 26 days with 24 hr harvests at days 4-5, 11-12 and 18-19 and a final harvest at day 26 (Section 3.2.1.6). Numbers of IJs emerging were assessed in three ways; estimation of total weekly harvest in each treatment (Section 3.3.1.4i), counts from individual White traps from the *pur1* and *yel3* treatments for day 4 and day 19 (section 3.3.1.4ii), and pooled counts of total emergence over a 26 day period from the *pur1* and *yel3* treatments (Section 3.3.1.4iii). There was a trend for first emergence of IJs to take longer from the *pur1* treatment compared to the other bacterial treatments (Table 3.5) Individual counts for four cadavers of emergence in the first four days as a proportion of total harvest for days 1- 4 and day 19 did not show a significant difference between *pur1* and *yel3* treatments although greater numbers of IJs did emerge from the *pur1* treatment in both experiments (Figure 3.9). The estimated emergence in the first week of emergence as a proportion of total emergence was highest in the *pur1* treatment compared to the *pur1/yel3* and the *yel3* treatments (Table 3.6), and there was a greater proportion of IJs in the *pur1* treatment in the first week compared to the other treatments (Table 3.8). The overall emergence data showed that more IJs were recovered over a 26 day period from cadavers in the *pur1* treatment than the *yel3* treatment (Table 3.10). Compared to the bacterial treatments, emergence was highest in the no bacteria treatment in week 1 and declined over the three week period whereas for all other treatments except *pur1* in experiment 2 IJ emergence increased in week 2. The mean number of IJs emerging in experiment 1 was greater than in experiment 2 (Table 3.10). Studies

with *Heterorhabditis megidis* have shown that invasion rate, emergence time and reproduction rate are related to number of IJs applied (Boff *et al.*, 2000; Ryder & Griffin, 2002). In both of these studies increasing densities of IJs were applied to live *G. mellonella* larvae. In the cross-compatibility experiment the larvae in the three bacterial treatments were already dead and colonised with either the homologous or the heterologous bacteria. The infection rate and the reproduction rate appear to be influenced by the bacterial load already established in the cadavers, as increasing numbers of IJs invaded with increasing bacterial load (Figure 3.6) and the overall yield was greater in experiment 1 than in experiment 2 (Table 3.10), suggesting that the readily available supply of nutrient in the cadavers was a factor. The bacteria also appear to have a role in the timing of emergence and this may indicate a difference in the quality of nutrients available. As was seen in Section 2.3.3.d there are differences in the metabolism of the two bacterial isolates, *pur1* and *yel3* and these differences may influence the timing of IJ emergence as bacterial type was found to be a highly significant factor influencing the proportion of IJs emerging early (Table 3.7). It has been shown that heterologous bacteria can support the growth and development of the non-host nematode in a number of *Heterorhabditis* spp. both i*n vitro* and *in vivo* (Han *et al.*, 1990; Gerritsen *et al.*, 1998; Han & Ehlers, 1999). In these studies axenic IJs were grown on heterologous bacteria *in vitro* and subsequently those combinations that were successful were tested in *G. mellonella* for the ability of the bacterium to support growth and reproduction of the nematode.

The novel combinations obtained in the cross-compatibility experiment were assessed to determine the bacterium carried by the emerging IJs. In the case of the single bacterial treatments *pur1* and *yel3* all IJs tested using 1 on 1 bioassays were found to be carrying the bacteria which had been injected into the *G. mellonella*

larva 3 days previously, even when this was different from the bacteria carried by the invading IJ. It is possible that if a larger number of IJs from each treatment were tested that some might have been found to be carrying the original bacteria. The level of bacterial colonisation of the injected *G. mellonella* was found to exceed 1 x 10^6 colony forming units in all cases and was nearly 1 x 10^8 colony forming units in the case of the *pur1/yel3* treatment in experiment 1 (Figure 3.2). While it is likely that the homologous bacteria carried by the invading nematodes would grow, as *pur1* and *yel3* were found not to inhibit each other's growth (see Chapter 2, Table 2.20) it seems that the already established bacteria would dominate and be more likely to be taken up by the developing nematodes. In the *pur1/yel3* treatment at both assessment times (days 1-4 and day 19 of emergence) more IJs carried the *yel3* than *pur1*. There was no difference in the number of bacteria carried by the IJs in either the homologous or heterologous combinations (Figure 3.13B) and in all cases some IJs carried no bacteria, with no difference between the homologous or heterologous combinations in the proportion of IJs with no bacteria (Appendix Table A 14). There appeared to be a shift in the bacteria taken up by the nematodes in the cadaver as in all five cadavers which produced both *pur* and *yel* carrying IJs there was a switch from *pur* or *pur/yel* at day 4 to exclusively *yel* at day 19. It may be the case that in the dual bacterial colonisation the *pur* and *yel* colour phenotypes differ in their nutritional value and ability to successfully infect and colonise the maternal nematode. In *Heterorhabditis* spp. pre-IJs develop and hatch inside the maternal body, rupturing the uterus and feeding on the maternal body in a process known as endotokia matricida (Johnigk & Ehlers, 1999). In a study on *H. bacteriophora* Ciche *et al.* (2008) showed that the symbiotic bacteria first infected the maternal nematode by adhering to the maternal intestine and then invaded the maternal body cavity

where they multiplied and then infected the pre-IJs developing inside the maternal body cavity. It is not clear if a single female can be infected by more than one bacterial type so in the IJs emerging from cadavers in the *pur/yel* bacterial treatment they may be the offspring of different females which were carrying the *pur* or *yel* subspecies or from females carrying both subspecies. However, if the latter were true I would have expected to detect both bacterial types in the IJs. It is possible that if a larger number of IJs were tested some might be found to carry both symbionts. The cross-compatibility experiment tested only one of nine possible pairings of PUR and YEL nematodes and their bacteria and it would be interesting to test other combinations in the same way to see if similar trends were observed.

The dynamics of the bacterial growth within the cadaver in terms of growth rate and metabolic capabilities may be having an effect on the availability of bacteria at different stages in the infection. A slower growth rate in the *pur1* isolate might explain why the time to first emergence of IJs in the *pur1* treatment was longer. Although there was no significant difference in growth rate between *pur1* and *yel3* in liquid cultures (Section 2.3.3b) the *pur1* isolate seemed to lag slightly in the initial stages of growth (see growth curves Appendix Figure A. 5). The differences in relative growth rate on different agars (Section 2.3.3c), and the metabolic capabilities (Section 2.3.3.d) of the two isolates might indicate different abilities to utilise resources within the cadaver and may allow that the *yel3* to outcompete the *pur1* isolate later in the infection.

The speed of kill and pathogenicity of the wild type and hybrid nematode/bacteria strains against *G. mellonella* was compared at three time points in order to assess the role of both the nematode and bacterial partner (Section 3.3.3). The greatest difference was seen at 40 hours. While the difference in the median number of

insects dead was not significant when comparing the four nematode/bacteria combinations, there was a significant difference depending on whether the nematodes carried the *pur1* or the *yel3* bacteria when data for the wild type and hybrid combinations were pooled (Table 3.18), with more dead in the *pur1* treatment. This is similar to the result of tests using *Tipula oleracea* where Gerritsen (1998) found that the heterologous combinations of *H. megidis/P. luminescens* strains NLH-E87.3 and NLH-jun had a higher mortality rate than the homologous combinations. As discussed earlier, the *pur1* isolate appeared to grow slightly slower in the earlier stages of growth in nutrient broth compared to MacConkey broth, perhaps indicating differing growth requirements for the two isolates, however by 40 hours both isolates had reached stationary phase when grown in broth. The growth of the bacteria in an insect is likely to be different from the growth in liquid culture, as growth in any medium depends on type and level of availability of nutrients, culturing conditions and competition from other microorganisms. The initial inoculum of bacteria depends on the number of nematodes invading and the number of bacteria each nematode carries in to the insect. While these two factors were not assessed in the cross-compatibility experiment they would not be expected to differ based on the results from the bacterial quantification from individual IJs (Section 3.3.5). The ability of *Photorhabdus* spp. to kill the insect host depends on the production of a range of proteases and toxins by the bacteria (Bowen *et al.*, 2000). Work by Crawford *et al.* (2010) suggested that a number of molecules linked to both virulence and antibiosis are upregulated upon recognition of the host by *P. luminescens* TTO1. The difference in mortality might be due to differences in growth rate and toxins produced by the two bacterial types. In antibiosis tests it was found that all three *pur* isolates had a greater inhibitory effect against a range of

bacteria and fungi than did any of the three *yel* isolates (Section 2.3.3.1) and it may be the case that the *pur1* isolate also produces a greater or more virulent range of compounds which are responsible for the greater rate of kill seen at the 40 hour time point.

The survival of IJs in water was found to vary both within and between bacterial subspecies (Section 3.3.4). The greatest rate of decline was seen between week 4 and week 10 for the PUR phenotype and between week 4 and week 8 for the YEL phenotype (Figure 3.11). While there were differences in percentage survival of the isolates at the various time points neither colour phenotype or isolate had a significant effect on the overall rate of survival from week 2 to week 8 of the experiment. The variation in survival rate was similar to that reported by Hass *et al.* (2001) for a number of *H. downesi* isolates from Ireland. Carrying the symbiotic bacteria appears to exert a cost on the nematode in terms of survival. Studies with *S. carpocapsae* found that IJs carrying their symbiotic bacteria had lower survival rates than IJs which had no symbionts (Mitani *et al.*, 2004; Emelianoff *et al.*, 2007). Similar studies do not appear to have been carried out with *Heterorhabditis* species. However, if there is a cost to *H. downesi* in carrying the symbiont it is the same for both subspecies of *P. temperata.*

The bacterial quantification (Section 3.3.5) showed that a small proportion of IJs carried no bacteria and this is consistent with the reported colonisation rate of over 90% of IJs (Lewis & Clarke, 2012). Overall there was no difference in the number of bacteria carried when PUR and YEL isolates were compared and in a subsequent experiment there was no difference in the number of bacteria carried by hybrid when compared to the wild type. These results are consistent with other studies, for example, Lewis *et al.* (1995) found that *H. bacteriophora* retained approximately

600 *P. luminescens* colony forming units during the first week of storage, with the number of bacteria retained by the IJs decreasing over time and the authors suggested that the bacteria may be utilised as a food source by the nematode. Studies with *H. bacteriophora* GPS11 and its symbiotic bacteria *P. temperata* showed that the number of bacterial cells in the nematode fell by approximately 50% in 30-day old IJs compared to freshly emerged IJs (An & Grewal, 2010a).

Chapter 4
Infectivity and development of *Heterorhabditis downesi* associated with two subspecies of *Photorhabdus* against a range of hosts

4.1 **Introduction**

Heterorhabditis downesi was found on North Bull Island to be associated with *P. temperata* subsp. *temperata* (*P.t temperata*) and *P. temperata* subsp. *cinerea* (*P.t cinerea*) (*yel* and *pur*, respectively). The natural host of *H. downesi* on North Bull Island are unknown but since the two colour phenotypes PUR and YEL are spatially distributed within the dune system it is possible that the two nematode-bacteria complexes are adapted to different hosts. Because the natural host of a particular EPN species is usually unknown laboratory-based studies are used to determine the efficacy of a particular nematode species against a range of hosts and to look at differences in virulence between strains of the same species. Different strains of the same species from different geographic locations may become adapted to particular hosts or indeed to different abiotic conditions (Mráček *et al.*, 1998). Both *Tenebrio molitor* and *Galleria mellonella* larvae are widely used in the laboratory to maintain cultures of nematodes. These insects are also widely used in bioassays to assess the ability of EPN species and strains to locate, infect and reproduce in insect hosts (Glazer & Lewis, 2000). Bioassays are also useful in determining the number of nematodes required to kill a particular host. It can also be useful to compare the infectivity of a particular EPN species against a range of life stages of a particular

host or against hosts from different orders to gain a better understanding of the fundamental ecology of a particular species and to predict the suitability of a particular EPN species or strain for use against a range of insect pests in the field.

In Ireland *H. downesi* K122, an indigenous Irish strain, has been shown to kill pine weevil, *H. abietis* (Dillon *et al.*, 2006; 2007), and black vine weevil larvae, *Otiorhynchus sulcatus* (Lola-Luz *et al.*, 2005) both in the laboratory and in field trials and this strain has been commercialised. Larvae of the garden chafer, *Phyllopertha horticola*, infected by *H. downesi* have been recovered on dunes (C Griffin, pers communication). A Hungarian strain, *H. downesi* strain 267 was found to be effective against the European cockchafer, *Melolontha melolontha* in the laboratory (Lakatos & Tóth, 2006). In chapter 3 the wild type (Pp, Yy) and hybrid (Py, Yp) combinations of *H. downesi*, produced in the cross-compatibility experiment, were tested against *G. mellonella* larvae. Some differences were found in the time to death for larvae infected with PUR1 or YEL3 nematodes, irrespective of the bacteria they carried, *pur1* or *yel3* and there were also differences in time to death between insects infected with *pur1* or *yel3* bacteria, irrespective of the nematode type (Section 3.3.3), indicating that both nematode and bacteria are involved in the mortality in this particular insect. However, *G. mellonella* is a very susceptible insect and is easily killed by many EPN species. Moreover, it is not an insect that the soil-dwelling EPN are likely to encounter as it is a pest of beehives and thus is of limited use in predicting the efficacy of a particular species or strain of EPN in the field. However, *G. mellonella* is of use in bioassays designed to look for differences between particular species or strain of EPN. As mentioned above, *H. downesi* K122 is effective against *H. abietis*. The symbiotic partner of K122 is *P. temperata* subsp. *temperata* strain K122. In this chapter the role of the bacteria in

infection will be investigated in more detail by challenging insects from three insect orders Lepidoptera (*G. mellonella*) Coleoptera (*T. molitor*, *H. Abietis*) Diptera (*Coelopa* spp). Both *G. mellonella* larvae and *T. molitor* larvae are commonly used in bioassays, as mentioned above. The large pine weevil, *H. abietis*, is an important pest of forestry and *H. abietis* larvae have been shown to be susceptible to the *H. downesi* K122 nematode strain which carries *P. temperata* subsp. *temperata* as its symbiont and is used as a proxy for other beetles (*Phyllopertha* spp. and *Otiorhynchus* spp.) that occur in sand dunes but are less readily available in the numbers required for bioassays. Two species of kelp fly, *Coelopa* species, *Coelopa frigida* and *C. pilipes* are commonly found in beds of seaweed (mainly *Laminaria* spp. and *Fucus* spp.) on the foreshore (Dobson, 1974) and the larvae feed mainly on the bacteria that are abundantly found in these clumps (Edward *et al.*, 2008). *Coelopa frigida* has been recorded on North Bull Island (Healy, 1975) and it is likely that *C. pilipes* occurs there also, since both species commonly occur together (Dobson, 1974), and might form part of the natural host range of *H. downesi* on North Bull Island. Since these dipterans occur in the strand line the PUR isolates might be expected to have a greater efficacy against these insects, given that the PUR isolates were found towards the front of the dune system.

The objective of this chapter is to investigate differences between *P.t. temperata* and *P.t. cinerea* in adaptation to different host species either in pathogenicity or in their ability to support *H. downesi* reproduction as evidenced by IJ production.

Specifically:

i) To test the wild type and hybrid nematode/bacterium combinations obtained in Chapter 3 against *G. mellonella* and *H. abietis*.

ii) To test the three PUR and three YEL nematode/bacterium isolates (Chapter 2) against *G. mellonella*, *H. abietis*, *T. molitor* and *Coelopa* spp.

4.2 Materials and Methods

4.2.1 Tests of wild type and hybrid combinations against *G. mellonella* (infectivity and infective juvenile yields)

A continuous exposure experiment was set up with the wild type and hybrid nematode/bacteria combinations established in Section 3.2.3.1, Table 3.2, consisting of a single nematode applied per insect using final instar *G. mellonella* larvae. The experiment included four blocks consisting of one replicate 24-well plate for each combination Pp, Py, Yy, Yp, plus one control plate per block (= 96 insects total per combination). The 24-well plates were prepared and stored as described in Section 3.2.2.2. Plates were checked after 48 hours and every eight hours thereafter up to 120 hours (5 days). Plates were checked daily from day 5 up to day 14. Insect mortality due to *P. temperata* infection (see Section 2.2.1.3) was recorded. Five cadavers per replicate were randomly chosen to assess emergence (= 20 cadavers total per combination). Fourteen days after infection a single White trap was set up for each replicate (= 5 cadavers per White trap). White traps were stored at 20^{o}C and each White trap was harvested twice at 14 day intervals following first emergence. All IJs were stored at 9^{o}C prior to counting as described in Section 3.2.1.6. Both harvests from each White trap were pooled for counting.

4.2.2 Tests of wild type and hybrid combinations against *Hylobius abietis* (infectivity and infective juvenile yields)

Pine weevil larvae were collected in the field from pine tree stumps and larvae were placed individually in the wells of 24-well plates. To prevent pupation and

desiccation the lid of each plate was lined with five layers of moist tissue paper and plates were stored at 9^{o}C. The tissue paper was replaced weekly and larvae were used in experiments within four weeks of collection. All larvae were weighed prior to use and larvae in the range 110 mg – 173 mg were used in all experiments.

A single *H. abietis* larva was added to a well of 24-well plates lined with three filter papers. IJs were added to each well in a total volume of 50 µl tap water at a range of application rates. Plates were randomly stacked in blocks consisting of one replicate of each application rate for each wild type or hybrid used, and one control plate, with three filter papers, 50 µl of tap water and one *H. abietis* larva per well. One empty plate with filter papers and 50 µl tap water only was placed on the top and bottom of each stack as a buffer. Plates were stored as described in Section 3.2.2.2 and checked daily and insect mortality recorded.

The experiment was run four times. In experiment 1 nematode treatments were Pp and Py, applied at a rate of 5, 10, 20 or 50 IJs per insect with 10 insects at each application rate. In experiment 2 nematode treatments were Pp and Py, applied at a rate of 5, 10, 20 or 50 IJs per insect with 7 insects at each application rate. In experiment 3 nematode treatments were Pp and Py, applied at a rate of 10, 20, 50 65 or 80 IJs with 10 insects at each application rate. In experiment 4 nematode treatments were Yy and Yp, applied at a rate of 10, 20, 50 65 or 80 IJs with 12 insects at each application rate and Pp and Py, applied at a rate of 50 IJs per insect with 12 insects per nematode treatment. All cadavers exhibiting evidence of death due to *P. temperata* infection (see Section 2.2.1.3) in the 50 IJ treatments for all nematode/bacterium combinations in experiments 2 - 4 were placed on individual White traps fourteen days after infection and each White trap was harvested

seventeen days after first emergence. All IJs were stored at 9°C prior to counting as described in Section 3.2.1.6.

4.2.3 Tests of PUR and YEL isolates against *G. mellonella* (infectivity and infective juvenile yields)

Infections were set up at an application rate of ten IJs per insect with six *H. downesi* nematode isolates from North Bull Island (PUR1, PUR2, PUR3, YEL1, YEL2, YEL3). Infections were set up as described in Section 3.2.2.2 with the following variations: three 1.5 cm filter papers were placed in each well; nematode suspensions were adjusted to 10 IJs/50 µl and 10 IJs were added to each of four wells at a time using a multichannel pipette. Sample counts were regularly carried out to check the consistency of IJ delivery. Plates were randomly stacked in blocks consisting of one plate of each isolate and one control plate with three filter paper, 50 µl of tap water and one *G. mellonella* larva per well. One plate with three filter papers and 50 µl tap water only was placed on the top and bottom of each stack as a buffer. Each stack was wrapped in tinfoil to ensure constant light conditions and stored at 20°C.

Experiment 1 consisted of four blocks of one replicate 24-well plate for each isolate (= 96 insects total per isolate). Plates were checked after 48 hours and insect mortality was recorded. Tap water (50 µl) was added to the wells as required to avoid the filter paper drying out. Insect mortality was recorded daily up to day 8, at which point the experiment was terminated. On days 2, 3 and 4 post infection up to three cadavers exhibiting signs of mortality due to EPN infection (Section 2.2.1.3) were chosen at random from each plate, stored for three days at 20°C and then frozen for dissection at a later date to assess the number of IJs invading.

In experiment 2 the procedure above was repeated with two replicate plates per isolate (= 48 insects total per isolate) and mortality was recorded after 29 hours and then at 12 hour (+/- 3 hrs) intervals up to 140 hours (day 6) post infection and daily up to 8 days post infection. At each time point on days 2, 3 and 4 of the experiment up to three cadavers exhibiting signs of mortality due to EPN infection were chosen at random from each plate and stored for dissection as described above. Fourteen days after infection all remaining cadavers exhibiting signs of mortality due to EPN infection were placed on individual White traps and stored at 20^{o}C. All White traps were harvested (see Section 3.2.1.6) 17 days following first emergence of IJs and IJs were stored at 9^{o}C prior to counting as described in Section 3.2.1.6. All IJs from each replication were pooled and counted.

4.2.4 Tests of PUR and YEL isolates against *H. abietis* (infectivity and infective juvenile yields)

Infections were set up at an application rate of 50 IJs per insect with six *H. downesi* nematode isolates from North Bull Island (PUR1, PUR2, PUR3, YEL1, YEL2, YEL3). Multiwell plates were prepared as described in Section 3.2.2.2, with the following variations: three filter papers were placed in each well; nematode suspensions were adjusted to 50 IJs/50 µl and 50 IJs were added to each of four wells at a time using a multichannel pipette; a single *H. abietis* larva was added to each well.

There were two experiments with sixteen and twenty four insects per isolate, respectively. Plates were stored as described in Section 4.2.3 with one control plate with three filter papers, 50 µl of tap water and one *H. abietis* larva per well. Plates were checked after 48 hours and insect mortality was recorded. Tap water (50 µl) was added to the wells as required to avoid the filter paper drying out. Insect

mortality was recorded daily for fourteen days at which point the experiment was terminated. Fourteen days after infection cadavers exhibiting evidence of death due to *P. temperata* infection (Section 2.2.1.3) were transferred to individual White traps and stored at 20^{o}C. White traps were harvested at day 17 following first emergence of IJs. All IJs were stored at 9^{o}C prior to counting as described in Section 3.2.1.6. In each experiment IJs from all White traps per isolate were pooled before counting.

4.2.5 Tests of PUR and YEL isolates against *T. molitor* (infectivity)

Infections were set up at an application rate of ten IJs per insect with six *H. downesi* nematode isolates from North Bull Island (PUR1, PUR2, PUR3, YEL1, YEL2, YEL3) as described in Section 3.2.2.2 with the following variations: 0.2 g moist coir (1:2.5 w/w coir:tap water) was added to each well and tamped down so that the well was half filled with coir; nematode suspensions were adjusted to 10 IJs/50 µl and added as described in Section 4.2.3. The experiment consisted of two blocks of one replicate 24-well plate for each isolate (= 48 insects total per isolate). Plates were stored as described in Section 4.2.3 with the following variations: control plate had moist coir, 50 µl of tap water and one *T. molitor* larva per well; buffer plates had moist coir, 50 µl of tap water. Plates were checked after 27 hours and then every 12 hours (+/- 3hrs) up to day 6 and then every 24 hours up to day 8 and insect mortality was recorded. Tap water (50 µl) was added to the wells as required to avoid the coir drying out. The experiment was terminated on day 8.

4.2.6 Tests of PUR and YEL isolates against Kelp fly (Infectivity and infective juvenile yields)

Kelp fly larvae (*Coelopa* sp.*)* were collected in the field from drifts of seaweed deposited by spring tides (wrack-beds) on Mullaghmore Strand, Co. Donegal. The larvae were stored in the dark in seaweed in 24 l plastic storage boxes with close

fitting lids (Tesco plc) at 9°C and used within one week of collection. A 48 hour infectivity test based on Peters (2005) was set up with six *H. downesi* nematode isolates from North Bull Island (PUR1, PUR2, PUR3, YEL1, YEL2, YEL3) as follows: one 9 cm filter paper was placed in the base of a 9 cm Petri dish, 0.5 ml of nematode suspension was pipetted evenly over the filter paper and ten kelp fly larvae were added to the dish. The larvae were covered with 12 g of air dried sterile sand (B & Q plc) mixed with 8% w/w ¼ strength sterile Ringer's solution (Figure 4.). A further 0.5 ml nematode suspension was evenly pipetted over the sand and the lid was replaced.

There were two experiments, with a dose of 1000 IJs/dish and 5000 IJs/dish, respectively. Each experiment consisted of five blocks. Dishes were randomly stacked in blocks consisting of one dish of each isolate and one control consisting of a 9 cm Petri dish with filter paper, moist sand, 10 kelp fly larvae and 2 x 0.5 ml aliquots of tap water added as described above. Two 9 cm Petri dishes with filter paper, moist sand and 2 x 0.5 ml aliquots of tap water were placed on the top and bottom of each stack and the dishes were securely taped together. All five blocks were placed in a plastic food container surrounded by eight stacks of empty dishes. The food containers were placed in closed polystyrene containers to maintain constant light and ventilation conditions and stored at 20°C. After 48 hours all insects were removed from the sand, rinsed in sterile ¼ strength Ringer's solution and transferred to 5 cm Petri dishes lined with a single filter paper moistened with 400 µl sterile ¼ strength Ringer's solution. Insect mortality was recorded and insects were stored at 20°C. In experiment 1 (1000 IJs/dish) one block was chosen at random and set aside for dissection. Ten days after infection all remaining cadavers in both experiments exhibiting evidence of death due to *P. temperata* infection

(Section 2.2.1.3) were transferred to individual White traps and stored at 20°C. Emerging IJs were allowed to accumulate in the White traps and a single harvest was carried out seventeen days following first emergence of infective juveniles. All IJs were stored at 9°C prior to counting as described in Section 3.2.1.6.

Figure 4.1: Kelp fly infections were set up in 9 cm Petri dishes, with 10 larvae per dish. Nematode concentration was 100 or 500 IJs/larva.

Numbers of IJs emerging were dealt with as follows:

i) Individual counts of emergence per cadaver were carried out for a single replication in each experiment.
ii) Counts of pooled total emergence for all remaining replications.

4.2.7 Statistical analysis

Routine statistical analysis, including tests for normality, was carried out using Minitab statistical software, version 16.1.1 (Minitab Inc., 2010). Normality of data

was tested using the Anderson-darling method. Analysis of variance was carried out using a one-way ANOVA or when groups had an uneven number of observations the general linear model with an unbalanced nested design with isolate nested within colour phenotype was used to compare isolate and colour phenotype. Analysis of variance was followed by pair-wise multiple comparisons using Tukey's test. In experiments where nematode type and bacterial type were factors wild type and hybrid results were analysed using a two-way ANOVA. Where no significant interactions occurred treatment means for each factor were compared using a one-way ANOVA and post-hoc Tukey's test for three or more treatments or 2-sample T-test for two treatments. When data could not be normalised treatment medians were compared using a Kruskal-Wallis test, followed by multiple pairwise comparisons using a Mann-Whitney U-test with Bonferroni 95% confidence intervals, obtained by calculating $(1 - \alpha / g)$ where α is the level of significance and g is the number of pairwise comparisons carried out. Lethal concentrations were calculated using Probit analysis. In all tests a P value of < 0.05 was taken as significant unless indicated otherwise.

4.3 **Results**

4.3.1 Tests of wild type and hybrid combinations against *G. mellonella*

4.3.1.1 Infectivity

In a single nematode continuous contact experiment using *G. mellonella* larvae the mortality was recorded at 8 hour intervals from 48 hours post infection to 120 hours post infection and at 24 hour intervals thereafter up to day 8 post infection. The data for the 48 hour, 56 hour and 80 hour time points were not normally distributed and could not be normalised. The mean or median percentage of insects dead at any time

point did not differ between the four strains tested (P > 0.05, Table 4.). Mortality increased from negligible levels at 48hrs to about 30% at 96hrs. Overall, the mean percentage mortality by day 8 of the experiment was approximately 35%. At each time point there was a higher percentage of insects dead in the *pur* treatments (Pp and Yp) than in the *yel* treatments (Yy and Py). This is dealt with below.

Table 4.1: Median† or mean‡ (± SE) percentage of *G. mellonella* dead at eight hour intervals for wild type and hybrid combinations of nematode and bacteria. All insects were infected with a single IJ in a continuous contact exposure. n = 96 per treatment. Results of Kruskal-Wallis test or one-way ANOVA comparison between treatment medians or means are shown for proportion dead at each time point.

	Time point (hrs)						
Nematode/ bacteria combination	48†	56†	64‡	72‡	80†	88‡	96‡
Pp	2.1	12.5	12.5 (2.95)	18.8 (2.69)	20.8	25.0 (2.95)	29.2 (4.50)
Py	0.0	4.2	8.3 (2.41)	12.5 (2.95)	18.8	22.9 (3.61)	26.0 (4.60)
Yp	0.0	12.5	17.7 (5.48)	25.0 (7.01)	27.1	30.2 (7.49)	31.3 (7.70)
Yy	2.1	8.3	13.5 (2.62)	19.8 (3.13)	27.1	26.0 (1.99)	28.1 (2.62)
P value	0.730	0.156	0.367	0.289	0.692	0.715	0.912
	H (3 df)	H (3 df)	$F_{3,12}$	$F_{3,12}$	H (3 df)	$F_{3,12}$	$F_{3,12}$
Test statistic	1.3	5.23	1.15	1.41	1.46	0.46	0.17

In order to explore the effect of the bacterial type more fully, the data were pooled by bacterial type and plotted. The data at each time point except 48 hours were normal. The data for the 48 hour time point were excluded and each of the remaining time points were analysed using a two-way ANOVA with nematode type and bacteria type as factors. The analysis showed there was no effect of nematode type or bacteria type at any time point at the 5% level, and no interaction between nematode type and bacteria type at any time point. However, bacteria type was significant at the 10% level at 56 hours post infection with more insects dead in the *pur* treatment

(two-way ANOVA, $F_{1,12}$ =3.87, P = 0.073) (Appendix Table A 15). There was no difference in the speed of kill between the *pur* and the *yel* bacterial treatments over the period from 48 hours post infection to 96 hours post infection. (Two-sample T-test on pairwise slopes, df = 7, T = -0.68, P = 0.516) (Figure 4.2).

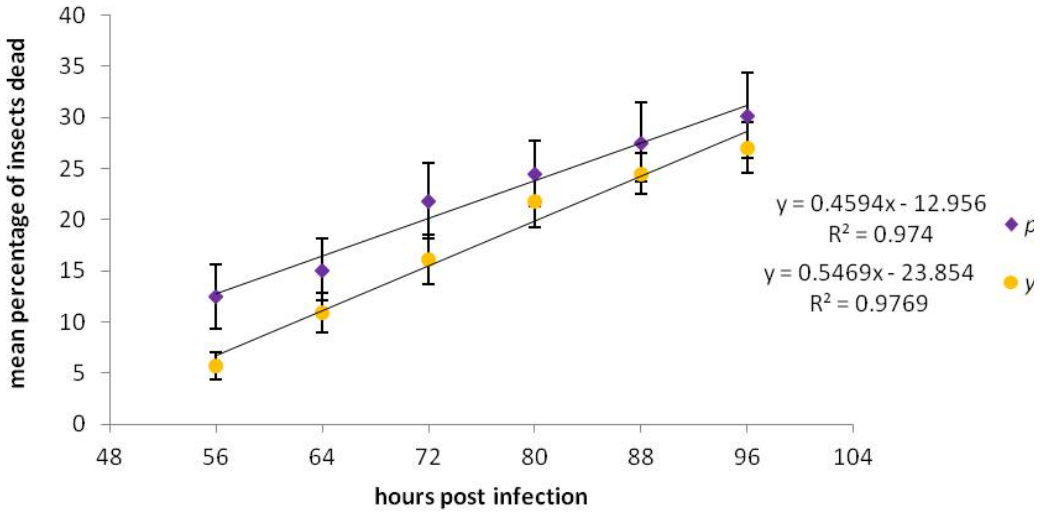

Figure 4.2: Mean percentage (± SE) of *G. mellonella* dead for the period 48 hours to 96 hours post infection in a single nematode continuous exposure experiment. Data were pooled by bacterial treatment.

4.3.1.2 Numbers of IJs emerging (*G. mellonella*)

A two-way ANOVA on number of IJs emerging with nematode type and bacterial type as factors showed that neither nematode type nor bacterial type had a significant effect on the total number of IJs emerging over the 28 day period (P > 0.05) (Table 4.2) and no further analysis was carried out on the emergence data..

Table 4.2: Mean number of IJs (±SE) emerging from *G. mellonella* over 28 days following an infection with a single wild type or hybrid IJ. Data shown is mean number of IJs emerging from a pooled harvest from five cadavers for each treatment, n = 4. Results of GLM analysis of data pooled by nematode type and by bacterial type are also given.

Nematode/bacteria combination	mean number of IJs in 4 pooled harvests
Pp	135767 (3658)
Py	120780 (5695)
Yy	116580 (5026)
Yp	108317 (13435)

Factor	Test statistic	P
Nematode type	$F_{1,12} = 0.10$	0.761
Bacterial type	$F_{1,12} = 1.16$	0.302
Interaction	$F_{1,12} = 2.15$	0.168

4.3.2 Virulence of wild type and hybrid combinations against *H. abietis*

In both experiment 1 and 2 of the continuous exposure test a concentration of 5 IJs per insect showed little or no kill (Table 4.3), and this concentration was omitted in experiments 3 and 4. While there was some evidence of a dose response this was not consistent. The LD_{50} was calculated for all four strains (using data from experiments 1-3 for Pp, Py, and from experiment 4 for Yy, Yp) (Table 4.4). There was a highly significant difference in the LD_{50} values between the Yy and Yp strains (experiment 4) (Probit analysis, Z = -3.18, P = 0.001). There was no difference in the slopes between strains in either comparison (experiments 1-3, $\chi^2 = 3.290$, df = 1, P = 0.070; experiment 4, $\chi^2 = 1.261$, df = 1, P = 0.261).

Table 4.3 Percentage mortality of *H. abietis* larvae exposed to a range of concentrations of wild type and hybrid nematode/bacteria combinations in four experiments.

		Experiment			
		1	2	3	4
		n			
nematode/bacteria combination	IJ concentration	10	7	10	12
Pp	5	0	0		
	10	10	14.29	10	
	20	20	14.29	30	
	50	50	57.14	50	83.33
	65			80	
	80			80	
Py	5	0	14.29		
	10	10	14.29	40	
	20	20	14.29	30	
	50	40	71.43	50	66.67
	65			80	
	80			40	
Yy	10				16.67
	20				41.67
	50				83.33
	65				75.00
	80				83.33
Yp	10				33.33
	20				75.00
	50				100
	65				100
	80				100

Table 4.4: Results of Probit analysis of mortality of *H. abietis* larvae exposed to a range of concentrations of wild type and hybrid nematode/bacteria combinations in four experiments. Day 8 LD_{50} values and lower and upper Fiducial confidence interval (Fc) values for the number of IJs required for wild type and hybrid nematode/bacteria combinations applied to *H. abietis* larvae are shown. Strains which do not share the same letter in each experiment(s) were significantly different.

Nematode/bacteria combination	LD_{50} (± SE)	Lower Fc – Upper Fc
Pp (experiments 1-3)	57.13 (10.50)	40.92 – 88.67
Py (experiments 1-3)	60.24 (11.01)	43.23 – 93.26
Yy (experiment 4†)	30.47 (5.45)a	19.07 – 41.39
Yp (experiment 4)	11.73 (2.67)b	6.50 – 17.42

† The Pp and Py data were not included in the analysis as there was only a single dose rate for each of these strains in experiment 4.

4.3.2.1 Numbers of IJs emerging (*H. abietis*) (fifty IJs per insect application rate)

In order to investigate the effect of nematode type and bacterial type on the number of IJs emerging two analyses were carried out. Firstly, the data from experiment 4 for all four strains (Pp, Py, Yy, Yp) were analysed using a GLM with nematode type and bacterial type as factors (Table 4.5). The analysis showed that neither nematode type nor bacterial type had a significant effect on the number of IJs emerging over the 17 day period, however there was a highly significant interaction effect, with the highest number of IJs per cadaver emerging from insects in the Yy treatment followed by the Pp treatment (Table 4.5) Secondly, the data from experiments 2 – 4 for strains Pp and Py were combined and analysed using a GLM with bacterial type as factor (Table 4.6). This analysis also showed that bacterial type did not have a significant effect on the total number of IJs emerging over the 17 day period, but that

experiment had a highly significant effect (Table 4.6). Overall the mean emergence per cadaver was 53551 IJs following infection with wild type or hybrid nematodes at dose rate of 50 IJs per insect (See Appendix Table A 16 for emergence data).

Table 4.5: Mean number of IJs (± SE) emerging from *H. abietis* over 17 days following infection with wild type or hybrid nematodes at an application rate of 50 IJs per insect. Data shown is for experiment 4. n = 5 for Pp and n = 6 for remaining treatments. Results of GLM analysis are also given.

Nematode/bacteria combination	Mean number of IJs per cadaver
Pp	68510 (7362)
Py	58709 (10670)
Yy	89086 (9432)
Yp	48488 (7146)

Factor	Test statistic	P
Nematode type	$F_{1,19} = 0.80$	0.382
Bacterial type	$F_{1,19} = 1.63$	0.218
Interaction	$F_{1,19} = 9.04$	0.007

Table 4.6: Mean number of IJs (± SE) emerging from *H. abietis* over 17 days following infection with wild type or hybrid nematodes at an application rate of 50 IJs per insect. Data shown is combined emergence from experiments 2-4 from insects infected with wild type or hybrid nematodes at a dose of 50 IJs per insect. n = 5 for Pp and n = 6 for remaining treatments. Results of GLM analysis are also given.

Experiment	nematode/bacteria combination	Mean number of IJs per cadaver	n
2	Pp	36369 (9131)	3
	Py	48316 (3947	4
3	Pp	23090 (1907)	4
	Py	29437 (4901)	6
4	Pp	68510 (7362)	5
	Py	58709 (10670)	6
Factor		Test statistic	P
Experiment		$F_{2,23} = 7.76$	0.003
Bacterial type		$F_{1,23} = 0.01$	9.350

4.3.3 Test of PUR and YEL isolates against *G. mellonella*

4.3.3.1 Overall mortality and time to death

In the assay with an application rate of ten IJs per insect with *G. mellonella* more insects died on day 3 of the experiment than on any other day in both experiment 1 and 2 (Appendix Table A 17). The proportion of insects dead due to EPN infection was tested at each time point for each experiment separately. The data were not normally distributed and were arcsine square root transformed for analysis. The data from each experiment were first analysed separately.

In experiment 1 there was a significant difference in insect mortality due to colour phenotypes at day 2, with a higher proportion of insects dead in the PUR treatment, (nested ANOVA with isolate nested within colour phenotype, $F_{1,4} = 15.44$, $P = 0.017$) and at days 5 – 8 the difference between colour phenotypes was significant

at the 10% level ($P < 0.1$). In experiment 2 there was no difference in mortality due to colour phenotype at any time point (Appendix Figure A. 8).

A nested ANOVA of the combined data with colour phenotype nested within experiment and isolate nested within colour phenotype showed that there was a difference in the insect mortality due to colour phenotype at the 10% level at day 2, ($F_{2,8} = 3.588$, $P = 0.077$), however, there was an interaction between colour and experiment at each time point day 3–day 8 (Figure 4.4).

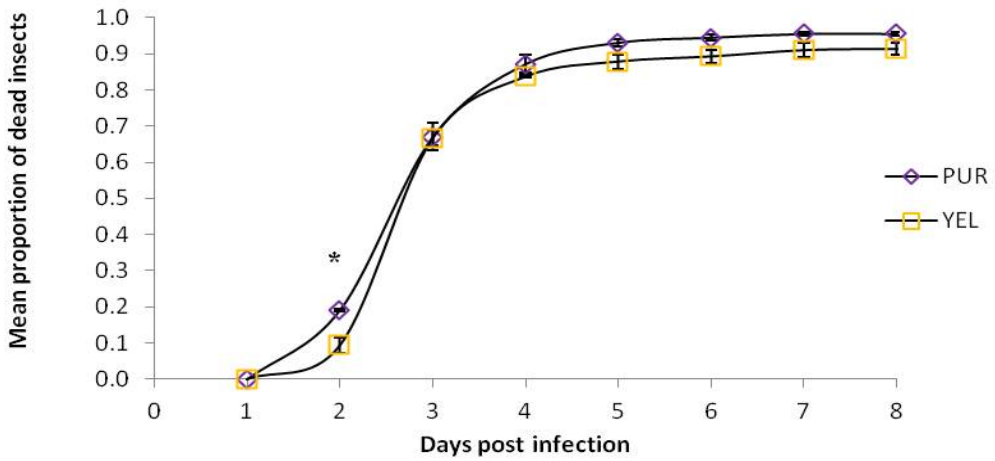

Figure 4.3: Mean proportion (± SE) of *G. mellonella* killed by PUR and YEL IJs over eight days at an application rate of ten IJs per insect. Data shown is from experiment 1 with three isolates of each colour phenotype in each trial. n per colour phenotype = 288. The proportion of insects killed by the PUR colour phenotype was significantly higher at day 2 post infection (nested ANOVA with isolate nested within phenotype and experiment nested within isolate, $P = 0.017$. Data was arcsine square root transformed for analysis)

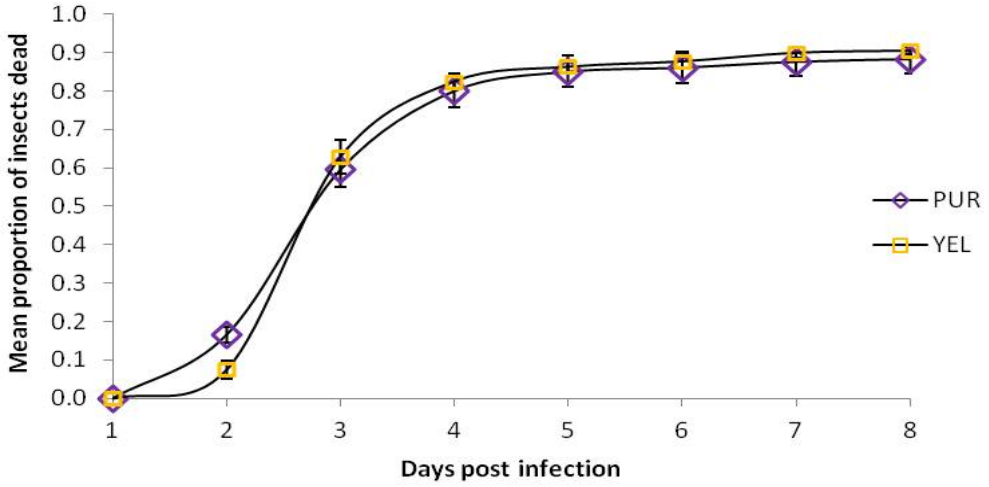

Figure 4.4: Mean proportion (± SE) of *G. mellonella* killed by PUR and YEL IJs over eight days at an application rate of ten IJs per insect. Data shown is mean of two experiments with three isolates of each colour phenotype in each trial. Total n per colour phenotype = 432. The proportion of insects killed by the PUR colour phenotype was significantly higher at the 10% level at day 2 post infection (nested ANOVA with isolate nested within phenotype and experiment nested within isolate, P = 0.077. Data was arcsine square root transformed for analysis).

4.3.3.2 Invasion rate (*G. mellonella)*

In both experiment 1 and 2 the median number of adult nematodes present in insects which were dead at day 2, day 3 and day 4 ranged from 2 to 4 nematodes per insect (Appendix Table A 18). The data were not normally distributed and could not be normalised by transformation. For each experiment the data were pooled by colour phenotype and the number of IJs of each colour phenotype invading on each day was compared (Table 4.7). In experiment 1 the median number of adult nematodes present at each time point was 3 in all cases except for the YEL IJs at day 3, when the median number of adults nematodes present in insects was 4 and this difference was highly significant (Kruskal-Wallis test, df = 5, H = 15.78, P = 0.008). A series of multiple pairwise comparisons were carried out which showed that the number of YEL IJs invading on day 3 was significantly higher than the number of PUR IJs

invading on day 3 or day 4. (Mann-Whitney U-test, 95% Bonferroni-adjusted significance level of $\alpha = 0.003$, P = 0.0009, and P = 0.0030 respectively) (Appendix Table A 19). There were no other differences between the number of PUR and YEL IJs invading at any other time point. There was no difference in experiment 2 (Kruskal-Wallis test, df = 5, P > 0.05).

Table 4.7: Median number of adult nematodes of each colour phenotype present in *G. mellonella* cadavers at three time points following infection with PUR or YEL nematodes at an application rate of ten IJs per insect. At each time point in each experiment up to twelve insects per isolate were randomly chosen for dissection. In each experiment treatments which do not share the same Section 4.3.3.2letter were significantly different. (Mann-Whitney U-test with a 95% Bonferroni confidence interval) (Appendix Table A 19)

Experiment	Phenotype	Day		
		2	3	4
1	PUR	3a,b	3a	3a
	YEL	3a,b	4b	3a,b
2	PUR	3	3	3
	YEL	3	3	3

A similar trend was seen when the data from both experiments were pooled by colour phenotype (Kruskal-Wallis test df = 5, H = 16.20, P = 0.006). Multiple pairwise comparisons showed that the number of YEL IJs invading was significantly higher on day 3 compared to the number of PUR IJs invading on day3 or day 4 (Table 4.8).

Table 4.8: Results of pairwise comparisons of median number of adult nematodes per cadaver in *G. mellonella* larvae which were dead at three time points post infection for each of two colour phenotypes of *H. downesi* nematodes from North Bull Island. Nematodes were applied at a rate of ten IJs per insect. Treatments which do not share the same letter were significantly different. (Mann-Whitney U-test with a 95% Bonferroni confidence interval) (Appendix Table A 20).

Phenotype	Day		
	2	3	4
PUR	3a,b	3a	3a
YEL	3a,b	4b	3a,b

4.3.3.3 Time of infective juvenile first emergence (*G. mellonella*)

In the second experiment all the remaining cadavers were placed on White traps fourteen days after infection. There was 100% emergence in all treatments except for the PUR3 isolate where there was no emergence from one cadaver (= 95% emergence). Time to first emergence ranged from 16 to 23 days post infection for the PUR isolates and from 16 to 25 days post infection for the YEL isolates with a median time to first emergence of 17 to 19 days for both colour phenotypes (Appendix Table A 21). The data were not normally distributed and could not be normalised. The data were pooled by colour phenotype and time to first emergence analysed using a Mann-Whitney U-Test. There was no difference in time to first emergence between colour phenotypes ($P > 0.05$) (Appendix Table A 22). A regression analysis carried out on time to first emergence vs time to death showed there was a positive correlation between time to death and time to first emergence for both colour phenotypes ($R^2 = 0.603$ for PUR and $R^2 = 0.487$ for YEL (see Appendix Figure A. 9A and Appendix Figure A. 9B).

4.3.3.4 Numbers of IJs emerging (*G. mellonella*)

The IJ harvests (Table 4.9, see also Appendix Table A 23) were compared using a nested ANOVA with isolate nested within colour. The analysis showed that neither colour phenotype nor isolate had a significant effect on the mean number of IJs emerging over the 17 day period ($P < 0.05$) (Appendix Table A 24) and no further analysis was carried out on the emergence data.

Table 4.9: Mean (± SE) number of IJs emerging for 3 PUR and 3 YEL isolates for *G. mellonella* over a 17 day period. n = 2 with 7 – 12 insects per replicate.

Isolate	Mean emergence per cadaver (± SE)
PUR1	131685 (4194)
PUR2	89918 (34300)
PUR3	136996 (31437)
YEL1	108103 (11543)
YEL2	114662 (9238)
YEL3	141245 (4141)

4.3.4 Test of PUR and YEL isolates against *H. abietis*

4.3.4.1 Overall mortality and time to death

In both experiment 1 and experiment 2 more insects died on day 5 of the experiment than on any other day and the total mortality reached approximately 90% by day 14 (Appendix Figure A. 10A and Appendix Figure A. 10B). The proportion of insects dead due to EPN infection at each time point for day 4 to 14 was analysed. The data were not normally distributed and were arcsine squareroot transformed for analysis. There were no differences between colour phenotypes in the proportion of insects dead at any time point in either experiment (nested ANOVA with isolate nested within colour, $P > 0.05$). Similarly, when the data from both experiments were

combined (Figure 4.5) there was no difference between colour phenotypes at any time point (nested ANOVA, P > 0.05), although the PUR 3 isolate killed significantly fewer insects by day 14 (one-way ANOVA $F_{5,6}$ = 4.51, P = 0.047) (Appendix Table A 25).

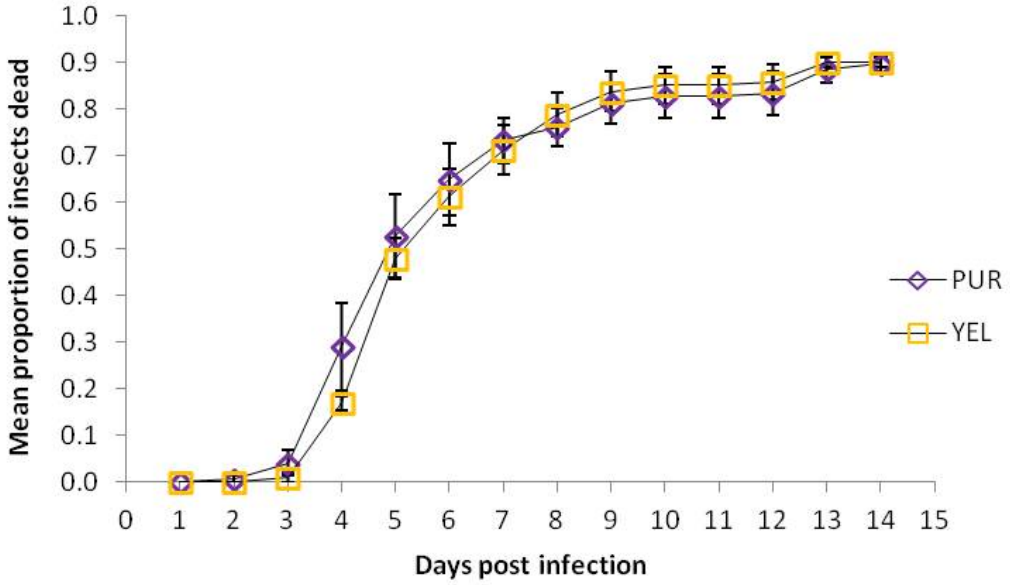

Figure 4.5: Mean proportion (± SE) of *H. abietis* larvae killed by PUR and YEL IJs over fourteen days. Data shown is mean of two experiments with three isolates of each colour phenotype in each experiment. n = 120 insects per colour phenotype.

4.3.4.2 Time of infective juvenile first emergence (*H. abietis*)

The percentage of cadavers with emergence of IJs ranged from 80% to 100% (Appendix Table A 26) with no difference due to colour phenotype in either experiment or when the data for both experiments were combined, (2-Sample T-test, P > 0.05 in all cases) (Appendix Table A 27). While there was a difference in time to first emergence at isolate level in both experiments separately (Table 4.10)., there was no overall pattern due to phenotype. Time to first emergence ranged from 19 to 54 days post infection in experiment 1 and from 18 to 50 days post infection in experiment 2.

Table 4.10: Mean (± SE) or median time post infection to first emergence of infective juveniles for each of six isolates of *H. downesi* nematodes from North Bull Island in two experiments using *H. abietis*. Treatments which do not share the same letter were significantly different.

Isolate	mean† (± SE) or median‡ time to first emergence of IJs (days post infection)	
	Experiment 1 †	Experiment 2‡
PUR1	26.0 $_{(1.25)}$ a b	18.0 a
PUR2	24.8 $_{(0.61)}$ a b	23.0 b
PUR3	32.5 $_{(3.81)}$ b	20.0 a b
YEL1	23.2 $_{(1.01)}$ a	22.0 a b
YEL2	28.1 $_{(1..20)}$ a b	21.0 a b
YEL3	27.8 $_{(0.90)}$ a b	21.5 b

† GLM, $F_{4,69} = 3.98$, $P = 0.006$

‡ Mann-Whitney U-test with a 95% Bonferroni confidence interval, (see Appendix Table A 28 for results of pairwise comparisons.

As was the case in the bioassay with *G. mellonella* (Section 4.3.3.3), there was a positive correlation between time to death and time to first emergence for both colour phenotypes in both experiments and this relationship was stronger for the PUR colour phenotype than the YEL in both experiments ($R^2 = 0.2335$ for PUR and $R^2 = 0.1355$ for YEL in experiment 1, $R^2 = 0.3375$ for PUR and $R^2 = 0.0488$ for YEL in experiment 2, see Figure A. 11A-D).

4.3.4.3 Numbers of IJs emerging (*H. abietis*)

Numbers of IJs emerging from *H. abietis* larvae ranged from approximately 30,000 per insect (YEL3 in experiment 1) to over 60,000 (PUR3 in experiment 2) (Table 4.11) (See also Appendix Table A 26). There was no difference in the percentage of cadavers with emergence between colour phenotypes in either experiment or when both experiments were combined (Appendix Table A 27). There was also no

difference between colour phenotypes in the mean number of IJs emerging in either experiment (2-sample T-Test, experiment 1: df = 2, T = 0.60, P = 0.610; experiment 2: df =2, T = 0.89, P = 0.468). The data from experiment 1 and experiment 2 were combined and analysed using a nested ANOVA, with isolate nested within colour and colour nested within experiment. There were no differences between colour phenotypes in number of IJs emerging over the 17 day period (nested ANOVA, $F_{2,8}$ = 0.506, P = 0.621)

Table 4.11: Number of IJs emerging from *H. abietis* cadavers for three PUR and three YEL isolates over a 17 day period in each of two experiments. In each experiment one pooled count was carried out for each isolate with 12 -14 cadavers per isolate in experiment 1 and 20 – 22 cadavers per isolate in experiment 2.

Experiment	Isolate	Mean emergence per cadaver
1	PUR1	47356
	PUR2	49977
	PUR3	45730
	YEL1	57755
	YEL2	45164
	YEL3	29528
2	PUR1	50305
	PUR2	45167
	PUR3	64285
	YEL1	50791
	YEL2	46545
	YEL3	46751

4.3.5 Host infectivity – *T. molitor*

In the assay with *T. molitor*, with an application rate of ten IJs per insect, more insects died on day 3 of the experiment than on any other day (Figure 4.6). The total mortality by day 8 ranged from 38% to 54%. The proportion of insects dead at each

time point was compared using a nested ANOVA with isolate nested within colour. The data were arcsine squareroot transformed for analysis. There was no difference in the mean proportion of insects dead for either factor at any time point.

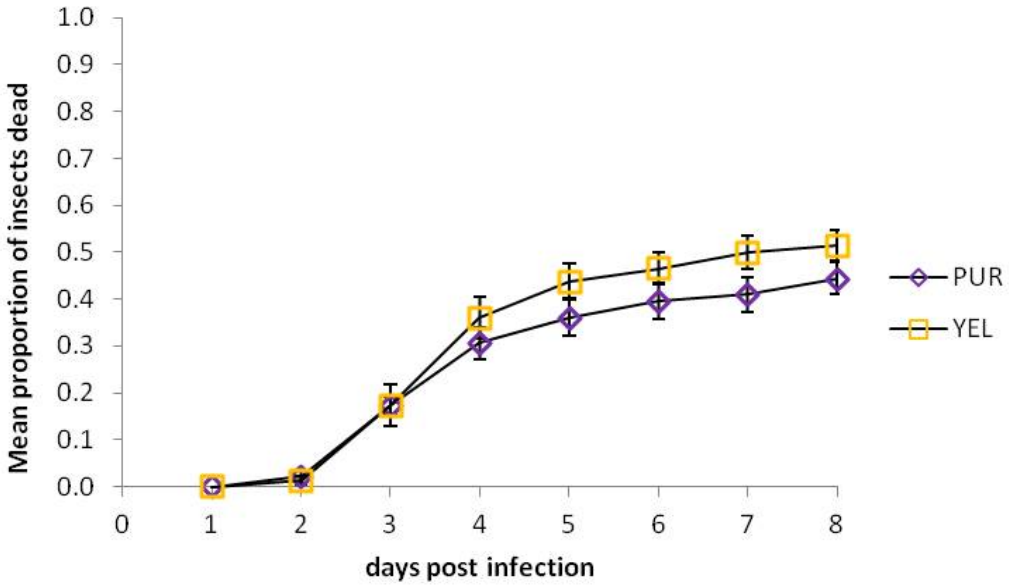

Figure 4.6: Mean proportion (± SE) of *T. molitor* killed by PUR and YEL IJs over eight days in a continuous exposure experiment. Nematodes were applied at a rte of en IJs per insect. Data shown is mean of three isolates of each colour phenotype. n = 144 insects per colour phenotype

4.3.6 Host infectivity – Kelp fly

4.3.6.1 Overall Mortality

There were two 48 hour exposure experiments, with a concentration of 1000 or 5000 IJs per 10 larva, respectively. In the 1000 IJ concentration experiment mortality due to EPN infection ranged from 60% to 100% after 48 hours and from 70% to 100% by day 6 (Appendix Table A 29). In the 5000 IJ concentration mortality due to EPN infection ranged from 20% to 80% after 48 hours and from 50

% to 100% by day 6 post infection. In each treatment a number of the larvae had pupated (Figure 4.7A) and in some instances adult flies had emerged. Nematodes developing within the larvae were easily observed (Figure 4.7B). There was no effect of colour phenotype or isolate on the proportion of insects dead for either concentration (nested ANOVA with isolate nested within phenotype, $P > 0.05$) (Figure 4.8). Interestingly, there was a lower mean mortality for each colour phenotype at the higher concentration (Figure 4.8B).

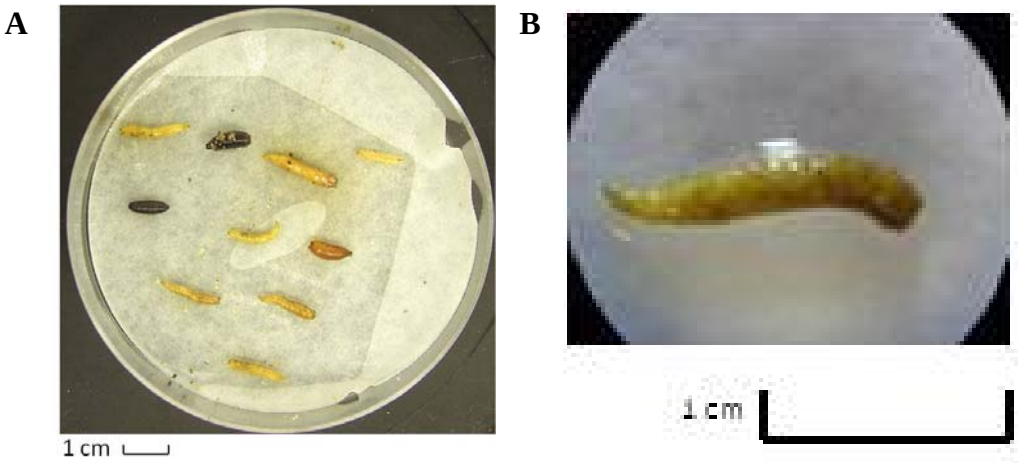

Figure 4.7: Kelp fly larvae infected with *H. downesi* isolates from North Bull Island. (A) Some larvae started to pupate prior to death. (B) Nematodes can be seen developing within the kelp fly larva. Image shown is a larva in the 1000 IJ /10 larvae treatment

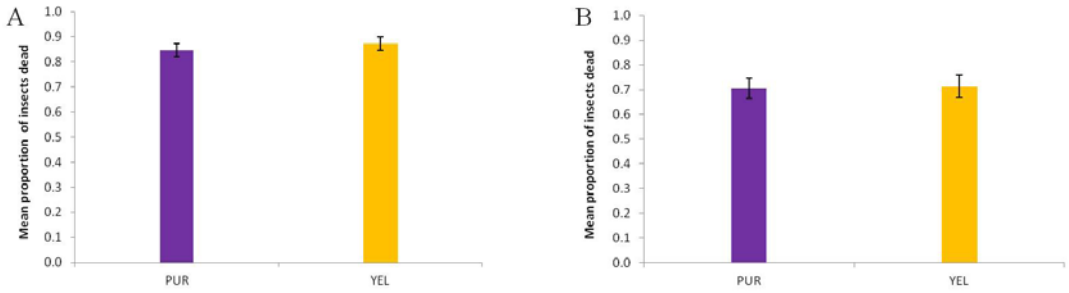

Figure 4.8: Mean proportion (± SE) mortality of kelp fly larvae in a single 48 hour exposure with (A) 1000 IJs / 10 insects and (B) 5000 IJs / 10 insects. n = 15 insects per colour phenotype in each experiment.

4.3.6.2 Assessment of colonisation of kelp fly larva by the entomopathogenic nematodes

All ten insects for each isolate from one block in the 1000 IJ / 10 insect experiment were sampled destructively to assess the success of the infection (Table 4.12). In a number of cadavers the number of nematodes observed was low (3/27 and 12/30 cadavers for PUR and YEL, respectively), with only dead nematodes present in two of the PUR cadavers. In the YEL treatment no nematodes were observed in 10/30 cadavers, despite evidence of bacterial colonisation, whereas no nematodes were observed in only 1/27 cadavers in the PUR treatment. The colonisation appeared more advanced in the YEL treatment as most of the cadavers contained pre IJs with few of the previous life stages present, whereas for two of the three PUR isolates there were many worms in the intermediate life stages still present in the cadavers.

Table 4.12: Colonisation of kelp fly by entomopathogenic nematode-bacterium complex. Ten insects were exposed to IJs for 48 hrs at a concentration of 1000 IJs/10 larvae and dissected fourteen days later.

		EPN lifestages present						Total number of EPN infections(/10)
Isolate	*C. frigida* (/10)	none/dead*	adults	adults & juveniles	adults, juveniles & bagged worms†	bagged worms & juveniles	pre IJs	
PUR1	10	1	1		1	6		9
PUR2	8	2	1	1	2	1	1	8
PUR3	10						10	10
YEL1	10	1	1				8	10
YEL2	10	5		1			4	10
YEL3	9	6	1				3	10

* none/dead was recorded where there was a large amount of bacterial biomass present, with either low numbers of dead nematodes or no nematodes.

† In a process known as *endotokia matricida*, in each generation some eggs hatch within the maternal body and consume it. These are referred to as bagged worms.

4.3.6.3 Numbers of IJs emerging (kelp fly)

In both experiments some emergence occurred in all treatments within 24 hrs of cadavers being placed on White traps. Emergence ranged from 37.5% to 100%, with no difference between isolates, colour phenotypes or IJ concentration (Appendix Table A 30A and Table A 30B).

i) Individual counts per cadaver

Emergence ranged from 304 IJs to 20832 IJs per cadaver. (See Appendix Table A 31 and Table A 32 for mean emergence per cadaver). The data for both concentrations were combined and analysed using a nested GLM with isolate nested within colour and concentration included as a factor. There was no difference in number of IJs emerging per cadaver between colour phenotypes and no effect of concentration on number of IJs emerging per cadaver ($P > 0.05$ for both factors), however there was a highly significant difference between isolates ($P < 0.001$) (Table 4.13).

Table 4.13: Mean emergence (± SE) over 17 days from kelp fly larvae. The data have been combined for two infection concentrations (1000 IJs and 5000 IJs) as there was no difference in number of IJs emerging due to concentration. n is the total number of cadavers with IJ emergence for each isolate. Isolates which do not share the same letter were significantly different (nested GLM, $F_{4,70} = 5.86$, $P < 0.001$)

Isolate	Mean number of IJs per isolate	n
PUR1	6273 (972) ab	16
PUR2	9960 (1789) a	12
PUR3	10326 (1760) a	10
YEL1	10664 (701) a	13
YEL2	4569 (812) b	16
YEL3	6817 (1053) ab	10

(ii) Counts of pooled emergence from all remaining cadavers

There was no difference in mean emergence per cadaver when the individual counts or the pooled counts were compared. All the emergence data for both concentrations was combined (Appendix Table A 33) and the mean emergence per cadaver was analysed using a GLM with isolate nested within colour and concentration included as a factor. There was no effect due to isolate, colour phenotype or concentration on the mean IJ emergence per cadaver (Table 4.14).

Table 4.14: Mean (± SE) emergence per cadaver over 17 days from kelp fly larvae infected with 1000 IJs per 10 larvae or 5000 IJs per 10 larvae. Nematodes used were three PUR and three YEL isolates from North Bull Island. Data shown is from pooled harvests for each isolate. There were three blocks in the 1000 IJ treatment and four blocks in the 5000 IJ treatment.

	Concentration	
Colour phenotype	1000	5000
PUR	6496 (821)	8075 (605)
YEL	5832 (1673)	7912 (706)

4.4 **Discussion**

At most, there were minor differences between *P.t. temperata* and *P.t. cinerea*. The ability of both PUR and YEL strains to utilise four species from three insect orders indicates that under laboratory conditions at least, nematode-bacterium complexes, *H. downesi-P.t. temperata* (YEL) and *H. downesi-P.t. cinerea* (PUR) have a broad host range as do many other EPN species (Peters, 1996). Mortality of the test insects ranged from 81% to 95% for *G. mellonella* larvae at an application rate of ten EPN per insect over an eight day period, 88% to 94% for *H. abietis* larvae at an application rate of fifty EPN per insect over a fourteen day period, 33% to 58% for *T. molitor* at an application rate of ten EPN per insect over an eight day period, and

70% to 100% at an application rate of 100 IJs per insect and 50% to 100% at an application rate of 500 IJs per insect for *Coelopa* spp. larvae over a six day period. The difference in the mortality rate of *G. mellonella* and *T. molitor,* a smaller host, at the same application rate and over the same time period is not surprising as *G. mellonella* are known to be very susceptible to EPN infection. On the other hand, coleopteran species are more difficult to kill and *H. abietis* larvae, which are similar in size to *G. mellonella* larvae, required a five-fold higher application rate and a longer time period to achieve the same mortality rate as seen in *G. mellonella.*

This is the first report of EPN being able to use *Coelopa* spp. as hosts. It is possible that the nematodes and *Coelopa* larvae on North Bull Island might encounter one another, particularly the *H. downesi-P.t. cinerea* (PUR) strains as these predominate at the front of the dunes system and *C. frigida* has been reported from the zone between the strandline and the embryonic dunes on North Bull Island (Healy, 1975). While there was no difference in mortality between *P.t. cinerea* and *P.t. temperata* subspecies, there were differences in the progression of the infection. The nematodes failed to develop in many cases in the *P.t. temperata* infections (Table 4.12), and where the IJs did develop there were with more pre-IJs present in cadavers infected with *P.t. temperata* (Table 4.12). This suggests that *P.t. temperata* is less able to utilise *Coelopa* cadavers, thus triggering a switch to production of infective juveniles earlier than is the case for the *P.t. cinerea* strain. However, there were no differences in overall IJ yields between the two subspecies. The characteristics of the bacteria were investigated in Chapter 2 and Chapter 3. There were differences in the growth of the *pur* phenotype but not the *yel* phenotype in nutrient broth and MacConkey broth, suggesting that the two phenotypes had different growth requirements (Section 2.3.3c). There were also some differences in the biochemical profiles of the

two phenotypes, suggesting differences in their metabolism. The differences in the time to switching development to infective juvenile production within the host cadaver may be due to differences in the ability of the two bacterial phenotypes to utilise the nutrients in this particular host. The lower mortality rate seen with the higher application rate may be due to competition within the host between the developing nematodes or between the bacteria for scarce resources.

In tests with the wild type (Pp, Yy) and hybrid (Py, Yp) combinations of nematode-bacterium against *G. mellonella* there was no significant difference between the combinations in the percentage of insects dead at any time point. However, when the data were compared by bacteria only the *pur1* strain of bacteria appeared to kill slightly quicker than the *yel3* strain, with higher percentage mortality in the *pur* treatments, irrespective of combination (Pp or Yp), although the difference was significant at the 10% level only and only at 56hrs post infection. These results confirm the effect seen in Chapter 3 (Section 3.3.3). In two tests against *G. mellonella* larvae with all six isolates, (three PUR and three YEL), this effect was also observed, with a higher mortality due to PUR nematodes at 48 hrs post infection, though again the effect was significant at the 10% level only. Similar results were obtained in further experiments with all six isolates (M Asaiyah, unpublished) and support the suggestion that the *pur* bacteria, *P.t. cinerea* kills *G. mellonella* larvae quicker than the *yel* bacteria, *P.t. temperata.* There was also some evidence that the *pur1* strain grew more quickly than the *yel3* strain in *G. mellonella* larvae (Section 3.3.1.1). It may be the case that early in the infection the growth rate of the *pur* bacteria is higher if this phenotype is better adapted to *G. mellonella* due to differences in the ability of the two phenotypes to utilise this particular host. Temperature is also a factor in speed of kill. In these experiments the insects were

incubated at 20°C following infection whereas the bacterial growth rate experiments were carried out at 28°C. The temperature profile for the growth of the two bacteria may be a factor in the quicker kill in the case of infection by the PUR nematode-bacterium combination.

In tests with the wild type and hybrid strains against *H. abietis* all strains were able to kill the larval stage at doses as low as 10 IJs per insect and in one experiment the *pur* bacteria was more virulent when a range of doses were compared (Section 4.3.2). However, this difference in speed of kill was not seen in tests with all six isolates against *H. abietis*, *T. molitor* or *Coelopa* spp. as there were no significant differences in percentage mortality at any stage in the infection between the colour phenotypes. The K122 strain of *H. downesi* has been shown to control *H. abietis* in forest situations (Dillon *et al.*, 2006; Williams *et al.*, 2013). This strain carries the *P.t. temperata* bacteria which is the subspecies carried by the *H. downesi* YEL strains in this study.

There is no evidence of differential adaptation of *P.t. temperata* and *P.t. cinerea* to different hosts. However, only four hosts were tested and none of these are known to be natural hosts of *H. downesi* in the sand dune system on North Bull Island, although *C. frigida* does occur in rotting clumps of seaweed deposited along the strandline.

Chapter 5
Effect of host desiccation on IJ survival and emergence

5.1 **Introduction**

It is clear from the results presented in Chapter 2 that both *P. temperata* subsp. *cinerea* (*pur*) and *P. temperata* subsp. *temperata* (*yel*) have persisted in the *H. downesi* nematodes in the sand dunes on North Bull Island for at least 10 years. There is some evidence of niche separation between the two nematode/bacteria combinations, with the *pur* phenotype predominating in areas of low soil moisture. In 2002, 2005 and 2008 nematodes carrying the *pur* subspecies were more prevalent in the front portion of the dunes, Rolston (2005; 2006), and Section 2.3.1.2. One possible reason for this spatial distribution is that differences in desiccation tolerance between the two bacterial subspecies are driving niche separation. Soil moisture content is likely to be more variable in the front than in the rear of the dunes, since sandy soil loses moisture quickly in dry or hot conditions due to its unstructured nature and low humic content (Rodwell *et al.*, 2000; Verhoeven, 2002) (see also Section 2.1).

A number of mechanisms may be responsible for this spatial separation. Nematode population survival in the soil depends primarily on the availability of suitable hosts. Although nothing is known about the natural host range of *H. downesi* on North Bull Island there is no evidence that the three PUR or three YEL phenotypes differ in their ability to kill or reproduce in different hosts (Sections 4.3.3 – 4.3.6). While the IJ can persist for a considerable time in the soil, it is subject to a range of biotic and

abiotic conditions, some of which can make the environment unfavourable and thus limit its survival. Among the challenging abiotic conditions are periods of low water availability, fluctuations in soil temperature, UV radiation, as well as variations in pH and salinity (reviewed in Section 2.1).

It is reasonable to assume that EPN, like other organisms isolated from a particular environment have, over the course of evolutionary time, adapted to survive the range of environmental conditions that pertain in that particular habitat (Hominick, 2002). For example, EPN isolated from regions and altitudes where sub-zero soil temperatures occur are able to survive freezing, either through freeze tolerance (Brown & Gaugler, 1996) or freeze avoidance (Wharton & Surrey, 1994), although survival rate is low for IJs directly exposed to sub-zero temperatures. Similarly, EPN species isolated from warmer regions or which have an ambusher foraging strategy can survive temperatures above 30^{o}C, although there is evidence that heat tolerance is not simply a question of adaptation to a geographic locality (reviewed in Grewal *et al*. (2006). A correlation has been observed between the heat or cold tolerance of the nematode and its symbiont, for example *P. luminescens akhurstii,* the symbiont of *H. indica* and *X. poinarii,* the symbiont of *S. glaseri and S. cubanum,* nematodes that are found in tropical and sub-tropical regions, have upper growth limits at 38-39^{o}C and 40^{o}C respectively, while *P. luminescens,* the symbiont of *H. megidis,* and *X. bovienii,* the symbiont of *S. feltiae* respectively, nematodes that are found in temperate regions, have upper growth limits at 34^{o}C and 32^{o}C respectively (Boemare, 2002).

A number of studies have looked at the role of the host cadaver in protecting the EPN from unfavourable environmental conditions over a prolonged time period. In a study using *S. carpocapsae* strain A10 Serwe-Rodriguez *et al.* (2004) found that IJs

emerged from *G. mellonella* that had been desiccated in air at 29% relative humidity (RH) for up to 56 days post-infection. The authors speculated that the A10 strain might be adapted to survive within a desiccated host as it was isolated from farmland in Wisconsin, USA, an area which can experience prolonged dry periods during the summer months, interspersed with heavy rainfall. Koppenhöfer *et al.* (1997) found that EPN (three *Steinernema* spp. and one *Heterorhabditis* sp.) could survive in *G. mellonella* cadavers stored in soil with 2% moisture for up to 91 days, with differences in survival between the *Steinernema* spp., possibly an adaptation due to their foraging strategy or to the habitat where they were isolated. The authors suggested two possible mechanisms for the survival of IJs within the desiccated host cadaver; first, that the drying of the host cuticle acts as a buffer against further moisture loss within the cadaver, and second, that the IJs become trapped within the hardened cuticle and are unable to emerge until the cadaver is rehydrated. This is similar to the views of Brown and Gaugler (1997), who found that while IJs (*S. carpocapsae)* could survive in *G. mellonella* cadavers for more than 40 days at 15^{o}C and 75% RH, the nematodes could only emerge following rehydration of the cadavers. Spence *et al.,* (2011) found that EPN (2 *Steinernema* spp. and 1 *Heterorhabditis* sp.) could survive within *G. mellonella* cadavers desiccated at 0% RH which had lost up to 70% of their body weight (desiccated for up to 15 days).

It is clear from the results presented in Chapter 2 (Section 2.3.1.2) that both *P. temperata* subsp. *cinerea* (*pur*) and *P. temperata* subsp. *temperata* (*yel*) (Chapter 2, Section 2.3.4) have persisted in the *H. downesi* nematodes in the sand dunes on North Bull Island for at least 10 years. There is some evidence of niche separation between the two nematode/bacteria combinations as in 2002, 2005 and 2008 nematodes carrying the *pur* subspecies were more prevalent in the front portion of

the dunes Rolston (2005; 2006) and Section 2.3.1.2, where soil moisture content is likely to be more variable than in the rear of the dunes, since sandy soil loses moisture quickly in dry or hot conditions due to its unstructured nature and low humic content (Rodwell *et al.*, 2000; Verhoeven, 2002) (see also Section 2.1).

In this chapter a hypothesis for the apparent spatial segregation of the two nematode/bacteria colour phenotypes from North Bull Island will be investigated through a series of experiments designed to test ability of the IJs carrying each bacterial subspecies to survive in desiccated hosts (*G. mellonella).* Using the wild and hybrid nematode/bacteria combinations produced in Chapter 3 (see Table 3.2) to focus on the role of the bacterial partner in IJ survival in hosts stored under desiccating conditions, I will test the hypothesis that the *pur* bacterial phenotype, *P. temperata* subsp. *cinerea*, protects nematodes in cadavers against desiccation better than the *yel* bacterial phenotype, *P. temperata* subsp. *temperata*.

The objectives of this chapter are:

i) To determine the ability of the wild type and hybrid nematode/bacteria combinations produced in Chapter 3 to survive and emerge from desiccated hosts.
ii) To look for differences between the *pur* and *yel* bacterial subspecies in terms of infective juvenile survival and number of IJs emerging.

5.2 **Materials and Methods**

All desiccation experiments were carried out using *G. mellonella* last instar larvae weighing between 201 and 366 mg as the host. Experiment 1 was set up to determine the response in cadavers stored in sand at a range of different moisture levels. Experiment 2 was set up to determine the response in cadavers stored at 0% relative

humidity (RH). Following on from these two initial experiments, experiments 3 and 4 examined in more detail nematode survival in cadavers stored for various periods in dry sand or at 0% RH. Table 5. lists details of percentage moisture (desiccation in sand) and duration of desiccation treatments for experiments 1 – 4. The experimental methods are described in more detail in Sections 5.2.1 and 5.2.2 below.

Table 5.1: Experimental set up to investigate the effect of host desiccation on IJ survival and emergence (E) from *G. mellonella.* Cadaver weight loss (W) was also monitored.

Experiment	Nematode/bacteria combination	n		Desiccation method					
				Sand			Glass desiccator		
				(moisture content w/w)	Duration (days)		RH[1]	Duration (days)	
		W	E		W	E		W	E
1†	Pp Py	3	8	0% 1% 2% 3% 8%	6 9 12 15 18	 14 53 61			
2‡	Pp Py Yy Yp	5-8	8				0%	3 6 9 28	3 6 9 28
3*	Pp Py Yy Yp		5	0%	28	14 28 42 56	0%	28	21 28 42 56
4+	Pp Py Yy Yp		25	0%	28	28 60	0%	28	28

1 All glass desiccators were at 0% relative humidity (RH).

† Experiment 1
Three cadavers per nematode/bacteria combination and sand treatment were weighed (W) at each of five time points (6, 9, 12, 15, and 18 days).
Eight cadavers per nematode/bacteria combination and sand treatment were assessed for nematode survival by recording IJ emergence (E) after 14 days. A further eight cadavers in the 0% sand treatment were assessed after 53 or 61 days.

‡ Experiment 2
(W) n = 5 (day 3), n = 6 (day 6, day 9), n = 8 (day 28) for cadaver weight. (E) On day 9 five cadavers per nematode/bacteria combination were returned to the experiment and reweighed before rehydrating and recording IJ emergence at day 28, along with a further three cadavers per nematode/bacteria combination.

* Experiment 3
(E) Five cadavers per nematode/bacteria combination were weighed (W) at each time point in each desiccation treatment before rehydrating and recording IJ emergence. In the case of the 0 % RH treatments the cadavers were weighed at day 7, returned to the experiment and reweighed before rehydrating and recording IJ emergence at the subsequent time points.

+ Experiment 4
25 cadavers per nematode/bacteria combination were weighed at each time point in each desiccation treatment before rehydrating and recording IJ emergence.

5.2.1 Desiccation in sand

An initial experiment was set up to examine the ability of the wild type and hybrid nematode/bacteria combinations produced in Chapter 3 to survive and emerge from desiccated cadavers. Host cadavers were stored for various time periods in sand at a range of moisture contents to determine the experimental conditions at which differences between the wild type and hybrid nematode/bacteria combinations might be apparent. Sand (Play-pit sand, B & Q Ireland Limited) was filter-washed to give a particle size in the range 150 – 850 μm, allowed to air dry then heat sterilised over night at 120 ^{o}C (0% moisture). The moisture content of the sand was adjusted to 1%, 2%, 3% or 8% w/w by adding sterile water, mixing thoroughly and equilibrating for three days. *Galleria mellonella* were infected in multiwell plates as described in Section 3.2.2.2, with the following variations: nematode suspensions were adjusted to 1000 IJs/ml, 40 IJs were added to each well and the weight of each insect was recorded before adding it to the multiwell plate. Three days after initial exposure all insects exhibiting evidence of nematode infection (Section 2.2.1.3) were randomly assigned to sand pots as follows: 60 cm^3 polypropylene pots (4 cm x 4.4 cm, H x D) were filled to a depth of 2 cm with sterile sand and a single cadaver was placed in each pot (Figure 5.A, B). The pot was then filled with sterile sand (Figure 5.C) and a lid placed on top with the initial weight of the insect recorded on the lid. The pots were double bagged in zip lock polythene sandwich bags, placed in polystyrene boxes and stored at 20 ^{o}C.

Weight loss during desiccation was assessed at each of five time points (6, 9, 12, 15 or 18 days) by removing the cadaver from the sand and weighing it. Three cadavers from each moisture treatment (0%, 1%, 2%, 3% or 8% w/w water) were assessed in this manner. Nematode survival within the cadavers removed for weighing was then

assessed by dissecting the insects as described in Section 3.2.1.5 and noting the abundance of nematodes within the cadaver and the life stages present. Infective juvenile emergence was assessed at each of three time points (14, 53 or 61 days) by removing the cadavers from the sand and placing them individually on a 7 cm filter paper on a 3.5 cm Petri dish lid in a 9 cm Petri dish (White trap). The filter paper was moistened with 1 ml of tap water and the traps were stored at 20 ^{o}C on trays covered with tinfoil for 24 hrs to rehydrate the cadavers. Water was then added to the traps and they were monitored daily for IJ emergence for up to four weeks. White traps where IJ emergence occurred were harvested and counted (Sections 2.2.1.4 & 2.2.1.6). At each assessment point sand pots from which the cadaver had been removed were returned to the experimental arena for the remainder of the experiment to minimise disruption to the storage conditions.

Experiments 1, 3 and 4 included sand treatments (Table 5.). All timings are from the start of the desiccation period with day 0 being the day the cadavers were placed in the sand pots. Experiment 1 consisted of 32 blocks of ten sand pots with one pot per treatment, at each of two infection types (Pp or Py) for each of five moisture treatments (0, 1, 2, 3 & 8 %). The blocks were randomly assigned to be assessed as follows: three blocks at each of five time points (6, 9, 12, 15 or 18 days) to monitor weight loss during desiccation; eight blocks to assess nematode survival in desiccated cadavers; nine blocks to monitor IJ emergence into sand at each of 3 intervals (14, 53 & 60 days).

In experiment 3 cadavers were desiccated in dry sand only (0 % moisture). There were five blocks of four sand pots, with one pot for each of four infection types (Pp, Py, Yy or Yp), for each time point (14, 28, 42 or 56 days). Nematode emergence was

assessed for all time points. White traps were harvested and counted nine days following first emergence in any trap and a further seven days later in the 28 day treatment only. All traps were harvested and counted eight days following first emergence in any trap and a further seven days later as previously described.

In experiment 4 cadavers were desiccated in dry sand only (0% moisture). There were five replicates with 5 pots per replicate, for each treatment, in a randomised block design. Treatments were four infection types, (Pp, Py, Yy or Yp) for each of two time points (28 or 61 days). Nematode emergence was assessed as previously described. Traps were harvested ten days following first emergence in any trap and a further seven days later. Harvests for all five cadavers per replicate were pooled and a single count carried out.

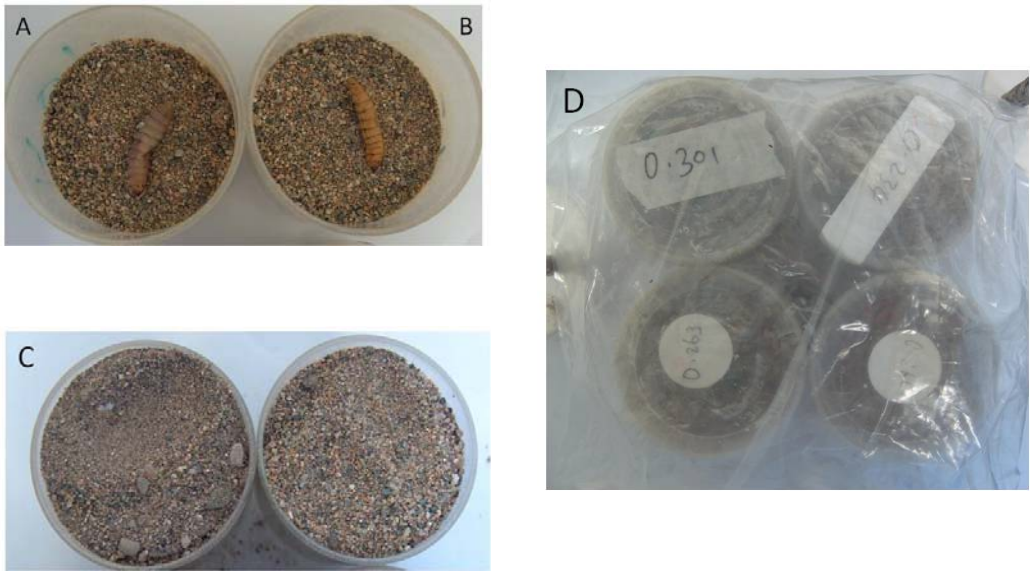

Figure 5.1: *G. mellonella* cadavers showing the characteristic colouration of infection with (A) *pur* or (B) *yel* bacteria were placed in sand pots, (C) covered with sand and (D) double bagged and stored at 20°C for up to 61 days.

5.2.2 Desiccation at 0% relative humidity

Desiccant cartridges containing silica gel beads (197 mm, DES 850-050G, Fisher Scientific) were dried at 50 ^{o}C overnight. The cartridges were placed in glass desiccators (200mm, FB35005, Fisher Scientific) which were allowed to equilibrate at 20 ^{o}C for three days before adding *G. mellonella* cadavers. Relative humidity (RH) was monitored using Humonitor® humidity indicator cards (Z163457, Sigma-Aldrich). *Galleria mellonella* were infected as described in Section 5.2.1. Three days after initial exposure all insects exhibiting evidence of nematode infection (Section 2.2.1.3) were randomly assigned to 4-well trays, made by cutting multiwell plates into segments (Figure 5.2A). Each well had three 3 mm diam. holes drilled in the base to allow for air movement. Cadavers were desiccated for up to 28 days at 0% RH in glass desiccators (Figure 5.2B). Weight loss and nematode emergence were assessed at intervals as described in Section 5.2.1. Experiments 2, 3 and 4 included desiccator treatments (Table 5.).

Experiment 2 consisted of five desiccators with four or five blocks, each block containing one cadaver of each infection type, (Pp, Py, Yy or Yp). At least one block per desiccator was removed at each of three time points (3, 6, or 9 days) and weight loss was assessed. The cadavers removed at day 3 and day 6 were stored on moist tissue paper and placed on White traps 14 days after the initial infection to assess nematode emergence. The cadavers removed on day 9 were returned to the desiccators and reweighed on day 28. All remaining cadavers were also removed on day 28 and nematode emergence was assessed. All traps in the 28 day treatment were harvested and counted eight days following first emergence in any trap and a further eight days later as previously described.

Experiment 3 consisted of five desiccators with five blocks, each containing one cadaver of each infection type, (Pp, Py, Yy or Yp), one block was removed at each of five time points (14, 21, 28, 42 or 56 days). All cadavers were weighed at day 7, returned to the desiccator and one block per desiccator was reweighed at the time points listed. Nematode emergence was assessed. The 28 day treatments were harvested and counted eight days following first emergence in any trap and a further seven days later as previously described.

Experiment 4 consisted of five desiccators with five replicate blocks, each containing one cadaver of each infection type, (Pp, Py, Yy or Yp). All cadavers were removed after 28 days and weight loss and nematode survival was assessed. White traps were harvested and counted eight days following first emergence in any trap and a further seven days later as previously described.

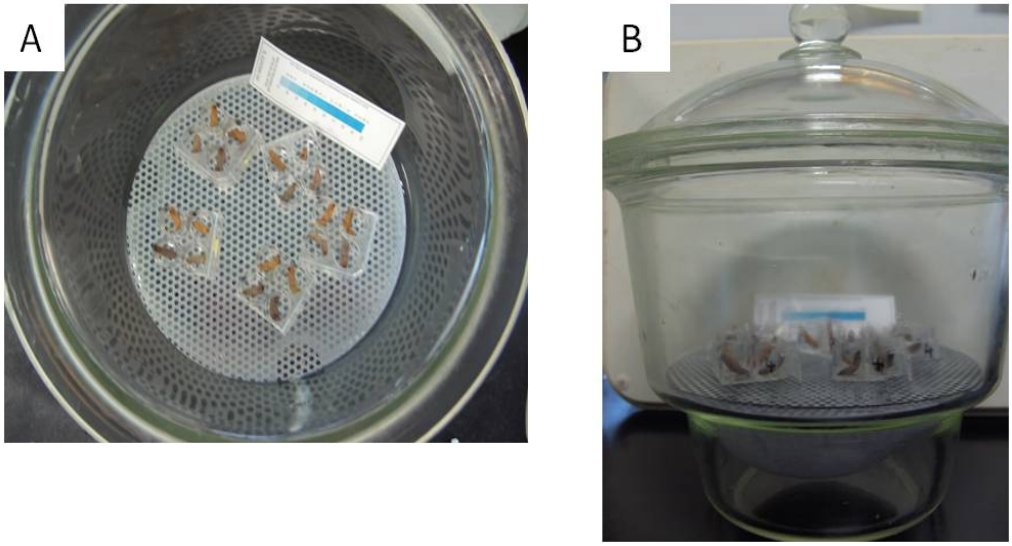

Figure 5.2: (A) *G. mellonella* cadavers infected with one of four nematode/bacteria combinations were placed in multiwell plate segments and (B) desiccated at 0% RH and 20^{o}C for 28 days.

5.2.3 Statistical analysis

Routine statistical analysis, including tests for normality, was carried out using Minitab statistical software, version 16.1.1 (Minitab Inc., 2010). Normality of data was tested using the Anderson-Darling method. Where the data were not normally distributed comparisons between two groups were carried out using a Mann-Whitney U test. Analysis of variance was carried out using a one-way ANOVA or when groups had an uneven number of observations the general linear model (GLM) with an unbalanced nested design was used to compare isolate and colour phenotype. Analysis of variance was followed by pair-wise multiple comparisons using Tukey's test where appropriate. In experiments where nematode type and bacterial type were factors, results were analysed using a two-way ANOVA. Where no significant interactions occurred treatment means for each factor were compared using a one-way ANOVA and post-hoc Tukey's test for three or more treatments. When data could not be normalised treatment medians were compared using a Kruskal-Wallis test, followed if necessary by multiple pairwise comparisons using a Mann-Whitney U-test test with Bonferroni 95% confidence intervals, obtained by calculating $(1 - \alpha / g)$ where α is the level of significance and g is the number of pairwise comparisons carried out. Proportional data were analysed using a Chi-square test. In all tests a P value of < 0.05 was taken as significant unless indicated otherwise.

5.3 **Results**

5.3.1 l Nematode survival in cadavers stored at a range of moisture levels (experiment 1)

In experiment 1 three cadavers per infection type (Pp & Py) from each moisture treatment (sand at 0%, 1%, 2%, 3% or 8% moisture content (w/w)) were dissected at

each of five time points post desiccation (6, 9, 12, 15 & 18 days). Numbers of nematodes in the cadavers were assessed semi-quantitatively (Table 5.2) and there was no difference in the estimated number of nematodes per cadaver between infection types due to moisture level at any time point (Kruskal-Wallis test, $P > 0.05$ at each time point). Both nematode types survived in cadavers in all moisture treatments and at all time points (Appendix Table A 34 - Table A 38). There was no difference between infection types in the number of cadavers with live nematodes. The estimated number of nematodes per cadaver ranged from less than 500 to more than 10000 by day 6 of the experiment. By day 18 of the experiment pre-infective juvenile nematodes were present in all cadavers. It was apparent during this initial experiment (experiment 1) that IJs were emerging into the sand in all treatments except 0% moisture and therefore in experiment 1 only cadavers from the dry sand treatment (0% moisture) were placed on White traps to assess IJ emergence. As the objectives of this chapter were to investigate differences between the *pur* and *yel* bacterial subspecies in terms of nematode survival and emergence from desiccated hosts subsequent experiments involving hosts desiccated in sand used only the dry sand treatment and the duration of the desiccation period was varied (experiment 3 and experiment 4).

Table 5.2: Estimated† median number of nematodes per *G. mellonella* cadaver dissected at each of five time points‡. Insects were infected with Pp or Py nematode/bacteria combinations and stored in sand at a range of different moisture levels prior to dissection. n = 3 for all treatments.

Nematode/bacteria combination	Moisture content (w/w)	Estimated median number of nematodes per cadaver				
		Day 6	Day 9	Day 12	Day 15	Day 18
Pp	0%	500	10000	10000	10000	10000
	1%	1000	10000	10000	10000	10000
	2%	10000	10000	10000	10000	10000
	3%	1000	10000	10000	10000	10000
	8%	10000	10000	10000	10000	10000
Py	0%	500	1000	10000	1000	10000
	1%	500	10000	10000	10000	10000
	2%	1000	10000	10000	10000	10000
	3%	1000	10000	10000	10000	10000
	8%	500	10000	10000	10000	1000

† The number of nematodes per cadaver was assessed semi-quantitatively as follows: 500 = up to 500 nematodes present; 1000 = 500 - 1000 nematodes present; 10000 = 1000 - 10000 nematodes present.

‡ By day 18 IJs were emerging into the sand in all moisture treatments except 0% (dry sand).

5.3.2 Emergence of infective juveniles from cadavers stored in dry sand (experiments 1, 3 & 4)

There was IJ emergence from 100% of the cadavers stored for 14 days in dry sand in both experiment 1 (Pp & Py only) and experiment 3 (Pp, Py, Yy & Yp) (Table 5.3). IJ emergence from cadavers stored in dry sand for 28 days ranged from 60 to 100% and 72 to 80% respectively in experiment 3 and experiment 4 (Pp, Py, Yy & Yp) (Table 5.3). There was IJ emergence from cadavers store for up to 61 days in dry sand in experiment 1 (Table 5.3). There was no emergence from any cadavers in the 42 day treatment except those infected with the Pp nematode/bacteria combination in experiment 3 and no emergence from any treatment after 56 days in experiment 3

(Table 5.3), and in experiment 4 there was no emergence from any cadaver after 60 days in dry sand (Table 5.3).

There was no clear trend in the time to first emergence following rehydration of the cadavers (Table 5.3) and in most cases IJs emerged 2-3 days after rehydration from cadavers stored for 14 days (experiments 1 & 3), although for four cadavers in the Pp infections IJs emerged after 4-5 days. For cadavers stored for 28 days (experiments 3 & 4) IJs emerged after 1-3 days but here, for two cadavers in the Yy infection and three cadavers in the Py infection, first emergence occurred after 7-8 days and 4, 6, and 9 days, respectively. In experiment 1 the bacterial type had a significant effect on the time to first emergence of IJs following rehydration from cadavers stored for 14 days in dry sand (Mann-Whitney U test, W = 88.0, P = 0.0269) while in experiment 3 there was an effect of bacteria type at the 10% level and an effect due to nematode type at the 5% level in this treatment (Table 5.4). In both experiments, IJs took longer to emerge from *pur* than from *yel* cadavers.

The mean numbers of IJs emerging from cadavers in experiment 1 are shown in Table 5.5. There was a significant difference due to moisture content (two-way ANOVA, $F_{1,28} = 4.85$, P = 0.036) but not due to bacterial phenotype (two-way ANOVA, P > 0.05) and no interaction between the two factors (two-way ANOVA, P > 0.05) following storage for 14 days in dry sand or sand at 8% moisture. Fewer IJs emerged from the dry treatment (approximately 13,000 overall average of *pur* and *yel* in the dry sand treatment compared to approximately 18,000 overall average of *pur* and *yel* in the 8% moisture sand treatment). Following storage of the cadavers in dry sand for up to 61 days the number of IJs emerging in the Pp nematode/bacteria combination was between 737 and 189455, while the number emerging in the Py nematode/bacteria combination was between 1 and 1507. The cadavers with no

emergence were excluded and data for emergence at days 53 and 61 were combined for analysis. There was a significant difference in the number of IJs emerging due to bacterial phenotype between the two nematode/bacterial combinations (one-way ANOVA, $F_{1,10} = 10.12$, $P = 0.010$) (Table 5.5), with the number of IJs emerging from Pp cadavers two orders of magnitude higher than from Py cadavers.

As day 28 was common to both experiment 3 and experiment 4 it is treated separately below.

Table 5.3: Percentage (±SE)† of White Traps with emergence and time to first emergence of IJs from *G. mellonella* cadavers following desiccation in dry sand for 14, 28, 42, 53 or 61 days.

Experiment	Nematode/ bacteria combination	Percentage of White traps with emergence					Median time to first emergence (days post rehydration)				
		days in sand at 0% moisture					days in sand at 0% moisture				
		14	28	42	53♦	61†	14	28	42*	53	61
1	Pp	100			100	100	3.5			4.5	1
	Py	100			50	50	2.0			1	1
3	Pp	100	60	20	0		3.0	3.0	2		
	Yp	100	100	0	0		2.0	1.0			
	Yy	100	80	0	0		2.0	2.0			
	Py	100	80	0	0		3.0	1.0			
4†	Pp		80 (12.6)			0		2.0			
	Yp		72 (12.0)			0		2.0			
	Yy		76 (16.0)			0		2.0			
	Py		76 (16.0)			0		2.0			

n = 8 for each nematode/bacteria combination at 14 days, n = 2 for each nematode/bacteria combination at 53 days, n = 6 for each nematode/bacteria combination at 61 days in experiment 1.
n = 5 for each nematode/bacteria combination at each time point in experiment 3
n = 25 for each nematode/bacteria combination in experiment 4.
† In experiment 4 percentages of White traps with emergence is a mean of 25 for each nematode/bacteria combination. The second time point was 60 days.
* In experiment 3 there was emergence from 1 cadaver only.
♦ In experiment 3 cadavers were removed from sand pots after 56 days.

Table 5.4: Results of Mann-Whitney U test on median time to first emergence of IJs from cadavers desiccated for 14 days in sand at 0% moisture. Data is from experiments 1 and 3 with n = 8 per bacterial type in experiment 1 and n = 10 per bacterial type or nematode type in experiment 3

		Median time to first IJ emergence (days)			
Experiment		Bacterial type		Nematode type	
1		*pur*	3.5		
		yel	2.0		
	P value	P = 0.0269			
	test statistic	W = 88.0			
3		*pur*	4.5	PUR	3.0
		yel	3.0	YEL	2.0
	P value	P = 0.0625		P = 0.0318	
	test statistic	W = 128.5		W = 130	

Table 5.5: Mean (± SE) numbers of IJs emerging from *G. mellonella* cadavers following desiccation for 14 days in dry (0%) or moist (8%) sand or 53-61 days in dry (0%) sand. n = 8 for each nematode/bacteria combination in all treatments. Data shown is for experiment 1. Results of Two-way ANOVA (14 days 0% and 8%) and One-way ANOVA (53-61 days) analysis of number of IJs emerging are also given.

Nematode/bacteria combination	Sand treatment			
	0%	8%	0%	
	Duration			
	14 days	14 days	53-61 days	
Pp	143372 (38627)	197099 (24664)	98841 (21328)	
Py	115878 (11547)	139767 (12500)	509 (346)	
	P value	P = 0.036	P value	P = 0.010
	Test statistic	$F_{1,28} = 4.85$	Test statistic	$F_{1,10} = 10.12$

5.3.3 Emergence of infective juveniles from cadavers stored for 28 days in dry sand (experiments 3 & 4)

Infective juvenile nematodes emerged from cadavers infected with each nematode/bacteria combination and stored for 28 days in dry sand in both experiments (Table 5.6). There was no effect of nematode type or bacteria on the percentage of cadavers with IJ emergence in either experiment (Chi-Square test, $P>0.05$ in all cases).

In experiment 3 the number of IJs emerging per cadaver over a 16 day period from first emergence was between 7000 and 126000 approximately, while in experiment 4 the mean number of IJs emerging over a 17 day period from first emergence was between 800 and 67000 approximately. The data from both experiments were combined and the mean numbers of IJs emerging per cadaver (cadavers with emergence only) were analyzed using a GLM with nematode and bacteria as fixed factors. A nematode by bacteria term was included in the model to test for interaction. The analysis showed that following desiccation of the cadavers for 28 days in dry sand more IJs emerged from insects infected with nematodes carrying the *pur* bacteria than from insects infected with nematodes carrying the *yel* bacteria and this difference was significant at the 10% level ($F_{1,32} = 4.10$, $P = 0.051$) (Figure 5.3). There was no effect of nematode on the numbers of IJs emerging and no evidence of any interaction ($P > 0.05$ in both cases).

Table 5.6: Mean emergence (±SE) per cadaver, percentage of cadavers with emergence, median time to first emergence of IJs following rehydration and mean emergence (±SE) per cadaver (cadavers with emergence only) from *G. mellonella* cadavers following desiccation for 28 days in dry sand in two experiments.

			All cadavers		Cadavers with emergence only			
Experiment	Nematode	Bacteria	Mean emergence per cadaver (±SE)	n	Percentage of cadavers with emergence	Median time to first emergence (days post rehydration)	Mean emergence per cadaver (±SE)	n
3	P	p	42238 (23010)	5	60	3.0	70396 (27816)	3
	Y	p	67321 (21714)	5	100	1.0	67321 (21714)	5
	Y	y	35793 (18940)	5	80	2.0	44742 (21551)	4
	P	y	44291 (14674)	5	80	1.0	55364 12432)	4
4	P	p	37273† (5356)	5†	80	2.0	47340‡ (1569)	5‡
	Y	p	30203† (5058)	5†	72	2.0	44183‡ (7304)	5‡
	Y	y	19724† (4869)	5†	76	2.0	25860‡ (2373)	5‡
	P	y	20424† (7930)	5†	76	2.0	21019‡ (7605)	5‡

Exp 3: 1 insect x 5 blocks, individual counts for each cadaver
Exp 4: 5 replicate insects x 5 blocks, 1 pooled count for each block
† mean emergence per cadaver is mean of 5 blocks (pooled count per block / 5 to give mean per cadaver for each block)
‡ mean emergence per cadaver is mean of 5 blocks (pooled count per block / n cadavers with emergence for each block)

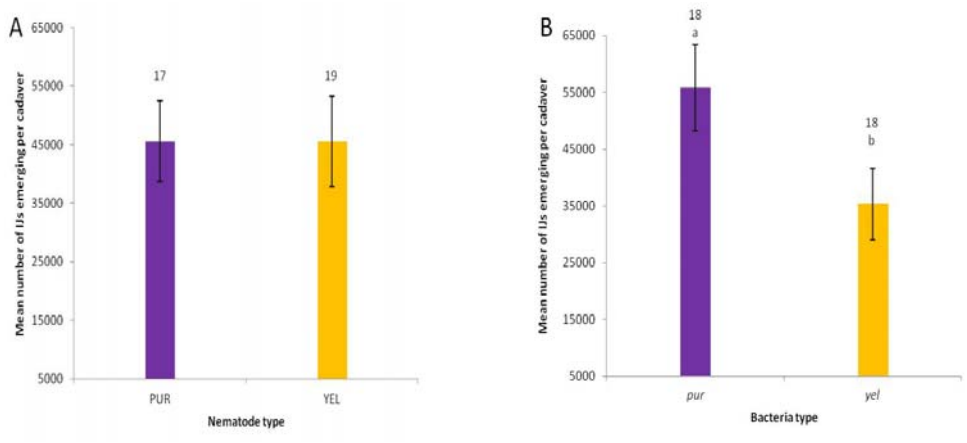

Figure 5.3: Mean (± SE) number of IJs emerging from *G. mellonella* cadavers (A) for each nematode type and (B) for each bacteria type. Insects were infected with 40 IJs of one of four nematode/bacteria combinations (Pp, Py, Y, Yp) and cadavers were desiccated for 28 days in dry sand. Data shown is from experiments 3 and 4 (cadavers with emergence only). n for each type is displayed above the bar. In each chart types which do not share the same letter were significantly different (GLM $F_{1,32} = 4.10$, $P = 0.051$) for bacteria type.

5.3.4 Emergence of infective juveniles from cadavers stored for 28 days at 0% relative humidity (experiment 2, 3 & 4)

In experiment 2 IJs emerged from all eight cadavers for all five nematode/bacteria combination stored for 3 or 6 days at 0% RH. In the 28 day treatment there was no emergence from any cadavers in the Py nematode/bacteria combination and emergence from cadavers in the other nematode/bacteria combinations ranged from 37.5 to 62.5% (Table 5.7). In experiment 3 IJs emerged from some cadavers in all four nematode/bacteria combinations in the 21 day treatment, ranging from 60 to 100% (data not shown) and this fell to between 20 and 40% in the 28 day treatment (Table 5.7). There was no IJ emergence from any cadavers in the 42 or 56 day treatments. After 28 days in experiment 4 there was no IJ emergence from cadavers in the Py nematode/bacteria combination and in one case (Pp) first emergence of IJs occurred 32 days after the cadaver was rehydrated. There was an extremely wide

variation across the three experiments in the 28 day treatment in numbers of IJs emerging from individual cadavers, ranging from 18 IJs in a Yy infection in experiment 2 to 117491 IJs in a Yp infection, also in experiment 2 (Appendix Table A 39).

The data for 28 days from experiments 2 - 4 were combined for analysis. The combined emergence data were not normal since in many cases no infective juveniles emerged from individual cadavers. A Kruskal-Wallis test on combined data showed a highly significant difference in numbers of IJs emerging between the nematode/bacteria combinations (H = 11.82, DF = 3, P = 0.008). The data were pooled by nematode type and by bacteria type and compared using a Mann-Whitney test. This showed that there was a highly significant difference in the number of IJs emerging depending on the bacteria the nematodes carried (Mann-Whitney U-Test, W = 6389.5, P = 0.0017), with ten times more IJs emerging where the infecting nematodes carried the *pur* bacterial phenotype than the *yel* bacterial phenotype (Appendix Table A 40). There was no difference in numbers of IJs emerging when the data were pooled by nematode type (P > 0.05).

The combined data for the cadavers with emergence only were analyzed using a GLM with nematode and bacteria as fixed factors and experiment as a random factor. A nematode by bacteria term was included in the model to test for interaction. The analysis showed that there was an effect of bacteria at 10% level on the number of IJs emerging from the cadavers stored for 28 days at 0% RH with more IJs emerging from insects infected with nematodes carrying the *pur* bacteria than from insects infected with nematodes carrying the *yel* bacteria (Figure 5.4) ($F_{1,22}$ = 3.37, P = 0.080). There was no effect of nematode and no evidence of a nematode-bacteria

interaction (P > 0.05 in both cases), however, experiment was also significant at the 10% level.

When the number of cadavers with emergence were grouped by nematode type or by bacteria type there was a highly significant difference between the *pur* and *yel* phenotypes in the number of cadavers with emergence with more cadavers infected with the *pur* bacteria producing IJs, (21/76 *pur* compared to 7/76 *yel*) (Chi-square Test, $\chi^2 = 8.581$, DF = 1, P = 0.003) but there was no difference between the PUR and YEL nematodes in the number of cadavers with emergence (Chi-square Test, P > 0.05).

Table 5.7: Mean emergence (±SE) per cadaver, percentage of cadavers with emergence, mean time to first emergence of IJs following rehydration and mean emergence (±SE) per cadaver from *G. mellonella* cadavers following desiccation for 28 days at 0% RH in three experiments.

			All cadavers		Cadavers with emergence only			
Experiment	Nematode	Bacteria	Mean emergence per cadaver (±SE)	n	Percentage of cadavers with emergence	Median time to first emergence (days post rehydration)	Mean emergence per cadaver (±SE)	n
2	P	p	37577 (13985)	8	62.5	†	60124 (14447)	5
	Y	p	47824 (18498)	8	50.0	†	95648 (8489)	4
	Y	y	3239 (3065)	8	37.5	†	8637 (8022)	3
	P	y	0	8	0.0			
3	P	p	9604 (8849)	5	40.0	10	24010 (20911)	2
	Y	p	209 (209)	5	20.0	8	1045	1
	Y	y	12567 (8138)	5	40.0	2.5	31417 (8368)	2
	P	y	26 (26)	5	20.0	3	129	1
4	P	p	6560 (3861)	25	16.0	10	41000 (16571)	4
	Y	p	7725 (4012)	25	20.0	4	38627 (13579)	5
	Y	y	747 (747)	25	4.0	4	18667	1
	P	y	0	25	0.0			

† In experiment 2 exact times to first emergence of infective juveniles were not recorded. In the Pp nematode/bacteria combination IJ emergence had occurred in 2/5 cadavers by 3 days post rehydration and in the remaining 3/5 cadavers by 7 days post rehydration. In the Yp nematode/bacteria combination IJ emergence had occurred in 2/4 cadavers by 3 days post rehydration and in the remaining 2/4 cadavers by 7 days post rehydration. In the Yy nematode/bacteria combination IJ emergence had occurred in 1/3 cadavers by 3 days post rehydration and in the remaining 2/3 cadavers by 7 days post rehydration.

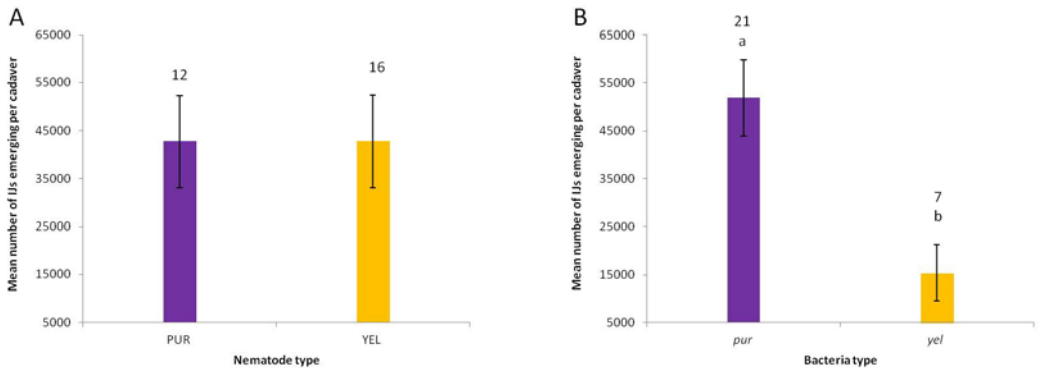

Figure 5.4: Mean (± SE) number of IJs emerging from *G. mellonella* cadavers (A) for each nematode type and (B) for each bacteria type. Insects were infected with 40 IJs of one of four nematode/bacteria combinations (Pp, Py, Yy, Yp) and cadavers were stored for 28 days at 0% RH. Data shown is from experiments 2 – 4. n for each type is displayed above the bar. (B) Bacterial types were significantly different at the 10% level (GLM $F_{1,22} = 3.37$, P =0.080).

5.3.5 Weight loss during desiccation

5.3.5.1 Desiccation in sand

In experiment 1 cadavers were weighed at each of five time points for each moisture treatment (Appendix Table A 41). In the dry sand treatment cadavers lost between 30% and 45% of their initial weight over a period of 18 days. There was no difference in the mean percentage weight loss between insects infected with either the wild type or hybrid nematode/bacteria combinations (Pp and Yp, respectively) at any time point (one-way ANOVA, P > 0.05 in all cases) (Figure 5.5).

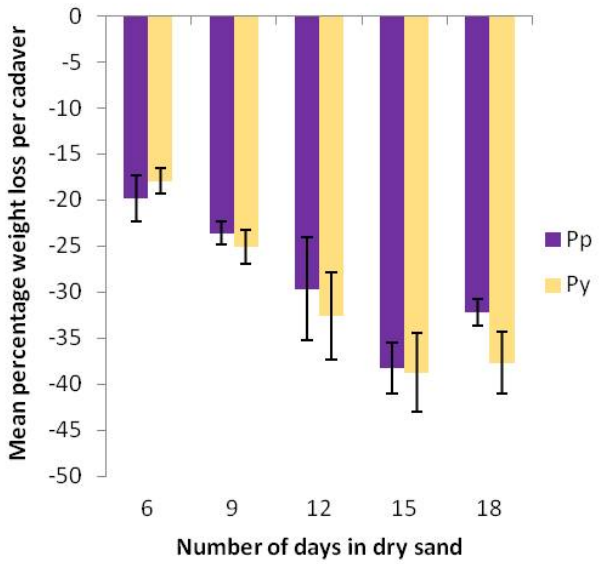

Figure 5.5: Mean (± SE) weight loss from *G. mellonella* cadavers stored in dry sand over an 18 day period. n = 3 at each time point.

In experiment 3 the percentage weight loss per cadaver ranged from approximately 34 to 53% at day 14, 61 to 74% at day 28 and 63 to 74% at day 42 (data not shown). In experiment 4 the percentage weight loss per cadaver ranged from approximately 50 to 75% at day 28. The weight loss data from experiment 3 and experiment 4 for the 28 day dry sand treatment were not normal and could not be normalised. The data from both experiments were pooled by nematode type or by bacteria type and were analysed by Mann-Whitney U test. In each case there was no difference in the percentage weight loss between the colour phenotypes (Table 5.8).

Table 5.8: Median percentage change in weight of *G. mellonella* cadavers stored in dry sand for 28 days. The data were pooled by nematode type and by bacteria type and were combined for two experiments with n = 10 per nematode type or bacteria type in experiment 3 and n = 50 per nematode type or bacteria type in experiment 4.

	Median percentage weight loss (n = 60)
Nematode type	
PUR	-68.97
YEL	-68.62
Bacteria type	
pur	-68.96
yel	-68.62

5.3.5.2 Desiccation at 0% RH

In experiment 2 weight loss was assessed at days 3, 6 and 9 (Figure 5.6). Percentage weight loss per cadaver ranged from approximately 7 – 12% at day 3, 15 – 27% at day 6 and 21 – 38% at day 9. The data at each time point were pooled by nematode type (PUR or YEL) or by bacteria type (*pur* or *yel*) and were tested using a two-way ANOVA of percentage weight loss per cadaver. There was no difference in the mean percentage weight loss between insects for either nematode type or bacteria type and no evidence of an interaction at any time point (two-way ANOVA, $P > 0.05$ in all cases).

The percentage weight loss per cadaver in the 28 day treatment ranged from approximately 60 – 77%, 61 – 78% and 50 – 81% in experiments 2, 3, and 4, respectively (Table 5.9). The data for the 28 day treatment from experiments 2 - 4 were combined for analysis and pooled by nematode type (PUR or YEL) or by bacteria type (*pur* or *yel*) and were tested using a two-way ANOVA of percentage

weight loss per cadaver. The analysis showed there was no effect of nematode or bacteria on the percentage weight loss per cadaver and no interaction effect and no further analysis was carried out on the data.

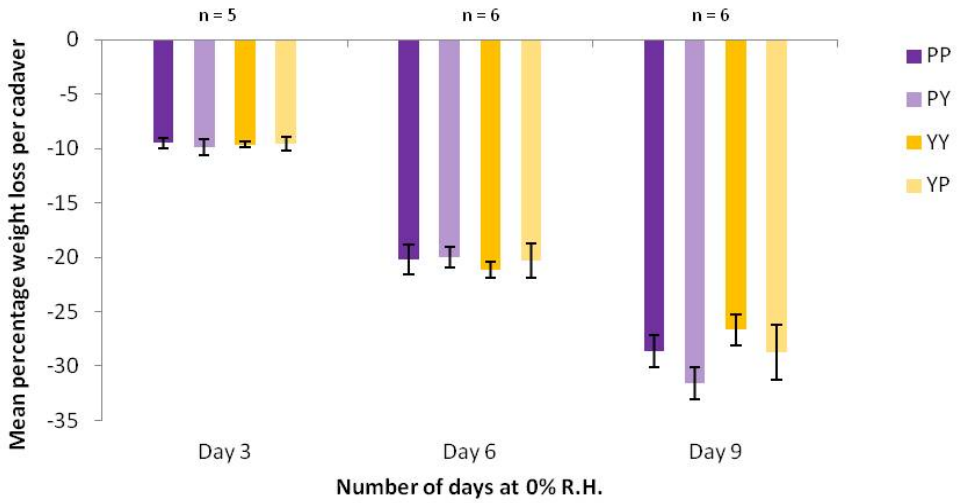

Figure 5.6: Mean (±SE) percentage weight loss from cadavers stored at 0% RH at 3 time points.

Table 5.9: Mean (± SE) percentage weight loss from *G. mellonella* infected with one of four nematode /bacteria combinations and stored for 28 days at 0% RH.

	Nematode/bacteria combination			
Experiment	Pp	Yp	Yy	Py
2	-70.73 (1.968)	-71.53 (1.153)	-73.45 (0.481)	-68.45 (2.368)
3	-71.05 (2.63)	-74.79 (0.68)	-71.56 (0.86)	-75.21 (1.04)
4	-72.41 (0.74)	-71.97 (0.40)	-70.61 (0.44)	-71.04 (0.56)

5.4 **Discussion**

Cadavers with *P. temperata* subsp. *cinerea* (*pur1*) subjected to desiccation produce more IJs than those with *P. temperata* subsp. *temperata* (*yel3*) so the hypothesis that the *pur* bacterial phenotype, *P. temperata* subsp. *cinerea*, protects nematodes in cadavers against desiccation better than the *yel* bacterial phenotype, *P. temperata* subsp. *temperata* is plausible. Koppenhöfer *et al.* (1997), using *G. mellonella* larvae as the host, found that for three *Steinernema* spp. (*S. carpocapsae* All strain, *S. glaseri* NC strain, *S. riobravis* Texas strain and one *Heterorhabditis* sp. (*H. bacteriophora* NC1 strain) there was no emergence from cadavers stored for 14 days in loamy sand at 1% moisture (equivalent to soil water potential -500 mPa) whereas for cadavers stored for 14 days in loamy sand at 2% moisture (equivalent to soil water potential -40 mPa) emergence ranged from 75 to 100%. The cadavers were not rehydrated. In a second experiment with the same species the authors found that IJs of all four species emerged from rehydrated cadavers that had been stored for up to four weeks in loamy sand at 2% moisture and for two to three months in the case of *H. bacteriophora* and *S. riobravis,* respectively and emergence at both the 21 and 35 day time points was 100% for *H. bacteriophora*.

From my results it is clear that a substantial proportion of *H. downesi* nematodes carrying either bacterial subspecies, *P. temperata* subsp. *cinerea* (*pur1)* or *P. temperata* subsp. *temperata* (*yel3*), can survive for at least 28 day in host cadavers stored in dry sand or glass desiccators at 0% RH. In my experiments I found that IJs emerged from all rehydrated cadavers at the 14 day time point and from 60 to 100% of rehydrated cadavers at the 28 day time point in the dry sand treatment. In the 0% RH treatment emergence was more variable, ranging from 0 to over 60% after 28 days. If we compare this result to that obtained by Kung *et al* (1991) cited above it is

clear that the host cadaver provides considerable protection to IJs under severe desiccating conditions. Similarly to the results obtained by Koppenhöfer *et al.* (1997), IJs emerged from rehydrated cadavers that had been stored in dry sand for up to 61 days, although this result was obtained in experiment 1 only. Koppenhöfer *et al.* (1997) noted that the *H. bacteriophora*-infected *G. mellonella* cadavers had a "gummous consistency" and speculated that this might be a factor in helping the IJs to survive in the cadavers under desiccating conditions by helping to retain moisture in the cadavers. In my first experiment the cadavers which were removed from the sand treatments at the five time points for weight loss assessment were dissected to assess nematode survival and there was no difference in the number of cadavers with live nematodes (Appendix Table A 34 – Table A 38). I observed that the *yel3*-infected cadavers had a stickier consistency than the *pur1*-infected ones, however, this gummy consistency of the *yel3*-infected cadavers does not appear to assist the IJ survival as there was a trend for greater emergence from *pur1*-infected cadavers. There was a difference in IJ emergence as early as the 14 day time point with fewer IJs emerging from *yel3*-infected cadavers compared to the *pur1*-infected ones and by days 53-61 emergence from cadavers in the Py treatment (*yel3*-infected cadavers) was approximately 0.5% of the emergence from cadavers in the Pp treatment (*pur1*-infected cadavers). Similarly, in experiments 3 and 4, more IJs emerged from *pur1*-infected cadavers than from *yel3*-infected ones following storage for 28 days in dry sand, although there was no difference in the number of cadavers with IJ emergence in any of these experiments at the same time points. Under severe conditions (0% RH) there was a highly significant difference in the IJ emergence from the cadavers following 28 days storage with emergence from more *pur1*-infected cadavers in experiments 2 and 4, although in experiment 2 there was emergence from equal

numbers of *pur1*-infected and *yel3*-infected cadavers. Here again, however, significantly more IJs emerged from *pur1*-infected cadavers than from *yel3*-infected ones.

In a study of *S. affine* in the Czech Republic Půža & Mráček (2005, 2007) observed a decrease in IJ numbers in the soil during dry periods followed by a rapid increase in IJ numbers in the soil as soil moisture increased following precipitation. The authors suggested IJ persistence in host cadavers in the soil during dry conditions as a possible reason for this observed increase and also speculated that this might be an adaptation for this particular EPN rather than a case of being trapped within the dried cuticle or having no choice as emergence would expose the IJs to low moisture conditions in the soil which they would be unlikely to survive for very long Koppenhöfer *et al.* (1997). Both groups have shown that IJs can persist for considerable lengths of time in cadavers under conditions that would be rapidly lethal to IJs in the soil. Neither the Koppenhöfer *et al.* (1997) study or the work by Půža & Mráček (2005, 2007) have however considered the role of the symbiotic bacteria in the IJ survival within the cadaver.

Across all infection types and both experiments the mean weight loss for cadavers stored in dry sand in experiments 3 and 4 was approximately 67%, while across all infection types and three experiments the mean weight loss for cadavers stored at 0% RH was approximately 72%. This is similar to the weight loss reported for *G. mellonella* infected with *S. carpocapsae* (All strain), *S. riobrave* Cabanillas, Poinar and Raulston, and *H. bacteriophora* HB1 strain and subsequently stored at 0% RH by Spence *et al.* (2011). Here the authors were able to show that IJs could persist in cadavers that had lost up to 70% of their original mass and it was noted that the number of cadavers with IJs emerging, and the number of IJs emerging per cadaver

were both species specific, with a greater proportion of emergence for both *Steinernema* spp. while the lowest IJ production was observed for *S. riobrave*. The Spence *et al.* (2011) study also noted that the rate of weight loss was slowest for cadavers infected with *S. carpocapsae* and suggested that differences in the rate of nematode development or in the activity of the bacterial symbionts on the host cuticle could be involved. From the results of my experiments with *H. downesi* nematodes it appears that the two symbiont subspecies differentially influence the survival of IJs within the host cadaver, although the precise mechanism is unknown. While there was a difference in the internal consistency of the EPN infected cadaver, the stickier condition of the *yel*-infected cadavers, which is presumably a function of differences in metabolism between the two subspecies (see Section 2.3.3.d), this does not seem to benefit the nematodes carrying this subspecies and there was no difference in weight loss of cadavers infected with either subspecies.

Soil-dwelling nematodes require a film of water of sufficient thickness (relative to their body size) or water filled pores that provide a continuous film of water within the soil for them to move freely. The thickness of the water film and its continuity will be related to the type and size of soil particle. The free draining nature and small particle size of sand means that sand at a low moisture content (<1% by weight) will probably lack sufficient moisture to allow a continuous film of water to be present (Molyneux & Bedding, 1984). On the other hand, soil with high moisture content will lack sufficient air filled pores for the nematodes and in addition a continuous gradient of carbon dioxide and other signal molecules that the nematodes follow are unlikely to form. In open dune habitat water availability can be quite low. For example, the water table is likely to be more than 2 m below the soil surface and in dry dune slacks it will typically be 1 to 2 m deep (Ranwell, 1972). In addition, the

moisture capacity of the soil can vary considerably from front to rear in a dune system with the moisture capacity of older dunes being as high as 33%, while that of younger dunes can be as low as 7%, with the very young fore dunes having a moisture capacity of less than 1% and soil moisture typically being around 4% by weight (McLachlan & Brown, 2006). This difference in moisture capacity is due to the higher organic content of the older dune soil. For sandy soils 1% moisture capacity represents a water potential of around -10kPa (Richards & Weaver, 1944). Molyneux and Bedding (1984) achieved water potentials of between -1kPa and -10.2 kPa for a fine sand mixture (25% coarse sand, 65% fine sand, 7% silt, 3% clay) with the addition of 13% - 1% water. They found that *H. bacteriophora* was able to infect *L. cuprina* larvae in fine sand within this range of water potentials, indicating that this species was able to move through the sand and locate the host insect at this range of moisture contents. Thus the emergence of nematodes from cadavers stored at the range of soil water contents (1 – 8%) in the first desiccation experiment is not surprising. Since the main aim of this chapter was to explore differences between the *pur* and *yel* bacterial subspecies in terms of nematode survival and emergence from desiccated hosts subsequent experiments involving hosts desiccated in sand used only the dry sand treatment and the duration of the desiccation period was varied (experiment 3 and experiment 4). In addition, nematode-infected cadavers were subjected to severe desiccation in the form of exposure to 0% RH as hosts infected at or near the soil surface might be expected to experience dry periods due to exposure to the sun or during periods of drought.

Chapter 6
General Discussion

Heterorhabditis rely on their symbiont to kill the insect host and provide nutrients for growth and reproduction. Variations in the attributes of the bacteria affect the fitness of the associated nematode partner, which may in turn allow them to exploit different habitats. Given the data in this and previous studies (Rolston *et al.*, 2005; Rolston *et al.*, 2006) there is evidence of spatial separation of the two colour phenotypes of *H. downesi* within the dune habitat on North Bull Island. The tendency in some years is for the PUR colour phenotype to predominate in the front of the dunes. Six *Heterorhabditis* isolates from North Bull Island were identified as *H. downesi* on the basis of analysis of a portion of the ITS1 region of the ribosomal DNA (Section 2.3.2). This analysis showed that although there were seven single nucleotide polymorphisms along a 1031 nucleotide sequence there was no pattern of variation due to colour phenotype, as only a single isolate varied at each site and all six nematode isolates were identified as *H. downesi* on the basis of comparison with sequences from the *H. downesi* type strain, K122. On the other hand the *pur* and *yel* colour phenotypes of the symbiotic bacteria were identified as *P. temperata* subsp. *cinerea* (*P.t. cinerea*) and *P. temperata* subsp. *temperata* (*P.t. temperata*) respectively on the basis of analysis of a portion of the gyrB gene (Section 2.3.4).

In 2001 and 2002 Rolston *et al*. (2005) found that the range of the *H. downesi* colour phenotypes on North Bull Island overlapped, with both colour phenotypes found in both the front 50 m and the rear 50 m of the dune system. In both years the PUR phenotype was more common towards the front of the dune system and in 2002 this trend was significant. In a 2005 targeted resurvey of the section of dunes where *H.*

downesi had previously been recorded the PUR phenotype was again significantly more common than the YEL in the front 30 m of the dunes (Rolston *et al.*, 2006). In contrast, in this study, there was no overlap of range in 2008, with the PUR phenotype occurring at the front and the YEL phenotype occurring at the rear of the dunes only (Section 2.3.1.2). In 2012 a resurvey of a single transect from the 2008 survey (transect 1) showed that once again the range of the two colour phenotypes overlapped. Overall the distribution of the two phenotypes recorded in this study was significantly different, with the PUR phenotype occurring more frequently in the front section and the YEL phenotype occurring more frequently in the rear section of the dune system (Chapter 2, Table 2.17 & Table 2.18). The continued occurrence of the *H. downesi* associated with both *P.t. cinerea* and *P.t. temperata* on North Bull Island, as demonstrated by five surveys between 2001 and 2012 and the tendency for the *P.t. cinerea* symbiont to predominate towards the front of the dune system points to the stability of the association of the nematode with two subspecies of the symbiotic bacteria and also to the stability of the distribution of the both subspecies of the symbiont within the dune system.

The co-existence at a single site of two different species or subspecies of *Photorhabdus* with a single *Heterorhabditis* species is unusual and has only previously been reported from an oak forest and a nearby clearing in Hungary (Tóth & Lakatos, 2009). However, it is likely that the purple and yellow colour variants of the symbiont associated with *H. downesi* reported from a survey of sites in Ireland and Britain (Griffin *et al.*, 1994) are in fact *P.t. cinerea* and *P.t. temperata*, respectively. Furthermore, a targeted survey of sandy soils in Ireland, Britain, Denmark, Estonia and Hungary (Griffin *et al.*, 1999) reported the occurrence of *H. downesi* with purple and yellow variants of the *Photorhabdus* symbiont, and both

colour variants co-occurred in two sites in Ireland, neither of which were on North Bull Island, two sites in Wales and one site in Denmark (C. Griffin, pers. comm.), and it is likely that these are also *P.t. cinerea* and *P.t. temperata*, respectively. Thus, it would seem that the co-existence of these two symbionts within *H. downesi* is a general occurrence and occurs throughout the range of this nematode species.

The association of *H. downesi* and its *Photorhabdus temperata* symbiont with sandy soils in Ireland (Griffin *et al.*, 1994; Rolston *et al.*, 2002), Britain and Denmark and its absence from sandy coastal sites in Estonia (Griffin *et al.*, 1999), where winters tend to be colder and wetter, suggest that the nematode-bacterium complex is adapted to drier, warmer conditions in the more northerly section of its range. In the more southerly section of its range, in Hungary, the nematode-bacterium complex tends to occur in shaded areas, such as under trees in roadside verges (Griffin *et al.*, 1999), or in forest clearings (Tóth & Lakatos, 2009), where the nematode-bacterium complex would be shaded from the more intense effects of UV radiation and higher temperatures associated with a more southerly latitude.

For species to coexist they must use different resources or respond to biotic and abiotic stresses differently (Chase & Leibold, 2003). In the dune system on North Bull Island biotic and abiotic factors vary along a gradient from front to back of the dunes. The North Bull Island dune system is in fact comprised a number of microhabitats, with loosely compacted, free draining sand with little ground cover at the front of the dunes progressing through a build-up of organic content to a more moisture-retentive grass dominated soil with a large component of humic matter towards the rear of the dune system. Along this soil gradient there is also a decrease in both salinity and pH due to the leaching of minerals and the build-up of organic matter in the soil. These abiotic changes result in an increase in the range and

diversity of both above and below ground fauna and plant communities from front to rear of the dune system. Competition from other biotic factors within the dune system such as *S. feltiae*, nematophagous fungi or scavengers will also play a role in the success of the two *H. downesi*-*Photorhabdus temperata* complexes. It is clear from the surveys carried out in 2008 and 2012 and from the earlier surveys (Rolston *et al.*, 2005; Rolston *et al.*, 2006) carried out that *S. feltiae* is much more widely distributed in the sand dune system on North Bull Island than is *H. downesi* (Section 2.3.1.1). Although nothing is known of the distribution of nematophagous fungi within the sand dune system of North Bull Island such fungi are common in most soil types and are undoubtedly present on North Bull Island. There is some evidence that the second-stage cuticle that is retained by the IJs helps to protect *Heterorhabditis* spp. from nematophagous fungi (Timper & Kaya, 1989)

A number of factors could contribute to the observed distribution of the symbiont types associated with *H. downesi* on North Bull Island. Host specialisation is an obvious factor. While little is known about its natural host preferences, *H. downesi*-infected larvae of the garden chafer, *Phyllopertha horticola* have been found in modified dune grassland in Co. Clare by R. Enright (C. Griffin, pers. comm.). A wide range of invertebrate species have been recorded from the habitats in the sand dune system in North Bull Island (reviewed in Section 1.4). Host adaptation may be influenced by the antimicrobial activity of the bacterial symbiote as well as a range of other pathogenicity factors. In Chapter 2, *P.t. cinerea* was shown to be significantly better than *P.t. temperata* at inhibiting a range of bacteria and fungi, and this may be an important factor in the adaptation of *P.t. cinerea* to the front section of the dune system, by enhancing the ability of the nematode-bacterium to exploit a wide range of hosts and also to scavenge already dead hosts, as is the case

in some *Heterorhabditis* and *Steinernema* species (San-Blas & Gowen, 2008; San-Blas *et al.*, 2012), although in those studies the insects were freeze-killed. Scavenging on epn-killed cadavers may be a feature of *H. downesi* behaviour as nematodes carrying either symbiont infected cadavers killed by their own or the other symbiont (Section 3.3.1). On the other hand, *H. downesi* and *S. feltiae* may avoid cadavers already infected with the other nematode as no bait insects infected with both *H. downesi* and *S. feltiae* were recovered from any of the North Bull Island soil samples (Section 2.3.1.1). This may be due to the presence of the other species of symbiont rendering the cadaver unattractive to the later nematode, although *in vitro* tests showed that both *P.t. cinerea* and *P.t. temperata* inhibit the growth of *X. bovienii*, the symbiont of *S. feltiae* (Section 2.3.3.1), however, this wasn't tested *in vivo* in the current study.

Insects vary in the diversity of their intestinal microbial community (Broderick *et al.*, 2004; Egert *et al.*, 2005; van der Hoeven *et al.*, 2008; Wang *et al.*, 2011; Hirsch *et al.*, 2012) and the invading nematode-bacterium complex has to overcome not only the host insect immune system but also the gut microbiota resident in the insect and opportunistic soil-dwelling microorganisms which may also try to invade the insect cadaver. One source of this difference in antimicrobial activity may be the pigments produced by the bacteria. The two subspecies of the *H. downesi* symbiotic bacteria caused infected larvae (*G. mellonella, H. abietis* and *Coelopa* spp.) to turn grey/purple or straw to golden yellow and produced different coloured culture in broth and different coloured colonies on nutrient or LB Millar agar. These pigments have been identified as anthraquinones, secondary metabolites produced by all *Photorhabdus* spp. (Bode, 2009). Several anthraquinones including a red pigment from *P. luminescens* strain HK (Richardson *et al.*, 1988), two yellow pigments from

P. luminescens strain C9 (Li *et al.*, 1995), and two anthraquinones from *P. luminescens* TT01 (Brachmann *et al.*, 2007) were all shown to have antimicrobial properties and while the exact mode of action of this class of compounds is not fully understood they are believed to play a role in protecting the insect cadaver from competing microorganisms. Since *P.t. cinerea* and *P.t. temperata* have different colour phenotypes it may well be the case that they each produce a different range of these antimicrobial pigments. Preliminary HPLC analysis (data not shown) indicated that there were differences in the range of compounds secreted by both subspecies and these compounds may include antimicrobial products that allow the EPN to exploit a range of hosts (Dillon & Charnley, 1988), and so play a role in the distribution of *P.t. cinerea* and *P.t. temperata* within the dune system on North Bull Island.

In plant–feeding insects, variation in the symbiotic microbes can allow the colonisation of new host plants (Douglas, 2009), increase the fitness of the insect host to abiotic conditions such as increased temperature (Chen *et al.*, 2000), and assist in overcoming plant defences (Paine *et al.*, 1997). The differences in growth dynamics *in vitro* and *in vivo* (Sections 2.3.3 and 3.3.1.1) and the results of the biochemical characterisation (Section 2.3.3) support this with, for example only *P.t. temperata* isolates testing positive for tryptophan deaminase, suggesting that *P.t. cinerea* and *P.t. temperata* differ in their ability to utilise resources. The growth dynamics within the host are particularly interesting (Chapter 3 Figure 3.2) as more colony forming units were recovered from insects co-infected with both bacterial subspecies than from hosts with either type on its own. It is possible that differences in the metabolic profile of the two subspecies allow them to partition the resources within the insect, thus allowing both subspecies to multiply and producing an

overall greater level of colonisation of the insect host suggesting a synergy between the two subspecies.

The observed *in vitro* differences in antimicrobial activity and growth rate appear to have only a slight impact on the behaviour of the nematode-bacterium complexes in hosts under normal laboratory conditions. No differences were found in virulence against a range of hosts (Chapter 3) but these were not necessarily typical of the biota used in North Bull Island. Using hosts from three insect orders (Lepidoptera (1), Coleoptera (2), Diptera (1)), there was no difference in the ability of *H. downesi* carrying either *P.t. cinerea* or *P.t. temperata* to kill the insect host or for the nematode to successfully complete its development. *Photorhabdus. t. cinerea* killed *G. mellonella* larvae quicker than *P.t. temperata* (Sections 3.3.3 Table 3.18 and Section 4.3 Table 4.1 and Figure 4.2), but there was no difference between subspecies in overall mortality of the four species of hosts from three orders or in numbers of IJs emerging from these hosts. There was also no difference in the number of bacteria of either subspecies carried by the six isolates from North Bull Island or by the recombined lines or in the proportion of IJs that had no bacteria (Section 3.3.5). Differences in number of bacteria carried could be either good (enhanced virulence) or bad (reduced IJ survival) (Mitani *et al.*, 2004; Emelianoff *et al.*, 2008a).

A difference however was found in the proportion of IJs carrying each subspecies of bacteria from cadavers that had been co-infected with both subspecies (Section 3.3.2 table 3.12). The bacteria carried by the IJs were assessed 4 and 19 days after emergence began. At both time points more IJs carried *P.t. temperata* and at the second time point in the case of 5/19 of the cadavers there was a switch in the subspecies of bacteria colonising the IJs from *P.t. cinerea* (3/5) or a mixture of both

subspecies (2/5) to *P.t. temperata* only. It is unclear if this is a result of a preponderance of *P.t. temperata* late in the infection as bacterial quantification from infected larvae was only carried out at one time point, 72 hours post injection of the bacteria, and at that time point *P.t. cinerea* was more plentiful (Section 3.3.1.1). Another possibility is that *P.t. temperata* is better at colonising the nematodes late in the infection. This might arise due to metabolic differences between the two subspecies disadvantaging *P.t. cinerea* in its ability to utilise resources late in the infection and produce components necessary for successful colonisation of the nematode. The *pur1* and *yel3* isolates did not inhibit each other in the antibiosis assays carried out in Chapter 2 (Section 2.3.3.1). This suggests that interference competition mediated by bacteriocins is not a factor, but rather that scramble competition is occurring for the resources within the host.

There is evidence that *P.t. cinerea* protects IJs in cadavers in dry sand. *Heterorhabditis downesi* associated with *P.t. cinerea* within insect cadavers survived dry conditions significantly better (assessed by number of IJs emerging) than *H. downesi* associated with *P.t. temperata*. While there was no difference in the number of IJs emerging from cadavers infected with either subspecies under normal conditions, significantly more IJs emerged from insects infected with *P.t. cinerea* and stored under desiccating conditions for an extended duration (Sections 5.3.1 – 5.3.3). As discussed in Chapter 2 (Section 2.1) and Chapter 5 (Section 5.1), sand dunes represent a challenging environment for the animals and plants which live there and this is particularly true for smaller organisms such as nematodes, (Koppenhöfer *et al.*, 1997). *Photorhabdus t. cinerea*, occurring at the front of the dune system, is most exposed to desiccation and appears to be adapted to these conditions as evidenced by the greater survival of IJs carrying this subspecies in dry

conditions. Differences between the symbiotic bacteria may alter the nematodes' stress tolerance. Under dry conditions a number of biochemical changes occur which allows the nematode to survive desiccation. For example, nematodes have been shown to accumulate sugars and polyols, as well as undergoing alterations in fatty acid composition and the synthesis of a number of low molecular weight proteins (Glazer, 2002; Grewal *et al.*, 2006) and any of these features may be influenced by diet. Dietary factors can influence resistance to desiccation or thermal stress (Andersen *et al.*, 2010; Sisodia & Singh, 2012; Colinet & Renault, 2014). In *Drosophila melanogaster* dietary live yeast altered protein synthesis as well as altering total lipids and metabolic profile of the insect and promoted thermal tolerance (Colinet & Renault, 2014). *Heterorhabditis* species have been shown to reproduce when fed on some *Photorhabdus* strains but not on others (Han *et al.*, 1991; Gerritsen & Smits, 1993, 1997; Han & Ehlers, 1998), presumably due to differences in the nutritional factors provided by their own symbionts or that of another nematode, although the precise nature of these differences are unclear (Clarke, 2008). There is also some evidence that nematodes may take up potential protectant molecules across the cuticle (Qiu *et al.*, 2000; Qiu & Bedding, 2002) and differences in the biochemical activity of *P.t. temperata* and *P.t. cinerea* could also be a factor in making potential protectants such as trehalose available within the insect host, resulting in differences in survival of the *H. downesi* nematode in desiccated insects infected with one or the other symbiont.

Whatever the precise mechanism for the differences in protection from desiccation provided by the two *P. temperata* subspecies to *H. downesi* within a cadaver, *P.t. cinerea* provides a clear advantage to its associated nematodes under dry conditions. Although it is unusual for extreme drought conditions to persist for a period of

several weeks in Ireland, the effects of drying on the cadaver and the nematodes within it may be intensified by other extremes such as high temperatures or fluctuations in salinity due to cycles of drought and re-wetting and diurnal fluctuations in temperature (Willis *et al.*, 1959). During a hot summer sand near the surface may reach temperatures as high as 40^{o}C (Huiskes, 1979). This would have the effect of adding to the thermal stress of the nematode and its symbiont and increasing the rate of drying of the cadaver.

Bacterial symbionts are mediators of ecologically important traits for their hosts. *Photorhabdus* provide a range of services for the *Heterorhabditis* hosts including entomopathogenicity, essential nutrition, and suppression of the host immune system and of competing microbes. While the trait differences between *P.t. cinerea* and *P.t. temperata* have not been fully identified and characterised the two subspecies differ in their ability to protect nematodes in cadavers under desiccating conditions and in their antimicrobial activity. Trait differences between the two symbionts *P.t. cinerea* and *P.t. temperata* leading to niche separation may enable the two symbionts to co-exist and allow *H. downesi* expands its niche by using two subspecies of *P. temperata* and may help to explain the continued co-existence of two distinct subspecies of symbiotic bacteria within the *H. downesi* population .in the sand dune system on North Bull Island. *Heterorhabditis* populations occur at low frequency and have a patchy distribution (Campbell *et al.*, 1996). Similarly, the host insects are a patchy resource (Crossan *et al.*, 2007) Co-infection of a host by *H. downesi* carrying the different bacterial symbionts will probably be infrequent due to the patchiness of the nematode and host populations, but may be facilitated by movement of the host prior to death, since it takes 72 hours or more for the nematode-bacterium complex to kill the host insect, depending on its size. Since

Heterorhabditis IJs always develop into self-fertile hermaphrodites in the first generation the nematodes can always reproduce. This allows the possibility for cross-fertilisation to occur in the second generation of worms. Under laboratory conditions, nematodes emerging from a co-infected host were found to be able to carry either host (Section 3.3.2). From the experiments investigating the role of the symbiont in nematode survival within a host (Section 5.3), it appears that under normal conditions *P.t. temperata* out-competes *P.t. cinerea* in forming a symbiotic association with the nematode when both subspecies are present in the same insect host, resulting in the *H. downesi-P.t. temperata* association predominating in the rear section of the dunes, where the increased organic content of the soil favours moisture retention. On the other hand the drier conditions that predominate in the sandier soil towards the front of the dune system appears to favour the *H. downesi-P.t. cinerea* association. There is an apparent lack of specialisation by *H. downesi* toward either *P. temperata* subspecies and presumably it can pick up the other symbiont by invading an already colonised host (Chapter 3).

A number of issues that merit further research have arisen from the work presented here. Relatively little is known about the natural host range of EPN. This study has shown that *H. downesi* North Bull Island isolates can infect, kill, reproduce in and emerge from the kelp flies *C. frigida* and *C. pilipes* but there were no differences between *P.t. cinerea* and *P.t. temperata*. Sicard *et al*. (2008) found that EPN were pathogenic to terrestrial isopods and Mauleon *et al.* (2006) reported that EPN were pathogenic to beach associated terrestrial isopods and amphipods. On North Bull Island *C. frigida* occurs in seaweed in the strandline (Healy, 1975) and it is likely that amphipods occur there also. Terrestrial isopods occur in the zone between the strand line and the embryonic dunes (Healy, 1975; Speight, 1997). These may be a

natural host of *P. t. cinerea* since they occur at the front of the dune system where *P.t.* cinerea predominates. Testing *P.t. temperata* and *P.t. cinerea* against invertebrate species known to occur in the dune grassland habit may reveal further differences between the two subspecies, in particular, the effect of the antimicrobial activity of the symbiont against the diversity of insect intestinal microbe communities, which can vary considerably (Dillon & Dillon, 2004). It would also be of interest to carry out a survey of microbiota associated with a range of natural hosts from North Bull Island and test the efficacy of the *P.t. temperata* and *P.t. cinerea* against these where possible. Antimicrobials produced by the symbiotic bacteria allow the EPN to exploit a range of hosts and HPLC analysis could be used to investigate the range of chemicals produced by the two subspecies and their antimicrobial effect could also be tested.

The growth dynamics within the host early and late in the infection may be a factor in the colonisation of the early and late emerging IJs. *Photorhabdus t. cinerea* appears to predominate in the insect cadaver early in the infection. Is this because it is better at colonising the insect? Is *P.t. temperata* dominating the cadaver later in the infection? If both subspecies are equally colonising the insect late in the infection what factors are favouring IJ colonisation by *P.t.* temperata?

Much of the work described in this study looked at a single pair of *P.t. temperata* and *P.t. cinerea* isolates and therefore further study needs to be carried out to determine whether the observed differences i.e. *P.t. temperata* outcompetes *P.t. cinerea* in co-infections in the same insect host while *P.t. cinerea* is more desiccation tolerant than *P.t. temperata,* is an isolate effect or applies at subspecies level. The promiscuous behaviour of *H. downesi* in relation to the two subspecies of

Photorhabdus from North Bull Island may be a survival strategy that enables *H. downesi* to persist.

Two *Photorhabdus* subspecies, *P.t. cinerea* and *P.t. temperata* have been shown to co-exist at a single site in association with *H. downesi* (this study and that of Toth & Lakatos (2009)), and in association with *H. downesi* and *H. megidis* (Tóth & Lakatos, 2009), suggesting that these two nematodes are promiscuous in their association with the bacterial partner and that the symbiosis is not under tight regulatory control. In this study the niche separation of the two *Photorhabdus* subspecies appears to involve as yet undiscovered traits related to nematode colonisation and resistance to abiotic stress and by switching bacterial partners *H. Downesi* may be able to extend its niche in the dune system. This co-existence of two bacterial subspecies with a nematode species is in contrast to Maneesakorn *et al*. (2011) (discussed in Chapter 1), where a study of five *Heterorhabditis* species, including *H. megidis* and *H. downesi,* and their associated symbiotic bacteria found evidence of cospeciation events between *Heterorhabditis* and *Photorhabdus*. However, that study did not include analysis of *H. downesi* - *P.t. cinerea* and *H. megidis* - *P.t. cinerea* associations. Within the sand dune system on North Bull Island it is likely that the *H. downesi – P.t. cinerea* and *H. downesi – P.t. temperata* nematode-bacteria complexes co-exist as a freely-interbreeding population, capable of exchanging symbionts whenever IJs carrying the different symbiont co-infect the same host. The work presented here suggests that the persistence and success of each symbiont in the different microhabitats within the North Bull Island dune system depends in part on the ability of the symbiont to protect its nematode host as well as its ability to utilise and defend the insect host cadaver.

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Appendix

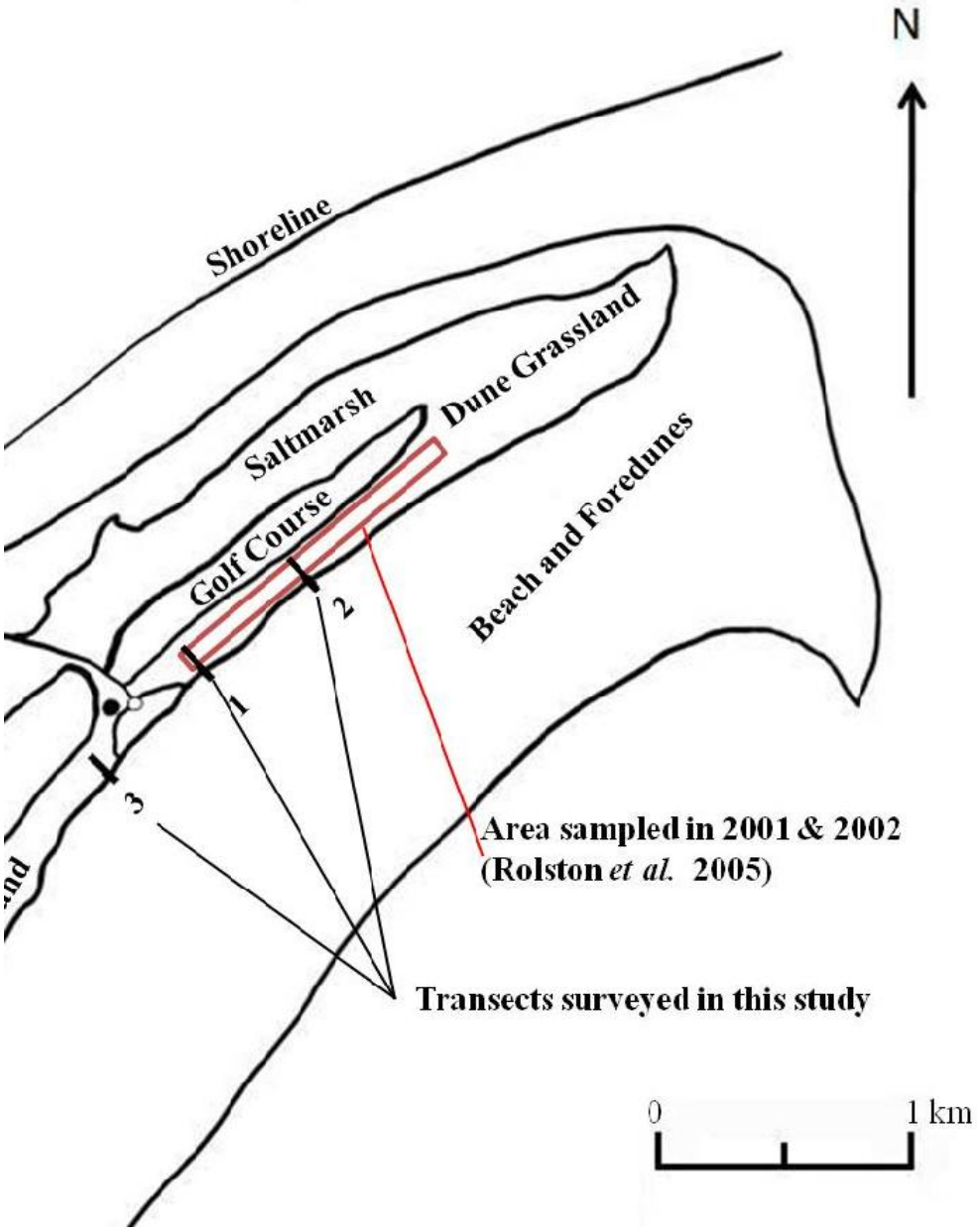

Figure A. 1: Overlap of sampling areas on North Bull Island, this study (Transect 1, 2, 3) and Rolston et al (2005) 2001 - 2002. (See Section 2.2.1.1, Figure 2.1).

Table A 1: TAE Buffer (See Section 2.2.2.2)

1 x Tris-Acetate (TAE) Buffer	**2 L**
50 x TAE	40 ml
adjust volume to 2 L with dH_2O	

50 x Tris-Acetate (TAE) Buffer	**1 L**
Trizma base (Sigma-Aldrich)	242 g
acetic acid (glacial) (Merck chemicals)	57.1 ml
0.5M EDTA (pH 8.0)	100 ml
adjust volume to 1 L with dH_2O	
Do not autoclave. Store at room temperature.	

0.5M EDTA	**1 L**
EDTA	146.1 g
dH_2O	800 ml
pH adjusted to 8.0 with 25g - 35 g NaOH pellets	
adjust volume to 1 L with dH_2O	
Sterilise by autoclaving and store at room temperature	

Table A 2: Parameters used for the Maximum Likelihood method for calculating the phylogeny of *Heterorhabditis downesi* isolates from North Bull Island (see Section 2.2.2.6 & Section 2.3.2, Figure 2.9). Phylogenetic analysis was carried out in MEGA5.

Analysis

Analysis ---------- Phylogeny Reconstruction

Statistical Method ---------- Maximum Likelihood

Phylogeny Test

Test of Phylogeny ---------- Bootstrap method

No. of Bootstrap Replications --------- 1000

Substitution Model

Substitutions Type ---------- Nucleotide

Model/Method ---------- Kimura 2-parameter model

Rates and Patterns

Rates among Sites ---------- Gamma Distributed (G)

No of Discrete Gamma Categories ---- 5

Data Subset to Use

Gaps/Missing Data Treatment --------- Use all sites

Tree Inference Options

ML Heuristic Method ---------- Nearest-Neighbor-Interchange (NNI)

Initial Tree for ML ---------- Make initial tree automatically

Codons Included ----------1st+2nd+3rd+Non-Coding

Number of Sequences: 29

Number of Sites: 1012

Table A 3: Culture media for *Photorhabdus temperata* subsp. (See Section 2.2.3.1)

A	**Nutrient agar**	g/l
	Lab-Lemco powder	1
	Yeast extract	2
	Peptone	5
	Sodium chloride	5
	Agar	15

B	**Nutrient agar plus bromothymol blue and triphenyltetrazolium chloride**	g/l
	Lab-Lemco powder	1
	Yeast extract	2
	Peptone	5
	Sodium chloride	5
	Agar	15
	Bromothymol blue	0.025
	triphenyltetrazolium chloride	0.04

C	**MacConkey agar**	g/l
	Peptone	20
	Lactose	10
	Bile salts	5
	Sodium chloride	5
	Neutral red	0.075
	Agar	12

D	**Luria broth †**	g/l
	Tryptone	10
	Yeast extract	5

† extra salt was added to Luria broth for differential growth investigation.

A.1 Generation of standard curve for *Photorhabdus temperata pur1* and *yel3* isolates

Three replicate liquid cultures were set up in NB as described in Section 2.2.3.2 and incubated for 45 hr at 27^{o} C with shaking at 200 rpm. A dilution series in ¼ strength sterile Ringer's solution was made from each culture. Initial serial dilutions of 1 in 10 and 1 in 5 yielded too few data points to construct a standard curve therefore a 1 in 2 (*pur1*) or a 1 in 3 (*yel*) serial dilution was used. The optical density of each dilution was read at 660 nm (OD_{660}). Direct cell counts were carried out in triplicate as described in Section 2.2.3.3b for each replicate culture using a suitable dilution and used to calculate the concentration of each dilution to generate the standard curves for *pur1* (Figure A.1A) and *yel3* (Figure A.1B). The standard curves were then used to calculate concentration of bacterial cells for DNA extraction (Section 2.2.4.1) and for the cross-compatibility experiment described in Section 3.2.1.

Figure A. 2: Standard curve for *pur1* (A) and *yel3* (B) *Photorhabdus temperata* isolates from North Bull Island (See section 2.2.4.1 and Section 3.2.1.1).

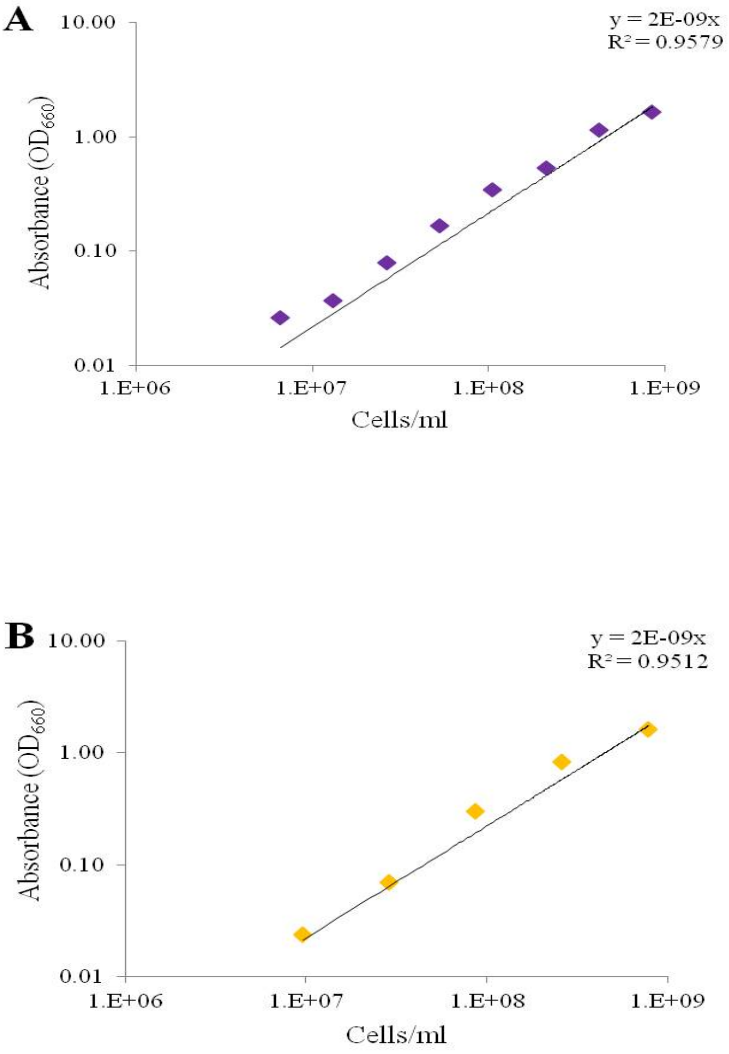

Table A 4: Parameters used for the Maximum Likelihood method for calculating the phylogeny of *Photorhabdus* isolates from North Bull Island (See Section 2.2.4.3 & Section 2.3.4, Figure 2.17). Phylogenetic analysis was carried out in MEGA5.

Analysis

Analysis ---------- Phylogeny Reconstruction

Statistical Method ---------- Maximum Likelihood

Phylogeny Test

Test of Phylogeny ---------- Bootstrap method

No. of Bootstrap Replications ----------1000

Substitution Model

Substitutions Type ---------- Nucleotide

Model/Method ---------- Kimura 2-parameter model

Rates and Patterns

Rates among Sites ---------- Gamma Distributed (G)

No of Discrete Gamma Categories ------ 5

Data Subset to Use

Gaps/Missing Data Treatment ---------- Use all sites

Tree Inference Options

ML Heuristic Method ---------- Nearest-Neighbor-Interchange (NNI)

Initial Tree for ML ---------- Make initial tree automatically

Codons Included ---------- 1st+2nd+3rd+Non-Coding

Number of Sequences: 33

Number of Sites: 847

Table A 5: The flora, soil temperature and pH recorded from three transects on North Bull Island in 2008 (see Section 2.3.1.5)

Transect 1				Transect 2			Transect 3		
Distance (m)	Habitat type†	soil temp ^{o}C (8 cm depth)	pH	Habitat type	soil temp ^{o}C (8 cm depth)	pH	Habitat type	soil temp ^{o}C (8 cm depth)	pH
0	E	15	8.38	E	20	8.17	E	20	8.28
10	E/M/SP	18	8.23	M/SFX/SP	15	8.17	E/M	15	8.37
20	M/SFX/SP	17	8.04	M/SFX/F/SP	14	8.19	M/SFX/SP	14	8.24
30	M/SFX/SP	16	7.89	M/SFX/F/SP	14	7.94	M/SFX/SP	14	8.11
40	M/SFX/SP	15	7.95	M/SFX/F	17	7.75	M/SFX/FX	17	8.02
50	M/SFX/SP	21	8.08	M/SFX/F/GP	14	7.89	M/SFX/SP	14	8.14
60	BO/M/SFX/F/ SP	17	7.95	M/SFX/F/GP	14	8.04	M/SFX/FX/GP	14	8.13
70	M/SFX/F/SP	15	8.23	M/SFX/F/GP	16	8.12	M/SFX/FX/GP	16	8.19
80	M/SFX/F/SP	13	7.85	M/SFX/F/GP	18	7.90	M/SFX/SP/GP	18	7.95
90	M/SFX/FX	14	7.99	M/SFX/F/SP/G P	15	7.87	M/SFX/FX/SP/ GP	15	7.90
100	SFX/FX	14	7.80	M/SFX/F/GP	14	7.87	M/SFX/SP/GP	14	7.89
110	SFX/FX/GP	13	7.75	M/SFX/F/GP	14	7.87	M/SFX/GP	14	7.91
120	SFX/FX/GP	16	7.82	M/SFX/F/SP	13	7.98	M/SFX/FX/GP	13	7.91
130	SFX/FX	16	7.81	M/SFX/F/GP	14	7.93	M/SFX/ /GP	14	7.91

† BO = Blowout, E = embryonic dunes, FX = fixed dunes, GP = grassy path, M = Marram dunes, SFX = Semi-fixed dunes, SP = sandy path (compacted soil)

```
PUR1            ------------TTGATTACGTCCCTGCCCTTTGTACACACCGCCCGTCGCTGTCCGGG 47
PUR2            ------------TTGATTACGTCCCTGCCCTTTGTACACACCGCCCGTCGCTGTCCGGG 47
PUR3            GGAATTCGCCCTTTTGATTACGTCCCTGCCCTTTGTACACACCGCCCGTCGCTGTCCGGG 60
YEL1            -------------TGATTACGTCCCTGCCCTTTGTACACACCGCCCGTCGCTGTCCGGG 46
YEL2            ------------TTGATTACGTCCCTGCCCTTTGTACACACCGCCCGTCGCTGTCCGGG 47
YEL3            ------------TTGATTACGTCCCTGCCCTTTGTACACACCGCCCGTCGCTGTCCGGG 47
                             *******************************************

PUR1            ACTGAGCTGTTTCGAGAAGAGTGGAGACTGCTGTATCGGGGCTTTCGGGCTCTGGTATGA 107
PUR2            ACTGAGCTGTTTCGAGAAGAGTGGAGACTGCTGTATCGGGGCTTTCGGGCTCTGGTATGA 107
PUR3            ACTGAGCTGTTTCGAGAAGAGTGGAGACTGCTGTATCGGGGCTTTCGGGCTCTGGTATGA 120
YEL1            ACTGAGCTGTTTCGAGAAGAGTGGAGACTGCTGTATCGGGGCTTTCGGGCTCTGGTATGA 106
YEL2            ACTGAGCTGTTTCGAGAAGAGTGGAGACTGCTGTATCGGGGCTTTCGGGCTCTGGTATGA 107
YEL3            ACTGAGCTGTTTCGAGAAGAGTGGAGACTGCTGTATCGGGGCTTTCGGGCTCTGGTATGA 107
                ************************************************************

PUR1            TGGAAACCATTTTAATCGCAATGGCTTGAACCGGGCAAAAGTCGTAACAAGGTATCTGTA 167
PUR2            TGGAAACCATTTTAATCGCAATGGCTTGAACCGGGCAAAAGTCGTAACAAGGTATCTGTA 167
PUR3            TGGAAACCATTTTAATCGCAATGGCTTGAACCGGGCAAAAGTCGTAACAAGGTATCTGTA 180
YEL1            TGGAAACCATTTTAATCGCAATGGCTTGAACCGGGCAAAAGTCGTAACAAGGTATCTGTA 166
YEL2            TGGAAACCATTTTAATCGCAATGGCTTGAACCGGGCAAAAGTCGTAACAAGGTATCTGTA 167
YEL3            TGGAAACCATTTTAATCGCAATGGCTTGAACCGGGCAAAAGTCGTAACAAGGTATCTGTG 167
                ************************************************************

PUR1            GGTGAACCTGCAGATGGATCATCGCTGAAAACCTTTATGGTTATGCTTTGGTCACGAGAG 227
PUR2            GGTGAACCTGCAGATGGATCATCGCTGAAAACC-TTATGGTTATGCTTTGGTCACGTGAG 226
PUR3            GGTGAACCTGCAGATGGATCATCGCTGAAAACC-TTATGGTTATGCTTTGGTCACGAGAG 239
YEL1            GGTGAACCTGCAGATGGATCATCGCTGAAAACT-TTATGGTTATGCTTTGGTCACGAGAG 225
YEL2            GGTGAACCTGCAGATGGATCATCGCTGAAAACT-TTATGGTTATGCTTTGGTCACGAGAG 226
YEL3            GGTGAACCTGCAGATGGATCATCGCTGAAAAC--TTATGGTTATGCTTTGGTCACGAGAG 225
                ********************************  ********************** ***

PUR1            ATCGGTGCTACTGGAATCAGGCTTGCTCTTGATTTCAATCGGTATCTCACCCCATCTAAG 287
PUR2            ATCGGTGCTACTGGAATCAGGCTTGCTCTTGATTTCAATCGGTATCTCACCCCATCTAAG 286
PUR3            ATCGGTGCTACTGGAATCAGGCTTGCTCTTGATTTCAATCGGTATCTCACCCCATCTAAG 299
YEL1            ATCGGTGCTACTGGAATCAGGCTTGCTCTTGATTTCAATCGGTATCTCACCCCATCTAAG 285
YEL2            ATCGGTGCTACTGGAATCAGGCTTGCTCTTGATTTCAATCGGTATCTCACCCCATCTAAG 286
YEL3            ATCGGTGCTACTGGAATCAGGCTTGCTCTTGATTTCAATCGGTATCTCACCCCATCTAAG 285
                ************************************************************

PUR1            CTCTCGGAGAGGTGTCTAATCGCAATCGGAGTCGCTTTGAGTGACGGCAATGAAGATTGT 347
PUR2            CTCTCGGAGAGGTGTCTAATCGCAATCGGAGTCGCTTTGAGTGACGGCAATGAAGATTGT 346
PUR3            CTCTCGGAGAGGTGTCTAATCGCAATCGGAGTCGCTTTGAGTGACGGCAATGAAGATTGT 359
YEL1            CTCTCGGAGAGGTGTCTAATCGCAATCGGAGTCGCTTTGAGTGACGGCAATGAAGATTGT 345
YEL2            CTCTCGGAGAGGTGTCTAATCGCAATCGGAGTCGCTTTGAGTGACGGCAATGAAGATTGT 346
YEL3            CTCTCGGAGAGGTGTCTAATCGCAATCGGAGTCGCTTTGAGTGACGGCAATGAAGATTGT 345
                ************************************************************

PUR1            GTGTGTTCCCCGTAAGGGTAGAGCATAGACTTTAAGAACAGTGCTGGGACTGTCCCCTCA 407
PUR2            GTGTGTTCCCCGTAAGGGTAGAGCATAGACTTTAAGAACAGTGCTGGGACTGTCCCCTCA 406
PUR3            GTGTGTTCCCCGTAAGGGTAGAGCATAGACTTTAAGAACAGTGCTGGGACTGTCCCCTCA 419
YEL1            GTGTGTTCCCCGTAAGGGTAGAGCATAGACTTTAAGAACAGTGCTGGGACTGTCCCCTCA 405
YEL2            GTGTGTTCCCCGTAAGGGTAGAGCATAGACTTTAAGAACAGTGCTGGGACTGTCCCCTCA 406
YEL3            GTGTGTTCCCCGTAAGGGTAGAGCATAGACTTTAAGAACAGTGCTGGGACTGTCCCCTCA 405
                ************************************************************

PUR1            CCAACCGTCGATTACTGGTGGCTATGAGTGACTTCGGTCACTCGAGATCTGCTACGCAGA 467
PUR2            CCAACCGTCGATTACTGGTGGCTATGAGTGACTTCGGTCACTCGAGATCTGCTACGCAGA 466
PUR3            CCAACCGTCGATTACTGGTGGCTATGAGTGACTTCGGTCACTCGAGATCTGCTACGCAGA 479
YEL1            CCAACCGTCGATTACTGGTGGCTATGAGTGACTTCGGTCACTCGAGATCTGCTACGCAGA 465
YEL2            CCAACCGTCGATTACTGGTGGCTATGAGTGACTTCGGTCACTCGAGATCTGCTACGCAGA 466
YEL3            CCAACCGTCGATTACTGGTGGCTATGAGTGACTTCGGTCACTCGAGATCTGCTACGCAGA 465
                ************************************************************

PUR1            GAGCCTCAATGAGTTGTTCGTCCAACCGCCGGTGTCGATAGAAATTTTTTCCTATTAACT 527
PUR2            GAGCCTCAATGAGTTGTTCGTCCAACCGCCGGTGTCGATAGAAATTTTTTCCTATTAACT 526
PUR3            GAGCCTCAATGAGTTGTTCGTCCAACCGCCGGTGTCGATAGAAATTTTTTCCTATTAACT 539
YEL1            GAGCCTCAATGAGTTGTTCGTCCAACCGCCGGTGTCGATAGAAATTTTTTCCTATTAACT 525
YEL2            GAGCCTCAATGAGTTGTTCGTCCAACCGCCGGTGTCGATAGAAATTTTTTCCTATTAACT 526
YEL3            GAGCCTCAATGAGTTGTTCGTCCAACCGCCGGTGTCGATAGAAATTTTTTCCTATTAACT 525
                ************************************************************

PUR1            TGTTTCTTGATTCGTGTTAATACATTTTGGCACAATGTATTAGCTTCAGCGATGGATCGG 587
PUR2            TGTTTCTTGATTCGTGTTAATACATTTTGGCACAATGTATTAGCTTCAGCGATGGATCGG 586
PUR3            TGTTTCTTGATTCGTGTTAATACATTTTGGCACAATGTATTAGCTTCAGCGATGGATCGG 599
YEL1            TGTTTCTTGATTCGTGTTAATACATTTTGGCACAATGTATTAGCTTCAGCGATGGATCGG 585
YEL2            TGTTTCTTGATTCGTGTTAATACATTTTGGCACAATGTATTAGCTTCAGCGATGGATCGG 586
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YEL3            TGTTTCTTGATTCGTGTTAATACATTTTGGCACAATGTATTAGCTTCAGCGATGGATCGG 585
                ************************************************************

PUR1            TTGATTCGCGTATCGATGAAAAACGCAGCAAGCTGCGTTATTTACCACGAATTGCAGACG 647
PUR2            TTGATTCGCGTATCGATGAAAAACGCAGCAAGCTGCGTTATTTACCACGAATTGCAGACG 646
PUR3            TTGATTCGCGTATCGATGAAAAACGCAGCAAGCTGCGTTATTTACCACGAATTGCAGACG 659
YEL1            TTGATTCGCGTATCGATGAAAAACGCAGCAAGCTGCGTTATTTACCACGAATTGCAGACG 645
YEL2            TTGATTCGCGTATCGATGAAAAACGCAGCAAGCTGCGTTATTTACCACGAATTGCTGACG 646
YEL3            TTGATTCGCGTATCGATGAAAAACGCAGCAAGCTGCGTTATTTACCACGAATTGCAGACG 645
                ************************************************** ****

PUR1            CTTAGGGTTGGTGAAGTTTTGAACGCACAGCGCCGTTGGGTTTTCCCTTCGGCACGTCTG 707
PUR2            CTTAGAGT-GGTGAAGTTTTGAACGCACAGCGCCGTTGGGTTTTCCCTTCGGCACGTCTG 705
PUR3            CTTAGAGT-GGTGAAGTTTTGAACGCACAGCGCCGTTGGGTTTTCCCTTCGGCACGTCTG 718
YEL1            CTTAGAGT-GGTGAAGTTTTGAACGCACAGCGCCGTTGGGTTTTCCCTTCGGCACGTCTG 704
YEL2            CTTAGAGT-GGTGAAGTTTTGAACGCACAGCGCCGTTGGGTTTTCCCTTCGGCACGTCTG 705
YEL3            CTTAGGGT-GGTGAAGTTTTGAACGCACAGCGCCGTTGGGTTTTCCCTTCGGCACGTCTG 704
                ***** ** **************************************************

PUR1            GCTCAGGGTTGTTTAAAGTACTGCGGTGTTGCTGCGAAAGTAGCAACACCGAATGTCGAA 767
PUR2            GCTCAGGGTTGTTTAAAGTACTGCGGTGTTGCTGCGAAAGTAGCAACACCGAATGTCGAA 765
PUR3            GCTCAGGGTTGTTTAAAGTACTGCGGTGTTGCTGCGAAAGTAGCAACACCGAATGTCGAA 778
YEL1            GCTCAGGGTTGTTTAAAGTACTGCGGTGTTGCTGCGAAAGTAGCAACACCGAATGTCGAA 764
YEL2            GCTCAGGGTTGTTTAAAGTACTGCGGTGTTGCTGCGAAAGTAGCAACACCGAATGTCGAA 765
YEL3            GCTCAGGGTTGTTTAAAGTACTGCGGTGTTGCTGCGAAAGTAGCAACACCGAATGTCGAA 764
                ************************************************************

PUR1            CGGCGTTAGTGTTACTACTGGCCCCGTTCTAGTTCCGAATATTGGCAACATGTCTTCTTC 827
PUR2            CGGCGTTAGTGTTACTACTGGCCCCGTTCTAGTTCCGAATATTGGCAACATGTCTTCTTC 825
PUR3            CGGCGTTAGTGTTACTACTGGCCCCGTTCTAGTTCCGAATATTGGCAACATGTCTTCTTC 838
YEL1            CGGCGTTAGTGTTACTACTGGCCCCGTTCTAGTTCCGAATATTGGCAACATGTCTTCTTC 824
YEL2            CGGCGTTAGTGTTACTACTGGCCCCGTTCTAGTTCCGAATATTGGCAACATGTCTTCTTC 825
YEL3            CGGCGTTAGTGTTACTACTGGCCCCGTTCTAGTTCCGAATATTGGCAACATGTCTTCTTC 824
                ************************************************************

PUR1            GTGAAGACGCCGTAGAGAGTATAAGTCTGTACCTATGGATGTGTCGCGTATGAAATATGA 887
PUR2            GTGAAGACGCCGTAGAGAGTATAAGTCTGTACCTATGGATGTGTCGCGTATGAAATATGA 885
PUR3            GTGAAGACGCCGTAGAGAGTATAAGTCTGTACCTATGGATGTGTCGCGTATGAAATATGA 898
YEL1            GTGAAGACGCCGTAGAGAGTATAAGTCTGTACCTATGGATGTGTCGCGTATGAAATATGA 884
YEL2            GTGAAGACGCCGTAGAGAGTATAAGTCTGTACCTATGGATGTGTCGCGTATGAAATATGA 885
YEL3            GTGAAGACGCCGTAGAGAGTATAAGTCTGTACCTATGGATGTGTCGCGTATGAAATATGA 884
                ************************************************************

PUR1            TGCTTCCCATACATAGCGAGGAGGTGCTTCTTATGCTATCTTGCTTATGCAACCTGAGCT 947
PUR2            TGCTTCCCATACATAGCGAGGAGGTGCTTCTTATGCTATCTTGCTTATGCAACCTGAGCT 945
PUR3            TGCTTCCCATACATAGCGAGGAGGTGCTTCTTATGCTATCTTGCTTATGCAACCTGAGCT 958
YEL1            TGCTTCCCATACATAGCGAGGAGGTGCTTCTTATGCTATCTTGCTTATGCAACCTGAGCT 944
YEL2            TGCTTCCCATACATAGCGAGGAGGTGCTTCTTATGCTATCTTGCTTATGCAACCTGAGCT 945
YEL3            TGCTTCCCATACATAGCGAGGAGGTGCTTCTTATGCTATCTTGCTTATGCAACCTGAGCT 944
                ************************************************************

PUR1            CAGTCGTGATTACCCGCCGAACTTAAGCATATCATTCAGCGGAGGAAAAGAAACTAACTA 1007
PUR2            CAGTCGTGATTACCCGCCGAACTTAAGCATATCATTCAGCGGAGGAAAAGAAACTAACTA 1005
PUR3            CAGTCGTGATTACCCGCCGAACTTAAGCATATCATTCAGCGGAGGAAAAGAAACTAACTA 1018
YEL1            CAGTCGTGATTACCCGCCGAACTTAAGCATATCATTCAGCGGAGGAAAAGAAACTAACTA 1004
YEL2            CAGTCGTGATTACCCGCCGAACTTAAGCATATCATTCAGCGGAGGAAAAGAAACTAACTA 1005
YEL3            CAGTCGTGATTACCCGCCGAACTTAAGCATATCATTCAGCGGAGGAAAAGAAACTAACTA 1004
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PUR1            GGATTCCCTTAGTAACGGCGAGTGAAA---------- 1034
PUR2            GGATTCCCTTAGTAACGGCGAGTGAAA---------- 1032
PUR3            GGATTCCCTTAGTAACGGCGAGTGAAAAAGGGCGAATT 1056
YEL1            GGATTCCCTTAGTAACGGCGAGTGAAA---------- 1031
YEL2            GGATTCCCTTAGTAACGGCGAGTGAAA---------- 1032
YEL3            GGATTCCCTTAGTAACGGCGAGTGAAA---------- 1031
                ***************************
```

Figure A. 3: ClustalW Multiple Sequence Alignment of a portion (1056 bases with 1031 shared positions) of the ITS1 region from three PUR and three YEL *Heterorhabditis downesi* isolates from North Bull Island (see Section 2.3.2).

Asterisk (∗) denotes conserved sites
Spaces denote nucleotide polymorphism.

```
PUR1            AGAGATC--GGTGCT--ACTGGAATCA-GGC---TTGCTCTT-GA----------TTTCA 41
PUR2            TGAGATC--GGTGCT--ACTGGAATCA-GGC---TTGCTCTT-GA----------TTTCA 41
PUR3            AGAGATC--GGTGCT--ACTGGAATCA-GGC---TTGCTCTT-GA----------TTTCA 41
YEL1            AGAGATC--GGTGCT--ACTGGAATCA-GGC---TTGCTCTT-GA----------TTTCA 41
YEL2            AGAGATC--GGTGCT--ACTGGAATCA-GGC---TTGCTCTT-GA----------TTTCA 41
YEL3            AGAGATC--GGTGCT--ACTGGAATCA-GGC---TTGCTCTT-GA----------TTTCA 41
D099            AGAGATC--GGTGCT--ACTGGAATCA-GGC---TTGCTCTT-GA----------TTTCA 41
H3107           AGAGATC--GGTGCT--ACTGGAATCA-GGC---TTGCTCTT-GA----------TTTCA 41
H3173           AGAGATC--GGTGCT--ACTGGATTCA-GGC---TTGCTCTT-GA----------TTTCA 41
AY321482        AGAGATC--GGTGCT--ACTGGAATCA-GGC---TTGCTCTT-GA----------TTTCA 41
K122            AGAGATC--GGTGCT--ACTGGAATCA-GGC---TTGCTCTT-GA----------TTTCA 41
OH10            AGAGATC--GGTGCT--ACTGGAATCA-GGC---TTGCTCTT-GA----------TTTCA 41
EF043441        AGAGATC--GGTGCT--ACTGGAATCA-GGC---TTGCTCTT-GA----------TTTCA 41
AGC             AGAGATC--GGTGCT--ACCGGAATCA-GGC---TTGCTCCTCGA----------TTTCG 42
Andong          AGAGATC--GGTGCT--ACCGGAATCA-GGC---TTGCTCCT-GA----------TTTCG 41
H3014           AGAGATC--GGTGCT--ACCGGAATCA-GGC---TTGCTCCTCGA----------TTTCG 42
HF85            AGAGATC--GGTGCT--ACCGGAATCA-GGC---TTGCTCCTCGA----------TTTCG 42
NIHHS-KHH05     AGAGATC--GGTGCT--ACCGGAATCA-GGC---TTGCTCCT-GA----------TTTCG 41
N-UK76          AGAGATC--GGTGCT--ACCGGAATCA-GGC---TTGCTCCTCGA----------TTTCG 42
KJ938577        AGAGATC--GGTGCT--ACCGGAATCA-GGC---TTGCTCCTCGA----------TTTCG 42
SF52            AGAGATC--GGTGCT--ACTGGAATCG-GGC---TTGCTCTT-GA----------TTTCA 41
SF670           AGAGATC--GGTGCT--ACTGGAATCG-GGC---TTGCTCTT-GA----------TTTCA 41
SF724           AGAGATC--GGTGCT--ACTGGAATCG-GGC---TTGCTCTT-GA----------TTTCA 41
EF488006        AGAGATC--GGTGCT--ACTGGAATCG-GGC---TTGCTCTT-GA----------TTTCA 41
Florida         AGCTATC--GGTGCT--CATGGAATCA-GGC---TCACGTTT-GA----------TTTCA 41
J37             AGCTATC--GGTGCT--CATGGAATCA-GGC---TCACGTTT-GA----------TTTCA 41
NZH3            AGCTATC--GGTGCT--CATGGAATCA-GGC---TCACGTTT-GA----------TTTCA 41
X1              AGCTATC--GGTGCT--CATGGAATCA-GGC---TCACGTTT-GA----------TTTCA 41
X03680          AAACCTTAAGATGCTCGACTGGCTTCACGGTCAGTTGAGTGTCGAAATGTCAACGTTCCA 60
                     *   * ****     **  **  **    *      * **          ** *

PUR1            ATCGGTATCTCACCCC-ATCTAAGCTCTCGGAGAGG-TGTCTAATCGC---AAT-CGG-A 94
PUR2            ATCGGTATCTCACCCC-ATCTAAGCTCTCGGAGAGG-TGTCTAATCGC---AAT-CGG-A 94
PUR3            ATCGGTATCTCACCCC-ATCTAAGCTCTCGGAGAGG-TGTCTAATCGC---AAT-CGG-A 94
YEL1            ATCGGTATCTCACCCC-ATCTAAGCTCTCGGAGAGG-TGTCTAATCGC---AAT-CGG-A 94
YEL2            ATCGGTATCTCACCCC-ATCTAAGCTCTCGGAGAGG-TGTCTAATCGC---AAT-CGG-A 94
YEL3            ATCGGTATCTCACCCC-ATCTAAGCTCTCGGAGAGG-TGTCTAATCGC---AAT-CGG-A 94
D099            ATCGGTATCTCACCCC-ATCTAAGCTCTCGGAGAGG-TGTCTAATCTC---AAT-TGG-A 94
H3107           ATCGGTATCTCACCCC-ATCTAAGCTCTCGGAGAGG-TGTCTAATCGC---AAT-CGG-A 94
H3173           ATCGGTATCTCACCCC-ATCTAAGCTCTCGGAGAGG-TGTCTAATCGC---AAT-CGG-A 94
AY321482        ATCGGTATCTCACCCC-ATCTAAGCTCTCGGAGAGG-TGTCTAATCGC---AAT-CGG-A 94
K122            ATCGGTATCTCACCCC-ATCTAAGCTCTCGGAGAGG-TGTCTAATCGC---AAT-CGG-A 94
OH10            ATCGGTATCTCACCTC-ATCTAAGCTCTCGGAGAGG-TGTCTAATCCC---AAT-TGG-A 94
EF043441        ATCGGTATCTCACCCC-ATCTAAGCTCTCGGAGAGG-TGTCTAATCCC---AAT-TGG-A 94
AGC             ATCGGTATCTCACCCCCATCTAAGCTCTCGGAGAGG-TGTCTAATCCC---AAT-CGG-A 96
Andong          ATCGGTGTCTCACCCCCATCTAAGCTCTCGGAGAGGGTGTCTAATCCC---AAT-CGGGA 97
H3014           ATCGGTATCTCACCCCCATCTAAGCTCTCGGAGAGG-TGTCTAATCCC---AAT-CGG-A 96
HF85            ATCGGTATCTCACCCCCATCTAAGCTCTCGGAGAGG-TGTCTAATCCC---AAT-CGG-A 96
NIHHS-KHH05     ATCGGTGTCTCACCCCCATCTAAGCTCTCGGAGAGG-TGTCTAATCCC---AAT-CGG-A 95
N-UK76          ATCGGTATCTCACCCCCATCTAAGCTCTCGGAGAGG-TGTCTAATCCC---AAT-CGG-A 96
KJ938577        ATCGGTATCTCACCCCCATCTAAGCTCTCGGAGAGG-TGTCTAATCCC---AAT-CGG-A 96
SF52            ATCGGTATCTCACCCC-ATCTAAGCTCTCGGAGAGG-TGTCTAATCCC---AAT-TGG-A 94
SF670           ATCGGTATCTCACCCC-ATCTAAGCTCTCGGAGAGG-TGTCTAATCCC---AAT-TGG-A 94
SF724           ATCGGTATCTCACCCC-ATCTAAGCTCTCGGAGAGG-TGTCTAATCCC---AAT-TGG-A 94
EF488006        ATCGGTATCTCACCCC-ATCTAAGCTCTCGGAGAGG-TGTCTAATCCC---AAT-TGG-A 94
Florida         ATCGGTAGCTCACCCC-ATCTAAGCTCTTGGTGAGG-TGTCTATTCCC---AAT-TGG-A 94
J37             ATCGGTAGCTCACCCC-ATCTAAGCTCTTGGTGAGG-TGTCTATTCCC---AAT-TGG-A 94
NZH3            ATCGGTAGCTCACCCC-ATCTAAGCTCTTGGTGAGG-TGTCTATTCCC---AAT-TGG-A 94
X1              ATCGGTAGCTCACCCC-ATCTAAGCTCTTGGTGAGG-TGTCTATTCCC---AAT-TGG-A 94
X03680          GTTGAGATGCCACAAC-AACTG-GCAAGAGTAGTGACTGTCCGACCCATGGAACGTGGCA 118
                 * *      ***  * * **  **    *  * *  ****    *     **   ** *

PUR1            GTCGCT------------TTGAGTGACGGC--AATGAAGATTGTGT-GTGTT---CCCCG 136
PUR2            GTCGCT------------TTGAGTGACGGC--AATGAAGATTGTGT-GTGTT---CCCCG 136
PUR3            GTCGCT------------TTGAGTGACGGC--AATGAAGATTGTGT-GTGTT---CCCCG 136
YEL1            GTCGCT------------TTGAGTGACGGC--AATGAAGATTGTGT-GTGTT---CCCCG 136
YEL2            GTCGCT------------TTGAGTGACGGC--AATGAAGATTGTGT-GTGTT---CCCCG 136
YEL3            GTCGCT------------TTGAGTGACGGC--AATGAAGATTGTGT-GTGTT---CCCCG 136
D099            GTCGCT------------TTGAGTGACGGC--AATGAAGGTTGGGT-GTGTT---CCCCG 136
H3107           GTCGCT------------TTGAGTGACGGC--AATGAAGATTGTGT-GTGTT---CCCCG 136
H3173           GTCGCT------------TTGAGTGACGGC--AATGAAGATTGTGT-GTGTT---CCCCG 136
AY321482        GTCGCT------------TTGAGTGACGGC--AATGAAGATTGTGT-GTGTT---CCCCG 136
K122            GTCGCT------------TTGAGTGACGGC--AATGAAGATTGTGT-GTGTT---CCCCG 136
OH10            GTCGCT------------TTGAGTGACGGC--AATGAAGGTTGGGT-GTGTT---CCCCG 136
EF043441        GTCGCT------------TTGAGTGACGGC--AATGAAGGTTGGGT-GTGTT---CCCCG 136
AGC             GTCGCT------------TTGAGTGACGGC--AATGAGGATTGGGT-GTGCCATACCCCA 141
Andong          GTCGCT------------TTGAGTGACGGC--AATGAGGATTGGGT-GTGCCATACCCCA 142
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H3014           GTCGCT-----------TTGAGTGACGGC--AATGAGGATTGGGT-GTGCCATACCCCA 141
HF85            GTCGCT-----------TTGAGTGACGGC--AATGAGGATTGGGT-GTGCCATACCCCA 141
NIHHS-KHH05     GTCGCT-----------TTGAGTGACGGC--AATGAGGATTGGGT-GTGCCATACCCCA 140
N-UK76          GTCGCT-----------TTGAGTGACGGC--AATGAGGATTGGGT-GTGCCATACCCCA 141
KJ938577        GTCGCT-----------TTGAGTGACGGC--AATGAGGATTGGGT-GTGCCATACCCCA 141
SF52            GTCGCG-----------TTGAGTGACGGC--AATGAAGGTTGGGT-GTGTT---CCCCG 136
SF670           GTCGCG-----------TTGAGTGACGGC--AATGAAGGTTGGGT-GTGTT---CCCCG 136
SF724           GTCGCG-----------TTGAGTGACGGC--AATGAAGGTTGGGT-GTGTT---CCCCG 136
EF488006        GTCGCG-----------TTGAGTGACGGC--AATGAAGGTTGGGT-GTGTT---CCCCG 136
Florida         GTCGCT-----------TGGAGTGACGGC--AGTGATGATTGGGT-GTGTTG---CCCG 136
J37             GTCGCT-----------TGGAGTGACGGC--AGTGATGATTGGGT-GTGTTG---CCCG 136
NZH3            GTCGCT-----------TGGAGTGACGGC--AGTGATGATTGGGT-GTGTTG---CCCG 136
X1              GTCGCT-----------TGGAGTGACGGC--AGTGATGATTGGGT-GTGTTG---CCCG 136
X03680          GTTATTCCAAAACTACTTTTGTGTCAGGGCTTAATGATGAGTGAAAAGTGTT---TGCTG 175
                **                * * ** * ***  * *** *  **    ***       *

PUR1            TA---AGGGTAGAGCATAGACTTTAAGAACAGTGCTG--GGACTGTCCCCTCA-C-CAA- 188
PUR2            TA---AGGGTAGAGCATAGACTTTAAGAACAGTGCTG--GGACTGTCCCCTCA-C-CAA- 188
PUR3            TA---AGGGTAGAGCATAGACTTTAAGAACAGTGCTG--GGACTGTCCCCTCA-C-CAA- 188
YEL1            TA---AGGGTAGAGCATAGACTTTAAGAACAGTGCTG--GGACTGTCCCCTCA-C-CAA- 188
YEL2            TA---AGGGTAGAGCATAGACTTTAAGAACAGTGCTG--GGACTGTCCCCTCA-C-CAA- 188
YEL3            TA---AGGGTAGAGCATAGACTTTAAGAACAGTGCTG--GGACTGTCCCCTCA-C-CAA- 188
D099            TA---AGGGTAGAGCATAGACTTTATGAACAGTGCTG--GGACTGTCGCCTCA-C-CAAA 189
H3107           TA---AGGGTAGAGCATAGACTTTAAGAACAGTGCTG--GGACTGTCCCCTCA-C-CAA- 188
H3173           TA---AGGGTAGAGCATAGACTTTAAGAACAGTGCTG--GGACTGTCCCCTCA-C-CAA- 188
AY321482        TA---AGGGTAGAGCATAGACTTTAAGAACAGTGCTG--GGACTGTCCCCTCA-C-CAA- 188
K122            TA---AGGGTAGAGCATAGACTTTAAGAACAGTGCTG--GGACTGTCCCCTCA-C-CAA- 188
OH10            TA---AGGGTAGAGCATAGACTTTATGAACAGTGCTG--GGACTGTCGCCTCA-C-CAAA 189
EF043441        TA---AGGGTAGAGCATAGACTTTATGAACAGTGCTG--GGACTGTCGCCTCA-C-CAAA 189
AGC             TACGTGGGGTAGAGCATAGACGTTAGGAACAGTACTC--GGACTGTCGCCTCA-C-CAA- 196
Andong          TACGTGGGGTAGAGCATAGACGTTAAGAACAGTGCTC--GGACTGTCGCCTCA-C-CAA- 197
H3014           TACGTGGGGTAGAGCATAGACGTTAGGAACAGTACTC--GGACTGTCGCCTCA-C-CAA- 196
HF85            TACGTGGGGTAGAGCATAGACGTTAGGAACAGTACTC--GGACTGTCGCCTCA-C-CAA- 196
NIHHS-KHH05     TACGTGGGGTAGAGCATAGACGTTAAGAACAGTGCTC--GGACTGTCGCCTCA-C-CAA- 195
N-UK76          TACGTGGGGTAGAGCATAGACGTTAGGAACAGTACTC--GGACTGTCGCCTCA-C-CAA- 196
KJ938577        TACGTGGGGTAGAGCATAGACGTTAGGAACAGTGCTC--GGACTGTCGCCTCA-C-CAA- 196
SF52            TA---AGGGTAGAGCATAGACTTTATGAACAGTGCTG--GGACTGTCGCCTCA-C-CAAA 189
SF670           TA---AGGGTAGAGCATAGACTTTATGAACAGTGCTG--GGACTGTCGCCTCA-C-CAAA 189
SF724           TA---AGGGTAGAGCATAGACTTTATGAACAGTGCTG--GGACTGTCGCCTCA-C-CAAA 189
EF488006        TA---AGGGTAGAGCATAGACTTTATGAACAGTGCTG--GGACTGTCGCCTCA-C-CAAA 189
Florida         TAT---GGGTGGCGCATAGACTTAATGAACAGTACTCTCGGACTGTTGCCTCAGC-CAAA 192
J37             TAT---GGGTGGCGCATAGACTTAATGAACAGTACTCTCGGACTGTTGCCTCAGC-CAAA 192
NZH3            TAT---GGGTGGCGCATAGACTTAATGAACAGTACTCTCGGACTGTTGCCTCAGC-CAAA 192
X1              TAT---GGGTGGCGCATAGACTTAATGAACAGTACTCTCGGACTGTTGCCTCAGC-CAAA 192
X03680          TTC----TGCAG----TAGACGTT-------GTTCAC---GA--GTCGTCTCAACACAAC 215
                *       *  *    ***** *        ** *     **  **   **** * ***

PUR1            ---CCGT-CGATTACTGGTGGCTATG-A-GT---GACTTCGGTCAC-TCG---AGATCT- 234
PUR2            ---CCGT-CGATTACTGGTGGCTATG-A-GT---GACTTCGGTCAC-TCG---AGATCT- 234
PUR3            ---CCGT-CGATTACTGGTGGCTATG-A-GT---GACTTCGGTCAC-TCG---AGATCT- 234
YEL1            ---CCGT-CGATTACTGGTGGCTATG-A-GT---GACTTCGGTCAC-TCG---AGATCT- 234
YEL2            ---CCGT-CGATTACTGGTGGCTATG-A-GT---GACTTCGGTCAC-TCG---AGATCT- 234
YEL3            ---CCGT-CGATTACTGGTGGCTATG-A-GT---GACTTCGGTCAC-TCG---AGATCT- 234
D099            AACTCAT-CGATAACTGGTGGCTATG-ATGT---GACTTCGGTCACATCA---AGATCT- 240
H3107           ---CCGT-CGATTACTGGTGGCTATG-A-GT---GACTTCGGTCAC-TCG---AGATCT- 234
H3173           ---CCGT-CGATTACTGGTGGCTATG-A-GT---GACTTCGGTCAC-TCG---AGATCT- 234
AY321482        ---CCGT-CGATTACTGGTGGCTATG-A-GT---GACTTCGGTCAC-TCG---AGATCT- 234
K122            ---CCGT-CGATTACTGGTGGCTATG-A-GT---GACTTCGGTCAC-TCG---AGATCT- 234
OH10            AACTCAT-CGATAACTGGTGGCTATG-ATGT---GACTTCGGTCGCATCG---AGATCC- 240
EF043441        AACTCAT-CGATAACTGGTGGCTATG-ATGT---GACTTCGGTCGCATCG---AGATCC- 240
AGC             ---CCGT-CGATAACTGGTGGCTATG-A-GT---GACTTCGGTCAC-TCG---AGATCT- 242
Andong          ---CCGT-CGATTACTGGTGGCTATG-A-GT---GACTTCGGTCAC-TCG---AGATCT- 243
H3014           ---CCGT-CGATAACTGGTGGCTATG-A-GT---GACTTCGGTCAC-TCG---AGATCT- 242
HF85            ---CCGT-CGATTACTGGTGGCTATG-A-GT---GACTTCGGTCAC-TCG---AGATCT- 242
NIHHS-KHH05     ---CCGT-CGATTACTGGTGGCTATG-A-GT---GACTTCGGTCAC-TCG---AGATCT- 241
N-UK76          ---CCGT-CGATAACTGGTGGCTATG-A-GT---GACTTCGGTCAC-TCG---AGATCT- 242
KJ938577        ---CCGT-CGATTACTGGTGGCTATG-A-GT---GACTTCGGTCAC-TCG---AGATCT- 242
SF52            AACTCAT-CGATAACTGGTGGCTATG-GTGT---GACTTCGGTCACATCA---AGATCT- 240
SF670           AACTCAT-CGATAACTGGTGGCTATG-GTGT---GACTTCGGTCACATCA---AGATCT- 240
SF724           AACTCAT-CGATAACTGGTGGCTATG-GTGT---GACTTCGGTCACATCA---AGATCT- 240
EF488006        AACTCAT-CGATAACTGGTGGCTATG-GTGT---GACTTCGGTCACATCA---AGATCT- 240
Florida         ---CTAT-CGTGGACCGGTGGCTGTG-ATGT---GACTTCGGTCACATCG---AGATCC- 240
J37             ---CTAT-CGTGGACCGGTGGCTGTG-ATGT---GACTTCGGTCACATCG---AGATCC- 240
NZH3            ---CTAT-CGTGGACCGGTGGCTGTG-ATGT---GACTTCGGTCACATCG---AGATCC- 240
X1              ---CTAT-CGTGGACCGGTGGCTGTG-ATGT---GACTTCGGCCACATCG---AGATCC- 240
X03680          CG-CTATGTGTCTCCTGGTGGCTATATGCGTCTAGGCTTCTTCTTCTTTGCGGAGAGGAA 274
                      *  *    * ******* *    **   * ****     * *     ***
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PUR1            GCTA--CGCAGAGAGCCTCAATGA-----GTT--GT--TCGTCCAACC-----GCCGGTG 278
PUR2            GCTA--CGCAGAGAGCCTCAATGA-----GTT--GT--TCGTCCAACC-----GCCGGTG 278
PUR3            GCTA--CGCAGAGAGCCTCAATGA-----GTT--GT--TCGTCCAACC-----GCCGGTG 278
YEL1            GCTA--CGCAGAGAGCCTCAATGA-----GTT--GT--TCGTCCAACC-----GCCGGTG 278
YEL2            GCTA--CGCAGAGAGCCTCAATGA-----GTT--GT--TCGTCCAACC-----GCCGGTG 278
YEL3            GCTA--CGCAGAGAGCCTCAATGA-----GTT--GT--TCGTCCAACC-----GCCGGTG 278
D099            GCTA--TGCAGAGAGCCTCAATGA-----GTT--GT--TTGTTCAACC-----GCCGGTG 284
H3107           GCTA--CGCAGAGAGCCTCAATGA-----GTT--GT--TCGTCCAACC-----GCCGGTG 278
H3173           GCTA--CGCAGAGAGCCTCAATGA-----GTT--GT--TCGTCCAACC-----GCCGGTG 278
AY321482        GCTA--CGCAGAGAGCCTCAATGA-----GTT--GT--TCGTCCAACC-----GCCGGTG 278
K122            GCTA--CGCAGAGAGCCTCAATGA-----GTT--GT--TCGTCCAACC-----GCCGGTG 278
OH10            GCTT--TGCGGCTAGCCTCAATGA-----GTT--GT--TTGCTCAACC-----GCCGGTG 284
EF043441        GCTT--TGCGGCTAGCCTCAATGA-----GTT--GT--TTGCTCAACC-----GCCGGTG 284
AGC             GCTA--TGCAGAGAGTCTCAATGA-----GTT--GTGTTCGTCCAACC-----GCCGGTG 288
Andong          GCTA--TGCAGAGAGTCTCAATGA-----GTT--GT--TCGTCCAACC-----GCCGGTG 287
H3014           GCTA--TGCAGAGAGTCTCAATGA-----GTT--GTGTTCGTCCAACC-----GCCGGTG 288
HF85            GCTA--TGCAGAGAGTCTCAATGA-----GTT--GTGTTCGTCCAACC-----GCCGGTG 288
NIHHS-KHH05     GCTA--TGCAGAGAGTCTCAATGA-----GTT--GT--TCGTCCAACC-----GCCGGTG 285
N-UK76          GCTA--TGCAGAGAGTCTCAATGA-----GTT--GTGTTCGTCCAACC-----GCCGGTG 288
KJ938577        GCTA--TGCAGAGAGTCTCAATGA-----GTT--GTGTTCGTCCAACC-----GCCGGTG 288
SF52            GCTA--TGCAGAGAGCCTCAATGA-----GTT--GT--TTGTTCAACC-----GCCGGTG 284
SF670           GCTA--TGCAGAGAGCCTCAATGA-----GTT--GT--TTGTTCAACC-----GCCGGTG 284
SF724           GCTA--TGCAGAGAGCCTCAATGA-----GTT--GT--TTGTTCAACC-----GCCGGTG 284
EF488006        GCTA--TGCAGAGAGCCTCAATGA-----GTT--GT--TTGTTCAACC-----GCCGGTG 284
Florida         GCTA--TGCGGAGAGCCTCAATGA-----GTT--GTTCC-GTCAA-CC-----GCC--TT 282
J37             GCTA--TGCGGAGAGCCTCAATGA-----GTT--GTTCC-GTCAA-CC-----GCC--TT 282
NZH3            GCTA--TGCGGAGAGCCTCAATGA-----GTT--GTTCC-GTCAA-CC-----GCC--TT 282
X1              GTTT--TGCGGAGAGCCTCAATGA-----GTT--GTTCCCGTCAAACC-----GCC--TT 284
X03680          GTTAGTTGACGATAGTCTTGATGATCGTGGTTCGGTTTCGGCCGAGCCAAGGCGCCAAAA 334
                * *    *  *  ** **  ****     ***  **    *   * **     ***

PUR1            TCG-ATAG-AAATTTTT-------TCCTATTA----ACTTGTT--------TCTTG---- 313
PUR2            TCG-ATAG-AAATTTTT-------TCCTATTA----ACTTGTT--------TCTTG---- 313
PUR3            TCG-ATAG-AAATTTTT-------TCCTATTA----ACTTGTT--------TCTTG---- 313
YEL1            TCG-ATAG-AAATTTTT-------TCCTATTA----ACTTGTT--------TCTTG---- 313
YEL2            TCG-ATAG-AAATTTTT-------TCCTATTA----ACTTGTT--------TCTTG---- 313
YEL3            TCG-ATAG-AAATTTTT-------TCCTATTA----ACTTGTT--------TCTTG---- 313
D099            TCG-ATA--AATCTTAT-------CTCAATTA----ACTTGTT--------TCTTG---- 318
H3107           TCG-ATAG-AAATTTTT-------TCCTATTA----ACTTGTT--------TCTTG---- 313
H3173           TCG-ATAG-AAATTTTT-------TCCTATTA----ACTTGTT--------TCTTG---- 313
AY321482        TCG-ATAG-AAATTTTT-------TCCTATTA----ACTTGTT--------TCTTG---- 313
K122            TCG-ATAG-AAATTTTT-------TCCTATTA----ACTTGTT--------TCTTG---- 313
OH10            TCG-ATA--AATCTTAT-------CTCAATTA----ACTTGTT--------TCTTG---- 318
EF043441        TCG-ATA--AATCTTAT-------CTCAATTA----ACTTGTT--------TCTTG---- 318
AGC             TCG-ATAG-AAATTTTT-------TCCTATTA----ACTTGTT--------TCCTG---- 323
Andong          TCG-ATAG-AAATTTTT-------TCCTATTA----ACTTGTT--------TCCTG---- 322
H3014           TCG-ATAG-AAATTTTT-------TCCTATTA----ACTTGTT--------TCCTG---- 323
HF85            TCG-ATAG-AAATTTTT-------TCCTATTA----ACTTGTT--------TCCTG---- 323
NIHHS-KHH05     TCG-ATAG-AAATTTTT-------TCCTATTA----ACTTGTT--------TCCTG---- 320
N-UK76          TCG-ATAG-AAATTTTT-------TCCTATCA----ACTTGTT--------TCCTG---- 323
KJ938577        TCG-ATAG-AAATTTTT-------TCCTATTA----ACTTGTT--------TCCTG---- 323
SF52            TCG-ATA--AATCTTAT-------CTCAATTA----ACTTGTT--------TCTTG---- 318
SF670           TCG-ATA--AATCTTAT-------CTCAATTA----ACTTGTT--------TCTTG---- 318
SF724           TCG-ATA--AATCTTAT-------CTCAATTA----ACTTGTT--------TCTTG---- 318
EF488006        TCG-ATA--AATCTTAT-------CTCAATTA----ACTTGTT--------TCTTG---- 318
Florida         GCG-ATATTTGATTTTTGGGTACGTATCGTTA----TCTTGTC--------TCTTGG--- 326
J37             GCG-ATATTTGATTTTTGGGTACGTATCGTTA----TCTTGTC--------TCTTGG--- 326
NZH3            GCG-ATATTTGATTTTTGGGTACGTATCGTTA----TCTTGTC--------TCTTGG--- 326
X1              GCG-ATATTTGATTTTTGGGTACGTATCGTTA----TCTTGTC--------TCTTGG--- 328
X03680          ACACATAAGCACCTTTTCATAT--TTGAATCAGTACACTGATTGCCAAAAGTCTTCACGG 392
                 *  ***      ** *           * *     **  *         ** *

PUR1            ----------ATTCGT-GTTAA----TACATTTTGGCACAATGTATTAGCTTCAGCGATG 358
PUR2            ----------ATTCGT-GTTAA----TACATTTTGGCACAATGTATTAGCTTCAGCGATG 358
PUR3            ----------ATTCGT-GTTAA----TACATTTTGGCACAATGTATTAGCTTCAGCGATG 358
YEL1            ----------ATTCGT-GTTAA----TACATTTTGGCACAATGTATTAGCTTCAGCGATG 358
YEL2            ----------ATTCGT-GTTAA----TACATTTTGGCACAATGTATTAGCTTCAGCGATG 358
YEL3            ----------ATTCGT-GTTAA----TACATTTTGGCACAATGTATTAGCTTCAGCGATG 358
D099            ----------AATCGT-GTTAA----TACATTTTGGCACAATGTATTAGCTTCAGCGATG 363
H3107           ----------ATTCGT-GTTAA----TACATTTTGGCACAATGTATTAGCTTCAGCGATG 358
H3173           ----------ATTCGT-GTTAA----TACATTTTGGCACAATGTATTAGCTTCAGCGATG 358
AY321482        ----------ATTCGT-GTTAA----TACATTTTGGCACAATGTATTAGCTTCAGCGATG 358
K122            ----------ATTCGT-GTTAA----TACATTTTGGCACAATGTATTAGCTTCAGCGATG 358
OH10            ----------ATTCGT-GTTAA----TACATTTTGGCACAATGTATTAGCTTCAGCGATG 363
EF043441        ----------ATTCGT-GTTAA----TACATTTTGGCACAATGTATTAGCTTCAGCGATG 363
AGC             ----------ATTCGT-GTTAA----TACATTTTGGCACAATGTATTAGCTTCAGCGATG 368
Andong          ----------ATTCGT-GTTAA----TACATTTTGGCACAATGTATTAGCTTCAGCGATG 367
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H3014           ----------ATTCGT-GTTAA----TACATTTTGGCACAATGTATTAGCTTCAGCGATG 368
HF85            ----------ATTCGT-GTTAA----TACATTTTGGCACAATGTGTTAGCTTCAGCGATG 368
NIHHS-KHH05     ----------ATTCGT-GTTAA----TACATTTTGGCACAATGTATTAGCTTCAGCGATG 365
N-UK76          ----------ATTCGT-GTTAA----TACATTTTGGCACAATGTATTAGCTTCAGCGATG 368
KJ938577        ----------ATTCGT-GTTAA----TACATTTTGGCACAATGTATTAGCTTCAGCGATG 368
SF52            ----------ATTCGT-GTTAA----TACATTTTGGCACAATGTATTAGCTTCAGCGATG 363
SF670           ----------ATTCGT-GTTAA----TACATTTTGGCACAATGTATTAGCTTCAGCGATG 363
SF724           ----------ATTCGT-GTTAA----TACATTTTGGCACAATGTATTAGCTTCAGCGATG 363
EF488006        ----------ATTCGT-GTTAA----TACATTTTGGCACAATGTATTAGCTTCAGCGATG 363
Florida         ----------ATACGT-GTTAA----TACATTTTGGCACAATGTATTAGCTTCAGCGATG 371
J37             ----------ATACGT-GTTAA----TACATTTTGGCACAATGTATTAGCTTCAGCGATG 371
NZH3            ----------ATACGT-GTTAA----TACATTTTGGCACAATGTATTAGCTTCAGCGATG 371
X1              ----------ATACGT-GTTAA----TACATTTTGGCACAATGTATTAGCTTCAGCGATG 373
X03680          ACATGCGGTGATTTGTTGTTGAGATTTGCATCTCGATAATACGTACTAGCTTCAGCGATG 452
                          *   ** *** *    * *** * *  *  * **  **************

PUR1            GATCGGTTGATTCGCGTATCGATGAAAAACGCAGCAAGCTGCGTTATTTACCACGAATTG 418
PUR2            GATCGGTTGATTCGCGTATCGATGAAAAACGCAGCAAGCTGCGTTATTTACCACGAATTG 418
PUR3            GATCGGTTGATTCGCGTATCGATGAAAAACGCAGCAAGCTGCGTTATTTACCACGAATTG 418
YEL1            GATCGGTTGATTCGCGTATCGATGAAAAACGCAGCAAGCTGCGTTATTTACCACGAATTG 418
YEL2            GATCGGTTGATTCGCGTATCGATGAAAAACGCAGCAAGCTGCGTTATTTACCACGAATTG 418
YEL3            GATCGGTTGATTCGCGTATCGATGAAAAACGCAGCAAGCTGCGTTATTTACCACGAATTG 418
D099            GATCGGTTGATTCGCGTATCGATGAAAAACGCAGCAAGCTGCGTTATTTACCACGAATTG 423
H3107           GATCGGTTGATTCGCGTATCGATGAAAAACGCAGCAAGCTGCGTTATTTACCACGAATTG 418
H3173           GATCGGTTGATTCGCGTATCGATGAAAAACGCAGCAAGCTGCGTTATTTACCACGAATTG 418
AY321482        GATCGGTTGATTCGCGTATCGATGAAAAACGCAGCAAGCTGCGTTATTTACCACGAATTG 418
K122            GATCGGTTGATTCGCGTATCGATGAAAAACGCAGCAAGCTGCGTTATTTACCACGAATTG 418
OH10            GATCGGTTGATTCGCGTATCGATGAAAAACGCAGCAAGCTGCGTTATTTACCACGAATTG 423
EF043441        GATCGGTTGATTCGCGTATCGATGAAAAACGCAGCAAGCTGCGTTATTTACCACGAATTG 423
AGC             GATCGGTTGATTCGCGTATCGATGAAAAACGCAGCAAGCTGCGTTATTTACCACGAATTG 428
Andong          GATCGGTAGATTCGCGTATCGATGAAAAACGCAGCAAGCTGCGTTATTTACCACGAATTG 427
H3014           GATCGGTTGATTCGCGTATCGATGAAAAACGCAGCAAGCTGCGTTATTTACCACGAATTG 428
HF85            GATCGGTTGATTCGCGTATCGATGAAAAACGCAGCAAGCTGCGTTATTTACCACGAATTG 428
NIHHS-KHH05     GATCGGTTGATTCGCGTATCGATGAAAAACGCAGCAAGCTGCGTTATTTACCACGAATTG 425
N-UK76          GATCGGTTGATTCGCGTATCGATGAAAAACGCAGCAAGCTGCGTTATTTACCACGAATTG 428
KJ938577        GATCGGTTGATTCGCGTATCGATGAAAAACGCAGCAAGCTGCGTTATTTACCACGAATTG 428
SF52            GATCGGTTGATTCGCGTATCGATGAAAAACGCAGCAAGCTGCGTTATTTACCACGAATTG 423
SF670           GATCGGTTGATTCGCGTATCGATGAAAAACGCAGCAAGCTGCGTTATTTACCACGAATTG 423
SF724           GATCGGTTGATTCGCGTATCGATGAAAAACGCAGCAAGCTGCGTTATTTACCACGAATTG 423
EF488006        GATCGGTTGATTCGCGTATCGATGAAAAACGCAGCAAGCTGCGTTATTTACCACGAATTG 423
Florida         GATCGGTTGATTCGCGTATCGATGAAAAACGCAGCTAGCTGCGTTATTTACCACGAATTG 431
J37             GATCGGTTGATTCGCGTATCGATGAAAAACGCAGCTAGCTGCGTTATTTACCACGAATTG 431
NZH3            GATCGGTTGATTCGCGTATCGATGAAAAACGCAGCTAGCTGCGTTATTTACCACGAATTG 431
X1              GATCGGTTGATTCGCGTATCGATGAAAAACGCAGCTAGCTGCGTTATTTACCACGAATTG 433
X03680          GATCGGTTGCATCGAGTATCGATGAAGAACGCAGCTTGCTGCGTTACTTACCACGAATTG 512
                ******* *  *** *********** ********  ********* *************

PUR1            CAGACGCTTAGGGTTGGTGAAGTTTTGAACGCACAGCGCCGTTGGGTTTTCCCTTCGGCA 478
PUR2            CAGACGCTTAGAGT-GGTGAAGTTTTGAACGCACAGCGCCGTTGGGTTTTCCCTTCGGCA 477
PUR3            CAGACGCTTAGAGT-GGTGAAGTTTTGAACGCACAGCGCCGTTGGGTTTTCCCTTCGGCA 477
YEL1            CAGACGCTTAGAGT-GGTGAAGTTTTGAACGCACAGCGCCGTTGGGTTTTCCCTTCGGCA 477
YEL2            CTGACGCTTAGAGT-GGTGAAGTTTTGAACGCACAGCGCCGTTGGGTTTTCCCTTCGGCA 477
YEL3            CAGACGCTTAGGGT-GGTGAAGTTTTGAACGCACAGCGCCGTTGGGTTTTCCCTTCGGCA 477
D099            CAGACGCTTAGAGT-GGTGAAGTTTTGAACGCACAGCGCCGTTGGGTTTTCCCTTCGGCA 482
H3107           CAGACGCTTAGAGT-GGTGAAGTTTTGAACGCACAGCGCCGTTGGGTTTTCCCTTCGGCA 477
H3173           CAGACGCTTAGAGT-GGTGAAGTTTTGAACGCACAGCGCCGTTGGGTTTTCCCTTCGGCA 477
AY321482        CAGACGCTTAGAGT-GGTGAAGTTTTGAACGCACAGCGCCGTTGGGTTTTCCCTTCGGCA 477
K122            CAGACGCTTAGAGT-GGTGAAGTTTTGAACGCACAGCGCCGTTGGGTTTTCCCTTCGGCA 477
OH10            CAGACGCTTAGAGT-GGTGAAGTTTTGAACGCACAGCGCCGTTGGGTTTTCCCTTCGGCA 482
EF043441        CAGACGCTTAGAGT-GGTGAAGTTTTGAACGCACAGCGCCGTTGGGTTTTCCCTTCGGCA 482
AGC             CAGACGCTTAGAGT-GGTGAAGTTTTGAACGCACAGCGCCGTTGGGTTTTCCCTTCGGCA 487
Andong          CAGACGCTTAGAGT-GGTGAAGTTTTGAACGCACAGCGCCGTCGGGTTTTCCCTTCGGCA 486
H3014           CAGACGCTTAGAGT-GGTGAAGTTTTGAACGCACAGCGCCGTTGGGTTTCCCCTTCGGCA 487
HF85            CAGACGCTTAGAGT-GGTGAAGTTTTGAACGCACAGCGCCGTTGGGTTTTCCCTTCGGCA 487
NIHHS-KHH05     CAGACGCTTAGAGT-GGTGAAGTTTTGAACGCACAGCGCCGTCGGGTTTTCCCTTCGGCA 484
N-UK76          CAGACGCTTAGAGT-GGTGAAGTTTTGAACGCACAGCGCCGTTGGGTTTTCCCTTCGGCA 487
KJ938577        CAGACGCTTAGAGT-GGTGAAGTTTTGAACGCACAGCGCCGTTGGGTTTTCCCTTCGGCA 487
SF52            CAGACGCTTAGAGT-GGTGAAGTTTTGAACGCACAGCGCCGTTGGGTTTTCCCTTCGGCA 482
SF670           CAGACGCTTAGAGT-GGTGAAGTTTTGAACGCACAGCGCCGTTGGGTTTTCCCTTCGGCA 482
SF724           CAGACGCTTAGAGT-GGTGAAGTTTTGAACGCACAGCGCCGTTGGGTTTTCCCTTCGGCA 482
EF488006        CAGACGCTTAAAGT-GGTGAAGTTTTGAACGCACAGGGCCGTTGGGTTTTCCCTTCGGCA 482
Florida         CAGACGCTTAGAGT-GGTAAAGTTTTGAACGCACAGCGCCGTTGGGTTTTCCCTTCGGCA 490
J37             CAGACGCTTAGAGT-GGTGAAGTTTTGAACGCACAGCGCCGTTGGGTTTTCCCTTCGGCA 490
NZH3            CAGACGCTTAGAGT-GGTAAAGTTTTGAACGCACAGCGCCGTTGGGTTTTCCCTTCGGCA 490
X1              CAGACGCTTAGAGT-GGTGAAGTTTTGAACGCACAGCGCCGTTGGGTTTTCCCTTCGGCA 492
X03680          CAGACGCTTAGAGT-GGTGAAATTTCGAACGCATAGCACCAACTGGGCCTCCAGTTGGTA 571
                * ********  ** *** ** *** ******* **  **    **    **  * ** *
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PUR1            CGTCTGGCTCAGGGTTGTTTA----AAGTACTGCGGTGTTGCTGCG-AAAGTAGCAACAC 533
PUR2            CGTCTGGCTCAGGGTTGTTTA----AAGTACTGCGGTGTTGCTGCG-AAAGTAGCAACAC 532
PUR3            CGTCTGGCTCAGGGTTGTTTA----AAGTACTGCGGTGTTGCTGCG-AAAGTAGCAACAC 532
YEL1            CGTCTGGCTCAGGGTTGTTTA----AAGTACTGCGGTGTTGCTGCG-AAAGTAGCAACAC 532
YEL2            CGTCTGGCTCAGGGTTGTTTA----AAGTACTGCGGTGTTGCTGCG-AAAGTAGCAACAC 532
YEL3            CGTCTGGCTCAGGGTTGTTTA----AAGTACTGCGGTGTTGCTGCG-AAAGTAGCAACAC 532
D099            CGTCTGGCTCAGGGTTGTTTG----AAGTACTACGGTGTTGCTGCG-AAAGTAGCAACAT 537
H3107           CGTCTGGCTCAGGGTTGTTTA----AAGTACTGCGGTGTTGCTGCA-AAAGTAGCAACAC 532
H3173           CGTCTGGCTCAGGGTTGTTTA----AAGTACTGCGGTGTTGCTGCG-AAAGTAGCAACAC 532
AY321482        CGTCTGGCTCAGGGTTGTTTA----AAGTACTGCGGTGTTGCTGCG-AAAGTAGCAACAC 532
K122            CGTCTGGCTCAGGGTTGTTTA----AAGTACTGCGGTGTTGCTGCG-AAAGTAGCAACAC 532
OH10            CGTCTGGCTCAGGGTTGTTTA----AAGTACTGCGGTGTTGCTGCG-AAAGTGGCAACAC 537
EF043441        CGTCTGGCTCAGGGTTGTTTA----AAGTACTGCGGTGTTGCTGCG-AAAGTGGCAACAC 537
AGC             CGTCTGGCTCAGGGTTGTATATA--GAGTACTGCGGTGTTGCTCCG-AGAGTAGCAATGT 544
Andong          CGTCTGGCTCAGGGTTGTTTA----GAGTACTGCGGTGTTGCTGCG-AAAGTAGCAATGT 541
H3014           CGTCTGGCTCAGGGTTGTCTATA--GAGTACTGCGGTGTTGCTCCG-AGAGTAGCAATGT 544
HF85            CGTCTGGCTCAGGGTTGTTTATATAGAGTACTGCGGTGTTGCTCCG-AGAGTAGCAATGT 546
NIHHS-KHH05     CGTCTGGCTCAGGGTTGTTTA----GAGTACTGCGGTGTTGCTGCG-AAAGTAGCAATGT 539
N-UK76          CGTCTGGCTCAGGGTTGTCTATA--GAGTACTGCGGTGTTGCTCCG-AGAGTAGCAATGT 544
KJ938577        CGTCTGGCTCAGGGTTGTTTATATAGAGTACTGCGGTGTTGCTCCG-AGAGTAGCAATGT 546
SF52            CGTCTGGCTCAGGGTTGTTTA----AAGTACTGCGGTGTTGCTGCG-AAAGTAGCATCAT 537
SF670           CGTCTGGCTCAGGGTTGTTTA----AAGTACTGCGGTGTTGCTGCG-AAAGTAGCATCAT 537
SF724           CGTCTGGCTCAGGGTTGTTTA----AAGTACTGCGGTGTTGCTGCG-AAAGTAGCATCAT 537
EF488006        CGTTTGGGTCAGGGTTGTTTA----AAGTACTGCGGTGTTGCTGCG-AAAGTAGCATCAT 537
Florida         CGTCTGGCTCAGGGTTGCTTA----AAGTACTGCGGTGTTGCTGCG-AAAGTGGCAACGC 545
J37             CGTCTGGCTCAGGGTTGCTTA----AAGTACTGCGGTGTTGCTGCG-AAAGTGGCAACGC 545
NZH3            CGTCTGGCTCAGGGTTGCTTA----AAGTACTGCGGTGTTGCTGCG-AAAGTGGCAACGC 545
X1              CGTCTGGCTCAGGGTTGCTTA----AAGTACTGCGGTGTTGCTGCG-AAAGTGGCAACGC 547
X03680          CGTCTGGTTCAGGGTTGTTTAACTCAATGCCTTAGGCTTCTCTTCGGAGAGT-----CTT 626
                *** *** *********  *      *   **  **  *  ** *  * ***

PUR1            CGA-ATGTCGAACGGCGTTAGTG--------TT-AC---------TACTGGCCCCGTTCT 574
PUR2            CGA-ATGTCGAACGGCGTTAGTG--------TT-AC---------TACTGGCCCCGTTCT 573
PUR3            CGA-ATGTCGAACGGCGTTAGTG--------TT-AC---------TACTGGCCCCGTTCT 573
YEL1            CGA-ATGTCGAACGGCGTTAGTG--------TT-AC---------TACTGGCCCCGTTCT 573
YEL2            CGA-ATGTCGAACGGCGTTAGTG--------TT-AC---------TACTGGCCCCGTTCT 573
YEL3            CGA-ATGTCGAACGGCGTTAGTG--------TT-AC---------TACTGGCCCCGTTCT 573
D099            CGA-ATGTCGAGCGGCGTTAGTG--------TT-AC---------TACTGGCCCCGTTCT 578
H3107           CGA-ATGTCGAACGGCGTTAGTG--------TT-AC---------TACTGGCCCCGTTCT 573
H3173           CGA-ATGTCGAACGGCGTTAGTG--------TT-AC---------TACTGGCCCCGTTCT 573
AY321482        CGA-ATGTCGAACGGCGTTAGTG--------TT-AC---------TACTGGCCCCGTTCT 573
K122            CGA-ATGTCGAACGGCGTTAGTG--------TT-AC---------TACTGGCCCCGTTCT 573
OH10            CGA-TTGTCGAACGGCGTTAGTG--------TT-AC---------TACTGGCCCCGTTCT 578
EF043441        CGA-TTGTCGAACGGCGTTAGTG--------TT-AC---------TACTGGCCCCGTTCT 578
AGC             CGA-GTGTCGAACGGCGTTAGTG--------TTGGT---------TACTGGCCCCGTTCT 586
Andong          CGA-GTGTCGAACGGCGTTAGTG--------TTTGC---------TACTGGCCCCGTTCT 583
H3014           CGA-GTGTCGAACGGCGTTAGTG--------TTGGT---------TACTGGCCCCGTTCT 586
HF85            CGA-GTGTCGAACGGCGTTAGTG--------TTGGC---------TACTGGCCCCGTTCT 588
NIHHS-KHH05     CGA-GTGTCGAACGGCGTTAGTG--------TTTGC---------TACTGGCCCCGTTCT 581
N-UK76          CGA-GTGTCGAACGGCGTTAGTG--------TTGGT---------TACTGGCCCCGTTCT 586
KJ938577        CGA-GTGTCGAACGGCGTTAGTG--------TTGGC---------TACTGGCCCCGTTCT 588
SF52            CGA-ATGTCGAACGGCGTTAGTG--------TT-AC---------TACTGGCCCCGTTCT 578
SF670           CGA-ATGTCGAACGGCGTTAGTG--------TT-AC---------TACTGGCCCCGTTCT 578
SF724           CGA-ATGTCGAACGGCGTTAGTG--------TT-AC---------TACTGGCCCCGTTCT 578
EF488006        CGA-ATGTCGAACGGCGTTAGTG--------TT-AC---------TACTGGCCCCGTTCT 578
Florida         CGA-ATGTCGAGCGGCGTTAGTG--------TT-AC---------TACTGGCCCCGTTCT 586
J37             CGA-ATGTCGAGCGGCGTTAGTG--------TT-AC---------TACTGGCCCCGTTCT 586
NZH3            CGA-ATGTCGAGCGGCGTTAGTG--------TT-AC---------TACTGGCCCCGTTCT 586
X1              CGA-ATGTCGAGCGGCGTTAGTG--------TT-AC---------TACTGGCCCCGTTCT 588
X03680          CGGCTTGTCGGGCAACATTAGTGAGCTGAGATTCGCGTCTCGGCATACTGATCTTGTCCT 686
                **   *****  *  * ******        **           *****  *  ** **

PUR1            AGTTCCGAA---------------------------------------------------- 583
PUR2            AGTTCCGAA---------------------------------------------------- 582
PUR3            AGTTCCGAA---------------------------------------------------- 582
YEL1            AGTTCCGAA---------------------------------------------------- 582
YEL2            AGTTCCGAA---------------------------------------------------- 582
YEL3            AGTTCCGAA---------------------------------------------------- 582
D099            AGTTCCGAA---------------------------------------------------- 587
H3107           AGTTCCGAA---------------------------------------------------- 582
H3173           AGTTCCGAA---------------------------------------------------- 582
AY321482        AGTTCCGAA---------------------------------------------------- 582
K122            AGTTCCGAA---------------------------------------------------- 582
OH10            AGTTCCGAA---------------------------------------------------- 587
EF043441        AGTTCCGAA---------------------------------------------------- 587
AGC             AGTTCCGAA---------------------------------------------------- 595
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Andong           AGTTCCGAA---------------------------------------------------- 592
H3014            AGTTCCGAA---------------------------------------------------- 595
HF85             AGTTCCGAA---------------------------------------------------- 597
NIHHS-KHH05      AGTTCCGAA---------------------------------------------------- 590
N-UK76           AGTTCCGAA---------------------------------------------------- 595
KJ938577         AGTTCCGAA---------------------------------------------------- 597
SF52             AGTTCCGAA---------------------------------------------------- 587
SF670            AGTTCCGAA---------------------------------------------------- 587
SF724            AGTTCCGAA---------------------------------------------------- 587
EF488006         AGTTCCGAA---------------------------------------------------- 587
Florida          AGTTCCGAA---------------------------------------------------- 595
J37              AGTTCCGAA---------------------------------------------------- 595
NZH3             AGTTCCGAA---------------------------------------------------- 595
X1               AGTTCCGAA---------------------------------------------------- 597
X03680           AGTCCAGAAGCATCACAAGTCAAGACAGAGCTTCACCGTTCTGTCTTGTAATGTGACGCG 746
                 *** * ***
PUR1             ---------TATTGG--------CAACATGTCTTC-----T-----------TCGTGAAG 610
PUR2             ---------TATTGG--------CAACATGTCTTC-----T-----------TCGTGAAG 609
PUR3             ---------TATTGG--------CAACATGTCTTC-----T-----------TCGTGAAG 609
YEL1             ---------TATTGG--------CAACATGTCTTC-----T-----------TCGTGAAG 609
YEL2             ---------TATTGG--------CAACATGTCTTC-----T-----------TCGTGAAG 609
YEL3             ---------TATTGG--------CAACATGTCTTC-----T-----------TCGTGAAG 609
D099             ---------TATTGG--------CAACATGTCTTC-----T-----------TCGTGAAG 614
H3107            ---------TATTGG--------CAACATGTCTTC-----T-----------TCGTGAAG 609
H3173            ---------TATTGG--------CAACATGTCTTC-----T-----------TCGTGAAG 609
AY321482         ---------TATTGG--------CAACATGTCTTC-----T-----------TCGTGAAG 609
K122             ---------TATTGG--------CAACATGTCTTC-----T-----------ACGTGAAG 609
OH10             ---------TATCGG--------CAACATGTCTTC-----T-----------TCGTGAAG 614
EF043441         ---------TATCGG--------CAACATGTCTTC-----T-----------TCGTGAAG 614
AGC              ---------TATTGG--------CAACATGTCATCACGTGT-----------TCGTGACG 627
Andong           ---------TATTGG--------CAACATGTCGTCACGTCT-----------TCGTGACG 624
H3014            ---------TATTGG--------CAACATGTCATCACGTGT-----------TCGTGACG 627
HF85             ---------TATTGG--------CAACATGTCATCACGTCT-----------TCGTGACG 629
NIHHS-KHH05      ---------TATTGG--------CAACATGTCGTCACGTCT-----------TCGTGACG 622
N-UK76           ---------TATTGG--------CAACATGTCATCACGTGT-----------TCGTGACG 627
KJ938577         ---------TATTGG--------CAACATGTCATCACGTCT-----------TCGTGACG 629
SF52             ---------TATTGG--------CAACATGTCTTC-----T-----------TCGTGAAG 614
SF670            ---------TATTGG--------CAACATGTCTTC-----T-----------TCGTGAAG 614
SF724            ---------TATTGG--------CAACATGTCTTC-----T-----------TCGTGAAG 614
EF488006         ---------TATTGG--------CAACATGTCTTC-----T-----------TCGTGAAG 614
Florida          ---------TATTGG--------CAACATGTCTTC-----T-----------TCGTGAAG 622
J37              ---------TATTGG--------CAACATGTCTTC-----T-----------TCGTGAAG 622
NZH3             ---------TATTGG--------CAACATGTCTTC-----T-----------TCGTGAAG 622
X1               ---------TATTGG--------CAACATGTCTTC-----T-----------TCGTGAAG 624
X03680           CTACTCTTATATCGGAGGAGCTGCCAAATGGCGTCCGACTTGACTCGGCTGATCATCAAG 806
                          *** **        * * *** * **     *            * * * *

PUR1             ACGC---------CGTAG---AGAGTAT-AAGT--CTGTACCTATG----GATGTGTCG- 650
PUR2             ACGC---------CGTAG---AGAGTAT-AAGT--CTGTACCTATG----GATGTGTCG- 649
PUR3             ACGC---------CGTAG---AGAGTAT-AAGT--CTGTACCTATG----GATGTGTCG- 649
YEL1             ACGC---------CGTAG---AGAGTAT-AAGT--CTGTACCTATG----GATGTGTCG- 649
YEL2             ACGC---------CGTAG---AGAGTAT-AAGT--CTGTACCTATG----GATGTGTCG- 649
YEL3             ACGC---------CGTAG---AGAGTAT-AAGT--CTGTACCTATG----GATGTGTCG- 649
D099             ACGC---------CGTGG---AGAGTAT-AAGT--CTGTACCTATG----GATGTGTCG- 654
H3107            ACCC---------CGTAG---AGAGTAT-AAGT--CTGTACCTATG----GATGTGTCG- 649
H3173            ACGC---------CGTAG---AGAGTAT-AAGT--CTGTACCTATG----GATGTGTCG- 649
AY321482         ACGC---------CGTAG---AGAGTAT-AAGT--CTGTACCTATG----GATGTGTCG- 649
K122             ACGC---------CGTAG---AGAGTAT-AAGT--CTGTACCTATG----GATGTGTCG- 649
OH10             ACGC---------CGTGG---AGAGTAT-AAGT--CTGTACCTGTG----GATGTGTCG- 654
EF043441         ACGC---------CGTGG---AGAGTAT-AAGT--CTGTACCTGTG----GATGTGTCG- 654
AGC              ACGC---------CGTAG---AGAGTAT-AGGT--CTGTACCTGTG----GATGTGTAG- 667
Andong           ACGC---------CGTAG---AGAGTAT-AAGT--CTGTACCTGTG----GATGTGTAG- 664
H3014            ACGC---------CGTAG---AGAGTAT-AGGT--CTGTACCTGTG----GATGTGTAG- 667
HF85             ACGC---------CGTAG---AGAGTAT-AAGT--CTGTACCTGTG----GATGTGTAG- 669
NIHHS-KHH05      ACGC---------CGTAG---AGAGTAT-AAGT--CTGTACCTGTG----GATGTGTAG- 662
N-UK76           ACGC---------CGTAG---AGAGTAT-AGGT--CTGTACCTGTG----GATGTGTAG- 667
KJ938577         ACGC---------CGTAG---AGAGTAT-AAGT--CTGTACCTGTG----GATGTGTAG- 669
SF52             ACGC---------CGTGG---AGAGTAT-AAGT--CTGTACCTATG----GATGTGTCG- 654
SF670            ACGC---------CGTGG---AGAGTAT-AAGT--CTGTACCTATG----GATGTGTCG- 654
SF724            ACGC---------CGTGG---AGAGTAT-AAGT--CTGTACCTATG----GATGTGTCG- 654
EF488006         ACGC---------CGTGG---AGAGTAT-AAGT--CTGTACCTATG----GATGTGTCG- 654
Florida          ACGC---------CGTAG---AGAGTAT-AAGT--CTGTACCTATG----GATGTGTCG- 662
J37              ACGC---------CGTAG---AGAGTAT-AAGT--CTGTACCTATG----GATGTGTCG- 662
NZH3             ACGC---------CGTAG---AGAGTAT-AAGT--CTGTACCTATG----GATGTGTCG- 662
X1               ACGC---------CGTAG---AGAGTAT-AAGT--CTGTACCTATG----GATGTGTCG- 664
X03680           ACGTGTTTAACTGCGTGTCTTATGATGTGAAGCTACTGCATCTATGTCAGAATGTGTCGT 866
                 **           ***     *   * * * *   *** * ** **     ****** *
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PUR1            --------------------------------CGTATGAA-----ATAT--GAT--GCT 668
PUR2            --------------------------------CGTATGAA-----ATAT--GAT--GCT 667
PUR3            --------------------------------CGTATGAA-----ATAT--GAT--GCT 667
YEL1            --------------------------------CGTATGAA-----ATAT--GAT--GCT 667
YEL2            --------------------------------CGTATGAA-----ATAT--GAT--GCT 667
YEL3            --------------------------------CGTATGAA-----ATAT--GAT--GCT 667
D099            --------------------------------CGTATGAA-----ATAT--GAT--GCT 672
H3107           --------------------------------CGTATGAA-----ATAT--GAT--GCT 667
H3173           --------------------------------CGTATGAA-----ATAT--GAT--GCT 667
AY321482        --------------------------------CGTATGAA-----ATAT--GAT--GCT 667
K122            --------------------------------CGTATGAA-----ATAT--GAT--GCT 667
OH10            --------------------------------CGTATGAA-----ATAT--GAT--GCT 672
EF043441        --------------------------------CGTATGAA-----ATAT--GAT--GCT 672
AGC             --------------------------------CGTATGAA-----ATAT--GAT--GCT 685
Andong          --------------------------------CGTATGAA-----ATAT--GAT--GCT 682
H3014           --------------------------------CGTATGAA-----ATAT--GAT--GCT 685
HF85            --------------------------------CGTATGAA-----ATAT--GAT--GCT 687
NIHHS-KHH05     --------------------------------CGTATGAA-----ATAT--GAT--GCT 680
N-UK76          --------------------------------CGTATGAG-----ATAT--GAT--GCT 685
KJ938577        --------------------------------CGTATGAA-----ATAT--GAT--GCT 687
SF52            --------------------------------CGTATGAA-----ATAT--GAT--GCT 672
SF670           --------------------------------CGTATGAA-----ATAT--GAT--GCT 672
SF724           --------------------------------CGTATGAA-----ATAT--GAT--GCT 672
EF488006        --------------------------------CGTATGAA-----ATAT--GAT--GCT 672
Florida         --------------------------------CGTATGAA-----ATAT--GAT--GCT 680
J37             --------------------------------CGTATGAA-----ATAT--GAT--GCT 680
NZH3            --------------------------------CGTATGAA-----ATAT--GAT--GCT 680
X1              --------------------------------CGTATGAA-----ATAT--GAT--GCT 682
X03680          CGCTCCTTTCGAATACTGGGATTCGTCTAGTCTCGTGTGTGTGTTGATATCGAATTAATT 926
                                                *** **       ****   **    *

PUR1            TCCCAT--ACATAGCG----------AGGAGGTGCTTCTTATGCTAT-CT 705
PUR2            TCCCAT--ACATAGCG----------AGGAGGTGCTTCTTATGCTAT-CT 704
PUR3            TCCCAT--ACATAGCG----------AGGAGGTGCTTCTTATGCTAT-CT 704
YEL1            TCCCAT--ACATAGCG----------AGGAGGTGCTTCTTATGCTAT-CT 704
YEL2            TCCCAT--ACATAGCG----------AGGAGGTGCTTCTTATGCTAT-CT 704
YEL3            TCCCAT--ACATAGCG----------AGGAGGTGCTTCTTATGCTAT-CT 704
D099            TCCCAT--ACATAGCG----------AGGAGGTGCTTCTTATACT-ATCT 709
H3107           TCCCAT--ACATAGCG----------AGGAGGTGCTTCTTATGGGAAACT 705
H3173           TCCCAT--ACATAGCG----------AGGAGGTGCTTCTTATGGGAAACT 705
AY321482        TCCCAT--ACATAGCG----------AGGAGGTGCTTCTTATGCTAT-CT 704
K122            TCCCAT--ACATAGCG----------AGGAGGTGCTTCTTATGCTAT-CT 704
OH10            TCACAT--ACATGGCG----------AGGAGGTGCTTCTTGTACT-ATCT 709
EF043441        TCACAT--ACATGGCG----------AGGAGGTGCTTCTTGTACT-ATCT 709
AGC             TCCCAT--ACATAGCG----------AGGAGGTGCCTCTTATACGTATCT 723
Andong          TCCCAT--ACATAGCG----------AGGAGGTGCTTCTTATACT-ATCT 719
H3014           TCCCAT--ACATAGCG----------AGGAGGTGCCTCTTATAGG-AACT 722
HF85            TCCCAT--ACATAGCG----------AGGAGGTGCCTCTTATACT-ATCT 724
NIHHS-KHH05     TCCCAT--ACATAGCG----------AGGAGGTGCTTCTTATACT-ATCT 717
N-UK76          TCCCAT--ACATAGCG----------AGGAGGTGCCTCTTATACT-ATCT 722
KJ938577        TCCCAT--ACATAGCG----------AGGAGGTGCCTCTTATACT-ATCT 724
SF52            TCCCAT--ACATAGCG----------AGGAGGTGCTTCTTATACT-ATCT 709
SF670           TCCCAT--ACATAGCG----------AGGAGGTGCTTCTTATACT-ATCT 709
SF724           TCCCAT--ACATAGCG----------AGGAGGTGCTTCTTATACT-ATCT 709
EF488006        TCCCAT--ACATAGCG----------AGGAGGTGCTTCTTATACT-ATCT 709
Florida         TCCCAT--ACATGGCT----------AGGAGGTGCTTCTTATGCT-ATCT 717
J37             TCCCAT--ACATGGCT----------AGGAGGTGCTTCTTATGCT-ATCT 717
NZH3            TCCCAT--ACATGGCT----------AGGAGGTGCTTCTTATGCT-ATCT 717
X1              TCCCAT--ACATGGCT----------AGGAGGTGCTTCTTATGCT-ATCT 719
X03680          TTCGATTGATGCGGCGCTGAGAAGAGAGACGGTGCGTGTCTTGCTAATCT 976
                *   **  *    **          **  ***** * *  *      **
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Figure A. 4: ClustalW Multiple Sequence Alignment of a portion of the ITS region from three PUR and three YEL *Heterorhabditis* isolates from North Bull Island, a selection of *Heterorhabditis* species and 1 outgroup (*C. elegans* strain XO3680). Asterisk (*) denotes conserved sites (see Section 2.3.2 Figure 2.9).

Spaces denote nucleotide polymorphism

A.2 Generation of growth curve for *P. temperata pur1* and *yel3* isolates

Liquid cultures were set up by inoculating single colonies from 36 - 48 hr plate cultures into 50 ml nutrient broth (NB) or MacConkey broth (MB) in a 250 ml flask and growing for 24 - 48 hr in the dark with shaking at 200 rpm at 27 ^{o}C. At a number of time points 3 x 1 ml aliquots were drawn from the flask and the optical density (OD) at 660 nm of liquid cultures was recorded as required using a Shimadzu UV mini 1240 Spectrophotometer (Fischer Scientific). The growth curve (Figure A. 5) was generated by plotting log of absorbance (OD_{660}) vs time.

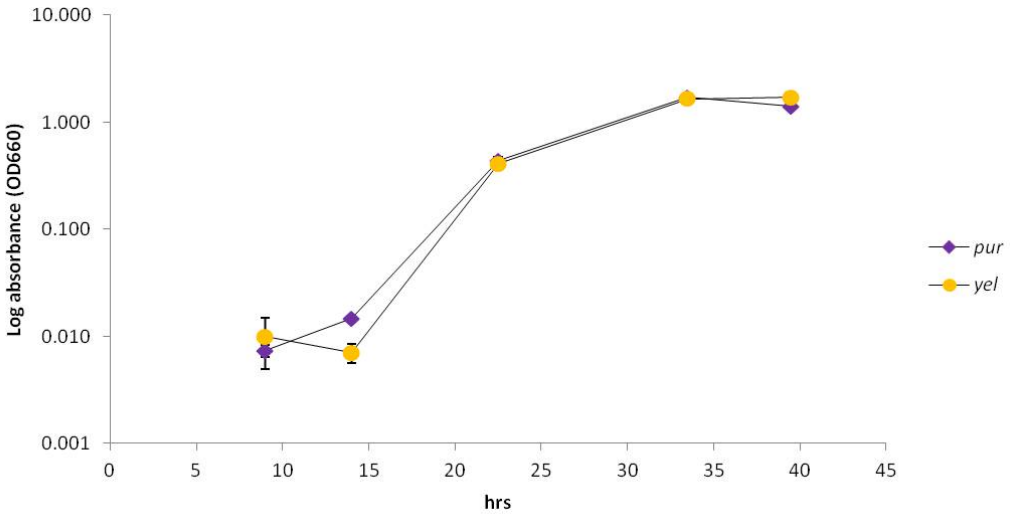

Figure A. 5: Growth curve for *pur1* and *yel3 Photorhabdus temperata* isolates from North Bull Island. Isolates were grown in MacConkey broth (See Section 2.3.3, & Section 3.4).

```
pur1            ATGACTGTGCTGCATGCGGGGGGTAAATTCGATGATAACTCTTATAAAGTCTCCGGCGGT 60
pur2            ATGACTGTGCTGCATGCGGGGGGTAAATTCGATGATAACTCTTATAAAGTCTCCGGCGGT 60
pur3            ATGACTGTGCTGCATGCGGGGGGTAAATTCGATGATAACTCTTATAAAGTCTCCGGCGGT 60
yel1            ATGACTGTGCTGCATGCGGGGGGTAAATTCGATGATAACTCTTATAAAGTTTCCGGCGGT 60
yel2            ATGACTGTGCTGCATGCGGGGGGTAAATTCGATGATAACTCTTATAAAGTTTCCGGCGGT 60
yel3            ATGACTGTGCTGCATGCGGGGGGTAAATTCGATGATAACTCTTATAAAGTTTCCGGCGGT 60
                ************************************************* *********

pur1            CTGCACGGTGTAGGGGTTTCTGTTGTTAACGCCTTGTCTGAAAAGCTGGAACTGATTATC 120
pur2            CTGCACGGTGTAGGGGTTTCTGTTGTTAACGCCTTGTCTGAAAAGCTGGAACTGATTATC 120
pur3            CTGCACGGTGTAGGGGTTTCTGTTGTTAACGCCTTGTCTGAAAAGCTGGAACTGATTATC 120
yel1            TTGCACGGCGTAGGGGTTTCTGTTGTTAACGCCTTGTCTGAAAAGCTGGAACTGGTTATC 120
yel2            TTGCACGGCGTAGGGGTTTCTGTTGTTAACGCCTTGTCTGAAAAGCTGGAACTGGTTATC 120
yel3            TTGCACGGCGTAGGGGTTTCTGTTGTTAACGCCTTGTCTGAAAAGCTGGAACTGGTTATC 120
                 ******* ************************************************* *****

pur1            CGCCGTGACGGCAAAGTTCATGAACAGACTTATCACCTTGGTGTGCCGCAAAGCCCACTA 180
pur2            CGCCGTGACGGCAAAGTTCATGAACAGACTTATCACCTTGGTGTGCCGCAAAGCCCACTA 180
pur3            CGCCGTGACGGCAAAGTTCATGAACAGACTTATCACCTTGGTGTGCCGCAAAGCCCACTA 180
yel1            CGTCGTGACGGCAAAGTTCATGAACAGACTTATCACCTTGGTGTGCCACAAAGCCCACTA 180
yel2            CGTCGTGACGGCAAAGTTCATGAACAGACTTATCACCTTGGTGTGCCACAAAGCCCACTA 180
yel3            CGTCGTGACGGCAAAGTTCATGAACAGACTTATCACCTTGGTGTGCCACAAAGCCCACTA 180
                ** ****************************************** ************

pur1            AAAGTTGTTGGTGAAACCGAACAGACAGGTACCCGTGTCCGCTTCTGGCCAAGCATGGAT 240
pur2            AAAGTTGTTGGTGAAACCGAACAGACAGGTACCCGTGTCCGCTTCTGGCCAAGCATGGAT 240
pur3            AAAGTTGTTGGTGAAACCGAACAGACAGGTACCCGTGTCCGCTTCTGGCCAAGCATGGAT 240
yel1            AAAGTCGTCGGTGAAACGGAGCAGACAGGTACCCGTGTCCGCTTCTGGCCGAGCATGGAC 240
yel2            AAAGTCGTCGGTGAAACGGAGCAGACAGGTACCCGTGTCCGCTTCTGGCCGAGCATGGAC 240
yel3            AAAGTCGTCGGTGAAACGGAGCAGACAGGTACCCGTGTCCGCTTCTGGCCGAGCATGGAC 240
                ********* ******** ** ***************************** ********

pur1            ACTTTCCGTAACAACACTGAATTCCAGCACGACATTCTGGCTAAACGTCTGCGTGAATTG 300
pur2            ACTTTCCGTAACAACACTGAATTCCAGCACGACATTCTGGCTAAACGTCTGCGTGAATTG 300
pur3            ACTTTCCGTAACAACACTGAATTCCAGCACGACATTCTGGCTAAACGTCTGCGTGAATTG 300
yel1            ACTTTCAGTAACAACACTGAATTCCAGCACGACATTCTGGCTAAACGTCTGCGTGAATTG 300
yel2            ACTTTCAGTAACAACACTGAATTCCAGCACGACATTCTGGCTAAACGTCTGCGTGAATTG 300
yel3            ACTTTCAGTAACAACACTGAATTCCAGCACGACATTCTGGCTAAACGTCTGCGTGAATTG 300
                ****** ************************************************

pur1            TCCTTTCTGAACTCAGGTGTATCTATCCGCCTGCTTGATAAACGCACCAATATTGAAGAC 360
pur2            TCCTTTCTGAACTCAGGTGTATCTATCCGCCTGCTTGATAAACGCACCAATATTGAAGAC 360
pur3            TCCTTTCTGAACTCAGGTGTATCTATCCGCCTGCTTGATAAACGCACCAATATTGAAGAC 360
yel1            TCCTTTCTGAACTCAGGTGTATCTATCCGCCTGCTTGATAAACGCACCAATATTGAAGAC 360
yel2            TCCTTTCTGAACTCAGGTGTATCTATCCGCCTGCTTGATAAACGCACCAATATTGAAGAC 360
yel3            TCCTTTCTGAACTCAGGTGTATCTATCCGCCTGCTTGATAAACGCACCAATATTGAAGAC 360
                ************************************************************

pur1            CATTTCCATTATGAAGGTGGTATTAAGGCGTTTGTCGAATTCTTAAATAAAAACAAAACA 420
pur2            CATTTCCATTATGAAGGTGGTATTAAGGCGTTTGTCGAATTCTTAAATAAAAACAAAACA 420
pur3            CATTTCCATTATGAAGGTGGTATTAAGGCGTTTGTCGAATTCTTAAATAAAAACAAAACA 420
yel1            CATTTCCATTACAAAGGTGGCATTAAAGCGTTTGTCGAATTCTTAAATAAAAACAAAACA 420
yel2            CATTTCCATTACAAAGGTGGCATTAAAGCGTTTGTCGAATTCTTAAATAAAAACAAAACA 420
yel3            CATTTCCATTACAAAGGTGGCATTAAAGCGTTTGTCGAATTCTTAAATAAAAACAAAACA 420
                *********** ******* ***** *********************************

pur1            CCAATCCATCCCAATGTTTTCTATTTCTCTACAGAAAAAGATGGCATCGGTGTTGAAGCC 480
pur2            CCAATCCATCCCAATGTTTTCTATTTCTCTACAGAAAAAGATGGCATCGGTGTTGAAGCC 480
pur3            CCAATCCATCCCAATGTTTTCTATTTCTCTACAGAAAAAGATGGCATCGGTGTTGAAGCC 480
yel1            CCAATCCATCCCAATGTTTTCTATTTCTCTACAGAAAAAGATAGCATCGGTGTTGAAGTC 480
yel2            CCAATCCATCCCAATGTTTTCTATTTCTCTACAGAAAAAGATAGCATCGGTGTTGAAGTC 480
yel3            CCAATCCATCCCAATGTTTTCTATTTCTCTACAGAAAAAGATAGCATCGGTGTTGAAGTC 480
                ******************************************* ***************

pur1            GCATTGCAATGGAACGATGGTTTCCAGGAAAATATTTACTGCTTTACCAACAACATCCCG 540
pur2            GCATTGCAATGGAACGATGGTTTCCAGGAAAATATTTACTGCTTTACCAACAACATCCCG 540
pur3            GCATTGCAATGGAACGATGGTTTCCAGGAAAATATTTACTGCTTTACCAACAACATCCCG 540
yel1            GCATTGCAGTGGAATGATGGTTTCCAGGAAAATATTTACTGCTTTACCAACAACATCCCG 540
yel2            GCATTGCAGTGGAATGATGGTTTCCAGGAAAATATTTACTGCTTTACCAACAACATCCCG 540
yel3            GCATTGCAGTGGAATGATGGTTTCCAGGAAAATATTTACTGCTTTACCAACAACATCCCG 540
                *********.***** ********************************************

pur1            CAACGTGACGGCGGTACTCACTTAGTGGGTTTCCGTACCGCGATGACTCGTACCCTTAAT 600
pur2            CAACGTGACGGCGGTACTCACTTAGTGGGTTTCCGTACCGCGATGACTCGTACCCTTAAT 600
pur3            CAACGTGACGGCGGTACTCACTTAGTGGGTTTCCGTACCGCGATGACTCGTACCCTTAAT 600
yel1            CAACGCGACGGCGGTACTCACTTAGTTGGTTTCCGTACTGCGATGACCCGTACCCTCAAC 600
yel2            CAACGCGACGGCGGTACTCACTTAGTTGGTTTCCGTACTGCGATGACCCGTACCCTCAAC 600
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yel3            CAACGCGACGGCGGTACTCACTTAGTTGGTTTCCGTACTGCGATGACCCGTACCCTCAAC 600
                ***** ******************** *********** ******** ******** **

pur1            AGCTACATGGATAAAGAGGGGTACAACAAGAAATCCAAAGTCAGCGCTACTGGTGATGAT 660
pur2            AGCTACATGGATAAAGAGGGGTACAACAAGAAATCCAAAGTCAGCGCTACTGGTGATGAT 660
pur3            AGCTACATGGATAAAGAGGGGTACAACAAGAAATCCAAAGTCAGCGCTACTGGTGATGAT 660
yel1            AGCTATATGGACAAAGAAGGGTACAACAAGAAATCCAAAGTCAGCGCCACCGGTGATGAT 660
yel2            AGCTATATGGACAAAGAAGGGTACAACAAGAAATCCAAAGTCAGCGCCACCGGTGATGAT 660
yel3            AGCTATATGGACAAAGAAGGGTACAACAAGAAATCCAAAGTCAGCGCCACCGGTGATGAT 660
                ***** ***** ***** ***************************** ** *********

pur1            GCCCGTGAAGGTTTAGTCGCCGTCATTTCCGTTAAAGTACCTGATCCAAAATTCTCTTCT 720
pur2            GCCCGTGAAGGTTTAGTCGCCGTCATTTCCGTTAAAGTACCTGATCCAAAATTCTCTTCT 720
pur3            GCCCGTGAAGGTTTAGTCGCCGTCATTTCCGTTAAAGTACCTGATCCAAAATTCTCTTCT 720
yel1            GCCCGCGAGGGTTTAGTCGCCGTCATTTCCGTTAAAGTACCTGATCCAAAATTCTCTTCT 720
yel2            GCCCGCGAGGGTTTAGTCGCCGTCATTTCCGTTAAAGTACCTGATCCAAAATTCTCTTCT 720
yel3            GCCCGCGAGGGTTTAGTCGCCGTCATTTCCGTTAAAGTACCTGATCCAAAATTCTCTTCT 720
                ***** ** ************************************************

pur1            CAGACTAAAGATAAACTAGTCTCTTCCGAAGTGAAAACCGCAGTTGAAACACTGATGAAT 780
pur2            CAGACTAAAGATAAACTAGTCTCTTCCGAAGTGAAAACCGCAGTTGAAACACTGATGAAT 780
pur3            CAGACTAAAGATAAACTAGTCTCTTCCGAAGTGAAAACCGCAGTTGAAACACTGATGAAT 780
yel1            CAGACCAAAGACAAACTGGTTTCTTCCGAGGTGAAAACGGCGGTTGAAACACTGATGAAC 780
yel2            CAGACCAAAGACAAACTGGTTTCTTCCGAGGTGAAAACGGCGGTTGAAACACTGATGAAC 780
yel3            CAGACCAAAGACAAACTGGTTTCTTCCGAGGTGAAAACGGCGGTTGAAACACTGATGAAC 780
                ***** ***** ***** ** ******** ******** ** *****************

pur1            GAGAAGCTGGTGGAATATCTGCTGGAAAACCCAAATGACGCCAAAACAGTCGTCAGTAAA 840
pur2            GAGAAGCTGGTGGAATATCTGCTGGAAAACCCAAATGACGCCAAAACAGTCGTCAGTAAA 840
pur3            GAGAAGCTGGTGGAATATCTGCTGGAAAACCCAAATGACGCCAAAACAGTCGTCAGTAAA 840
yel1            GAGAAGCTGGTGGAATACCTGCTGGAAAACCCGAATGACGCTAAAACCGTCGTCGGTAAA 840
yel2            GAGAAGCTGGTGGAATACCTGCTGGAAAACCCGAATGACGCTAAAACCGTCGTCGGTAAA 840
yel3            GAGAAGCTGGTGGAATACCTGCTGGAAAACCCGAATGACGCTAAAACCGTCGTCGGTAAA 840
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pur1            ATTATTGATGCTGCTCGTGCCCGTGAAGCAGCCCGTAAAGCACGCGAAATGACACGCCGC 900
pur2            ATTATTGATGCTGCTCGTGCCCGTGAAGCAGCCCGTAAAGCACGCGAAATGACACGCCGC 900
pur3            ATTATTGATGCTGCTCGTGCCCGTGAAGCAGCCCGTAAAGCACGCGAAATGACACGCCGC 900
yel1            ATTATCGACGCTGCCCGCGCCCGTGAAGCAGCCCGTAAAGCACGTGAAATGACACGCCGC 900
yel2            ATTATCGACGCTGCCCGCGCCCGTGAAGCAGCCCGTAAAGCACGTGAAATGACACGCCGC 900
yel3            ATTATCGACGCTGCCCGCGCCCGTGAAGCAGCCCGTAAAGCACGTGAAATGACACGCCGC 900
                ***** ** ***** ** ************************** ***************

pur1            AAAGGGGCTCTTGATCTGGCTGGCTTACCAGGCAAACTAGCTGATTGTCAGGAACGTGAC 960
pur2            AAAGGGGCTCTTGATCTGGCTGGCTTACCAGGCAAACTAGCTGATTGTCAGGAACGTGAC 960
pur3            AAAGGGGCTCTTGATCTGGCTGGCTTACCAGGCAAACTAGCTGATTGTCAGGAACGTGAC 960
yel1            AAAGGGGCTCTTGATCTGGCTGGCCTGCCAGGCAAACTGGCGGATTGTCAGGAACGTGAT 960
yel2            AAAGGGGCTCTTGATCTGGCTGGCCTGCCAGGCAAACTGGCGGATTGTCAGGAACGTGAT 960
yel3            AAAGGGGCTCTTGATCTGGCTGGCCTGCCAGGCAAACTGGCGGATTGTCAGGAACGTGAT 960
                ************************ * ************ ** *****************

pur1            CCGGCATTGTCCGAACTCTACTTAGTGGAAGGGGACTCGGCGGGCGGTTCTGCAAAACAG 1020
pur2            CCGGCATTGTCCGAACTCTACTTAGTGGAAGGGGACTCGGCGGGCGGTTCTGCAAAACAG 1020
pur3            CCGGCATTGTCCGAACTCTACTTAGTGGAAGGGGACTCGGCGGGCGGTTCTGCAAAACAG 1020
yel1            CCGGCACTGTCCGAACTCTACTTAGTGGAAGGGGACTCTGCGGGTGGTTCCGCAAAACAG 1020
yel2            CCGGCACTGTCCGAACTCTACTTAGTGGAAGGGGACTCTGCGGGTGGTTCCGCAAAACAG 1020
yel3            CCGGCACTGTCCGAACTCTACTTAGTGGAAGGGGACTCTGCGGGTGGTTCCGCAAAACAG 1020
                ****** ******************************* ***** ***** *********

pur1            GGCCGTAACCGCAAAAATCAGGCAATCTTGCCATTGAAAGGTAAAATCCTAAACGTTGAA 1080
pur2            GGCCGTAACCGCAAAAATCAGGCAATCTTGCCATTGAAAGGTAAAATCCTAAACGTTGAA 1080
pur3            GGCCGTAACCGCAAAAATCAGGCAATCTTGCCATTGAAAGGTAAAATCCTAAACGTTGAA 1080
yel1            GGGCGTAACCGCAAAAATCAGGCAATTTTGCCACTGAAAGGTAAAATCCTGAACGTTGAA 1080
yel2            GGGCGTAACCGCAAAAATCAGGCAATTTTGCCACTGAAAGGTAAAATCCTGAACGTTGAA 1080
yel3            GGGCGTAACCGCAAAAATCAGGCAATTTTGCCACTGAAAGGTAAAATCCTGAACGTTGAA 1080
                ** ********************** ****** ***************** *********

pur1            AAAGCGCGTTTCGATAAAATGCTCTCTTCTCAGGAAGTCGCAACACTGATCACTGCATTG 1140
pur2            AAAGCGCGTTTCGATAAAATGCTCTCTTCTCAGGAAGTCGCAACACTGATCACTGCATTG 1140
pur3            AAAGCGCGTTTCGATAAAATGCTCTCTTCTCAGGAAGTCGCAACACTGATCACTGCATTG 1140
yel1            AAAGCGCGTTTCGATAAAATGCTCTCTTCTCAGGAAGTGGCAACCCTGATCACTGCGTTG 1140
yel2            AAAGCGCGTTTCGATAAAATGCTCTCTTCTCAGGAAGTGGCAACCCTGATCACTGCGTTG 1140
yel3            AAAGCGCGTTTCGATAAAATGCTCTCTTCTCAGGAAGTGGCAACCCTGATCACTGCGTTG 1140
                ************************************ ***** *********** ***

pur1            GGTTGTGGTATTGGCCGTGACGAATACAACCCGGATAAGCTGCGTTATCACAGTATCATT 1200
pur2            GGTTGTGGTATTGGCCGTGACGAATACAACCCGGATAAGCTGCGTTATCACAGTATCATT 1200
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pur3            GGTTGTGGTATTGGCCGTGACGAATACAACCCGGATAAGCTGCGTTATCACAGTATCATT 1200
yel1            GGTTGCGGTATTGGCCGTGACGAATACAACCCGGATAAGCTGCGTTATCACAGTATTATT 1200
yel2            GGTTGCGGTATTGGCCGTGACGAATACAACCCGGATAAGCTGCGTTATCACAGTATTATT 1200
yel3            GGTTGCGGTATTGGCCGTGACGAATACAACCCGGATAAGCTGCGTTATCACAGTATTATT 1200
                ***** ************************************************** ***

pur1            ATCATGACAGATGCGGACGTTGATGGTTCTCATATCCGTACTCTATTGTTAACTTTTTTC 1260
pur2            ATCATGACAGATGCGGACGTTGATGGTTCTCATATCCGTACTCTATTGTTAACTTTTTTC 1260
pur3            ATCATGACAGATGCGGACGTTGATGGTTCTCATATCCGTACTCTATTGTTAACTTTTTTC 1260
yel1            ATCATGACAGATGCGGACGTCGATGGTTCTCATATCCGTACTCTGTTGTTGACTTTCTTC 1260
yel2            ATCATGACAGATGCGGACGTCGATGGTTCTCATATCCGTACTCTGTTGTTGACTTTCTTC 1260
yel3            ATCATGACAGATGCGGACGTCGATGGTTCTCATATCCGTACTCTGTTGTTGACTTTCTTC 1260
                ******************* ********************** ***** ***** ***

pur1            TATCGTCAAATGCCAGAAATCATTGAACGTGGTCACGTGTTTATAGCTCAGCCACCTCTG 1320
pur2            TATCGTCAAATGCCAGAAATCATTGAACGTGGTCACGTGTTTATAGCTCAGCCACCTCTG 1320
pur3            TATCGTCAAATGCCAGAAATCATTGAACGTGGTCACGTGTTTATAGCTCAGCCACCTCTG 1320
yel1            TATCGTCAAATGCCAGAAATCATTGAACGTGGTCATGTGTTTATTGCACAGCCGCCACTG 1320
yel2            TATCGTCAAATGCCAGAAATCATTGAACGTGGTCATGTGTTTATTGCACAGCCGCCACTG 1320
yel3            TATCGTCAAATGCCAGAAATCATTGAACGTGGTCATGTGTTTATTGCACAGCCGCCACTG 1320
                ********************************** ******** ** ***** ** ***

pur1            TATAAAGTGAAAAAAGGCAAACAGGAACAGTATATTAAAGATGATGAAGCAATGGATGAG 1380
pur2            TATAAAGTGAAAAAAGGCAAACAGGAACAGTATATTAAAGATGATGAAGCAATGGATGAG 1380
pur3            TATAAAGTGAAAAAAGGCAAACAGGAACAGTATATTAAAGATGATGAAGCAATGGATGAG 1380
yel1            TATAAAGTGAAAAAAGGCAAGCAGGAACAATATATTAAAGATGATGAAGCAATGGATGAG 1380
yel2            TATAAAGTGAAAAAAGGCAAGCAGGAACAATATATTAAAGATGATGAAGCAATGGATGAG 1380
yel3            TATAAAGTGAAAAAAGGCAAGCAGGAACAATATATTAAAGATGATGAAGCAATGGATGAG 1380
                ******************* ******** ******************************

pur1            TA 1382
pur2            TA 1382
pur3            TA 1382
yel1            TA 1382
yel2            TA 1382
yel3            TA 1382
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Figure A. 6: ClustalW Multiple Sequence Alignment of a portion (1382 bases) of the gyrB gene from three *pur* and three *yel Photorhabdus temperata* isolates from North Bull Island. Six single nucleotide polymorphisms resulting in non-synonymous amino acids in the *pur* and yel isolates have been outlined in red (See Section 2.3.4).

Asterisk (∗) denotes conserved sites
Spaces denote nucleotide polymorphism.

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H3107           ACTTATCACCTTGGTGTGCCGCAAAGCCCACTAAAAGTTGTTGGTGAAACCGAACAGACA 60
H3240           ACTTATCACCTTGGTGTGCCGCAAAGCCCACTAAAAGTTGTTGGTGAAACCGAACAGACA 60
H3014           ACTTATCACCTTGGTGTGCCGCAAAGCCCACTAAAAGTTGTTGGTGAAACCGAACAGACA 60
pur1            ACTTATCACCTTGGTGTGCCGCAAAGCCCACTAAAAGTTGTTGGTGAAACCGAACAGACA 60
pur2            ACTTATCACCTTGGTGTGCCGCAAAGCCCACTAAAAGTTGTTGGTGAAACCGAACAGACA 60
pur3            ACTTATCACCTTGGTGTGCCGCAAAGCCCACTAAAAGTTGTTGGTGAAACCGAACAGACA 60
yel1            ACTTATCACCTTGGTGTGCCACAAAGCCCACTAAAAGTCGTCGGTGAAACGGAGCAGACA 60
yel2            ACTTATCACCTTGGTGTGCCACAAAGCCCACTAAAAGTCGTCGGTGAAACGGAGCAGACA 60
yel3            ACTTATCACCTTGGTGTGCCACAAAGCCCACTAAAAGTCGTCGGTGAAACGGAGCAGACA 60
K122            ACTTATCACCTTGGTGTGCCACAAAGCCCACTAAAAGTCGTCGGTGAAACGGAGCAGACA 60
HF85            ACTTATCACCTTGGTGTGCCACAAAGCCCACTAAAAGTCGTCGGTGAAACGGAGCAGACA 60
HL81            ACTTATCACCTTGGTGTGCCACAAAGCCCACTAAAAGTCGTCGGTGAAACGGAGCAGACA 60
HW79            ACTTATCACCTTGGTGTGCCACAAAGCCCACTAAAAGTCGTCGGTGAAACGGAGCAGACA 60
XlLit           ACTTATCACCTTGGTGTGCCACAAAGCCCACTAAAAGTCGTCGGTGAAACGGAGCAGACA 60
XlNach          ACTTATCACCTTGGTGTGCCACAAAGCCCACTAAAAGTCGTCGGTGAAACGGAGCAGACA 60
H3016           ACTTATCACCTTGGTGTGCCACAAAGCCCACTAAAAGTCGTCGGTGAAACGGAGCAGACA 60
H3173           ACTTATCACCTTGGTGTGCCACAAAGCCCACTAAAAGTCGTCGGGGAAACGGAGCAGACA 60
H3179           ACTTATCACCTTGGTGTGCCACAAAGCCCACTAAAAGTCGTCGGTGAAACGGAGCAGACA 60
H3182           ACTTATCACCTTGGTGTGCCACAAAGCCCACTAAAAGTCGTCGGTGAAACGGAGCAGACA 60
C1              ACTTATCACCTTGGTGTGCCACAAAGCCCACTAAAAGTCGTTGGTGAAACCGAACAGACC 60
Meg             ACTTATCACCTTGGTGTGCCACAAAGCCCACTAAAAGTCGTTGGTGAAACCGAACAGACC 60
Habana          ACTTATCACCTTGGTGTGCCACAAAGCCCACTAAAAGTCGTTGGTGAAACCGAACAGACC 60
NZH3            ACTTATCACCTTGGTGTGCCGCAAAGCCCACTAAAAGTTGTCGGTGAAACCGAACAGACT 60
T327            ACTTATCACCTTGGTGTGCCGCAAAGCCCACTAAAAGTTGTCGGTGAAACCGAACAGACT 60
USCA01          ACTTATCACCTTGGTGTGCCGCAAAGCCCACTAAAAGTTGTCGGTGAAACCGAACAGACT 60
H3210           ACTTATCACCTTGGTGTGCCGCAAAACCCACTAAAAGTTGTCGGTGAAACCGAACAGACA 60
CIP108426       ACTTATCACCTTGGTGTGCCGCAAAACCCACTAAAAGTTGTCGGTGAAACCGAACAGACA 60
FR32            ACTTATCACCTTGGTGTGCCGCAAAACCCACTAAAAGTTGTCGGTGAAACCGAACAGACA 60
H111            ACTTATCACCTTGGTGTGCCACAAAGCCCACTAAAAGTCGTCGGTGAAACGGAGCAGACA 60
H267            ACTTATCACCTTGGTGTGCCGCAAAGCCCACTAAAAGTTGTTGGTGAAACCGAACAGACA 60
H295            ACTTATCACCTTGGTGTGCCACAAAGCCCACTAAAAGTCGTCGGTGAAACGGAGCAGACA 60
H3086           ACTTATCACCTTGGTGTGCCGCAAAGCTCACCCAAAGTTGTTGGTGAAACCGAACAGACA 60
AN6             ACTTACCATCATGGTGTTCCACAGTCTCCGCTGAACATTGTCGGTGATACCGAGCAGACA 60
                ***** ** * ****** ** **     * *  **  * ** ** ** ** ** *****

H3107           GGTACCCGTGTCCGCTTCTGGCCAAGCATGGATACTTTCCGTAACAACACTGAATTCCAG 120
H3240           GGTACCCGTGTCCGCTTCTGGCCAAGCATGGATACTTTCCGTAACAACACTGAATTCCAG 120
H3014           GGTACCCGTGTCCGCTTCTGGCCAAGCATGGATACTTTCCGTAACAACACTGAATTCCAG 120
pur1            GGTACCCGTGTCCGCTTCTGGCCAAGCATGGATACTTTCCGTAACAACACTGAATTCCAG 120
pur2            GGTACCCGTGTCCGCTTCTGGCCAAGCATGGATACTTTCCGTAACAACACTGAATTCCAG 120
pur3            GGTACCCGTGTCCGCTTCTGGCCAAGCATGGATACTTTCCGTAACAACACTGAATTCCAG 120
yel1            GGTACCCGTGTCCGCTTCTGGCCGAGCATGGACACTTTCAGTAACAACACTGAATTCCAG 120
yel2            GGTACCCGTGTCCGCTTCTGGCCGAGCATGGACACTTTCAGTAACAACACTGAATTCCAG 120
yel3            GGTACCCGTGTCCGCTTCTGGCCGAGCATGGACACTTTCAGTAACAACACTGAATTCCAG 120
K122            GGTACCCGTGTCCGCTTCTGGCCGAGCATGGACACTTTCAGTAACAACACTGAATTCCAG 120
HF85            GGGACCCGTGTCCGCTTCTGGCCGAGCATGGACACTTTCAGTAACAACACTGAATTCCAG 120
HL81            GGGACCCGTGTCCGCTTCTGGCCGAGCATGGACACTTTCAGTAACAACACTGAATTCCAG 120
HW79            GGGACCCGTGTCCGCTTCTGGCCGAGCATGGACACTTTCAGTAACAACACTGAATTCCAG 120
XlLit           GGGACCCGTGTCCGCTTCTGGCCGAGCATGGACACTTTCAGTAACAACACTGAATTCCAG 120
XlNach          GGGACCCGTGTCCGCTTCTGGCCGAGCATGGACACTTTCAGTAACAACACTGAATTCCAG 120
H3016           GGGACCCGTGTCCGCTTCTGGCCGAGCATGGACACTTTCAGTAACAACACTGAATTCCAG 120
H3173           GGGACCCGTGTCCGCTTCTGGCCGAGCATGGACACTTTCAGTAACAACACTGAATTCCAG 120
H3179           GGTACCCGTGTCCGCTTCTGGCCGAGCATGGACACTTTCAGTAACAACACTGAATTCCAG 120
H3182           GGTACCCGTGTCCGCTTCTGGCCGAGCATGGACACTTTCAGTAACAACACTGAATTCCAG 120
C1              GGTACCCGTGTCCGCTTTTGGCCGAGCATGGATACTTTCAGTAACAACACTGAATTCCAG 120
Meg             GGTACCCGTGTCCGCTTTTGGCCGAGCATGGATACTTTCAGTAACAACACTGAATTCCAG 120
Habana          GGTACCCGTGTCCGCTTTTGGCCGAGCATGGATACTTTCAGTAACAACACTGAATTCCAG 120
NZH3            GGTACTCGTGTCCGCTTTTGGCCGAGCATGGATACTTTCAGTAACAACACTGAATTCCAG 120
T327            GGTACTCGTGTCCGCTTTTGGCCGAGCATGGATACTTTCAGTAACAACACTGAATTCCAG 120
USCA01          GGTACTCGTGTCCGCTTTTGGCCGAGCATGGATACTTTCAGTAACAACACTGAATTCCAG 120
H3210           GGTACCCGTGTCCGCTTCTGGCCAAGCATGGATACTTTCCGTGACAACACTGAATTCCAG 120
CIP108426       GGTACCCGTGTCCGCTTCTGGCCAAGCATGGATACTTTCCGTGACAACACTGAATTCCAG 120
FR32            GGTACCCGTGTCCGCTTCTGGCCAAGCATGGATACTTTCCGTGACAACACTGAATTCCAG 120
H111            GGTACCCGTGTCCGCTTCTGGCCGAGCATGGACACTTTCAGTAACAACACTGAATTCCAG 120
H267            GGTACCCGTGTCCGCTTCTGGCCAAGCATGGATACTTTCCGTAACAACACTGAATTCCAG 120
H295            GGGACCCGTGTCCGCTTCTGGCCGAGCATGGACACTTTCAGTAACAACACTGAATTCCAG 120
H3086           GGTACCCGTGTCCGCTTCTGGCCAAGCATGGATACTTTCCGTAACAACACTGAATTCCAG 120
AN6             GGAACCACGGTTCGTTTCTGGCCAAGCATGGATACGTTCAAGATTAACACTGAATTCGAA 120
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H3107           CACGACATTCTGGCTAAACGTCTGCGTGAATTGTCCTTTCTGAACTCAGGTGTATCTATC 180
H3240           CACGACATTCTGGCTAAACGTCTGCGTGAATTGTCCTTTCTGAACTCAGGTGTATCTATC 180
H3014           CACGACATTCTGGCTAAACGTCTGCGTGAATTGTCCTTTCTGAACTCAGGTGTATCTATC 180
pur1            CACGACATTCTGGCTAAACGTCTGCGTGAATTGTCCTTTCTGAACTCAGGTGTATCTATC 180
pur2            CACGACATTCTGGCTAAACGTCTGCGTGAATTGTCCTTTCTGAACTCAGGTGTATCTATC 180
pur3            CACGACATTCTGGCTAAACGTCTGCGTGAATTGTCCTTTCTGAACTCAGGTGTATCTATC 180
yel1            CACGACATTCTGGCTAAACGTCTGCGTGAATTGTCCTTTCTGAACTCAGGTGTATCTATC 180
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yel2            CACGACATTCTGGCTAAACGTCTGCGTGAATTGTCCTTTCTGAACTCAGGTGTATCTATC 180
yel3            CACGACATTCTGGCTAAACGTCTGCGTGAATTGTCCTTTCTGAACTCAGGTGTATCTATC 180
K122            CACGACATTCTGGCTAAACGTCTGCGTGAATTGTCCTTTCTGAACTCAGGTGTATCTATC 180
HF85            CACGACATTCTGGCTAAACGTCTGCGTGAATTGTCCTTTCTGAACTCAGGTGTATCTATC 180
HL81            CACGACATTCTGGCTAAACGTCTGCGTGAATTGTCCTTTCTGAACTCAGGTGTATCTATC 180
HW79            CACGACATTCTGGCTAAACGTCTGCGTGAATTGTCCTTTCTGAACTCAGGTGTATCTATC 180
XlLit           CACGACATTCTGGCTAAACGTCTGCGTGAATTGTCCTTTCTGAACTCAGGTGTATCTATC 180
XlNach          CACGACATTCTGGCTAAACGTCTGCGTGAATTGTCCTTTCTGAACTCAGGTGTATCTATC 180
H3016           CACGACATTCTGGCTAAACGTCTGCGTGAATTGTCCTTTCTGAACTCAGGTGTATCTATC 180
H3173           CACGACATTCTGGCTAAACGTCTGCGTGAATTGTCCTTTCTGAACTCAGGTGTATCTATC 180
H3179           CACGACATTCTGGCTAAACGTCTGCGTGAATTGTCCTTTCTGAACTCAGGTGTATCTATC 180
H3182           CACGACATTCTGGCTAAACGTCTGCGTGAATTGTCCTTTCTGAACTCAGGTGTATCTATC 180
C1              CACGACATTCTGGCTAAACGTCTGCGTGAGCTGTCCTTTCTGAACTCAGGTGTATCTATC 180
Meg             CACGACATTCTGGCTAAACGTCTGCGTGAGCTGTCCTTTCTGAACTCAGGTGTATCTATC 180
Habana          CACGACATTCTGGCTAAACGTCTGCGTGAGCTGTCCTTTCTGAACTCAGGTGTATCTATC 180
NZH3            CACGACATTCTGGCTAAACGTCTGCGTGAACTGTCCTTTCTGAATTCAGGTGTATCTATC 180
T327            CACGACATTCTGGCTAAACGTCTGCGTGAACTGTCCTTTCTGAATTCAGGTGTATCTATC 180
USCA01          CACGACATTCTGGCTAAACGTCTGCGTGAACTGTCCTTTCTGAATTCAGGTGTATCTATC 180
H3210           CACGACATTCTGGCTAAACGTCTGCGTGAATTGTCCTTTCTGAACTCAGGTGTATCTATC 180
CIP108426       CACGACATTCTGGCTAAACGTCTGCGTGAATTGTCCTTTCTGAACTCAGGTGTATCTATC 180
FR32            CACGACATTCTGGCTAAACGTCTGCGTGAATTGTCCTTTCTGAACTCAGGTGTATCTATC 180
H111            CACGACATTCTGGCTAAACGTCTGCGTGAATTGTCCTTTCTGAACTCAGGTGTATCTATC 180
H267            CACGACATTCTGGCTAAACGTCTGCGTGAATTGTCCTTTCTGAACTCAGGTGTATCTATC 180
H295            CACGACATTCTGGCTAAACGTCTGCGTGAATTGTCCTTTCTGAACTCAGGTGTATCTATC 180
H3086           CACGACATTCTGGCTAAACGTCTGCGTGAATTGTCCTTTCTGAACTCAGGTGTATCTATC 180
AN6             TACGATATTCTGGCTAAGCGCCTGCGTGAATTATCATTCCTGAATTCCGGTGTTTCTATT 180
                 **** *********** ** ********  * ** ** ***** ** ***** *****

H3107           CGCCTGCTTGATAAACGCACCAATATTGAAGACCATTTCCATTATGAAGGTGGTATTAAG 240
H3240           CGCCTGCTTGATAAACGCACCAATATTGAAGACCATTTCCATTATGAAGGTGGTATTAAG 240
H3014           CGCCTGCTTGATAAACGCACCAATATTGAAGACCATTTCCATTATGAAGGTGGTATTAAG 240
pur1            CGCCTGCTTGATAAACGCACCAATATTGAAGACCATTTCCATTATGAAGGTGGTATTAAG 240
pur2            CGCCTGCTTGATAAACGCACCAATATTGAAGACCATTTCCATTATGAAGGTGGTATTAAG 240
pur3            CGCCTGCTTGATAAACGCACCAATATTGAAGACCATTTCCATTATGAAGGTGGTATTAAG 240
yel1            CGCCTGCTTGATAAACGCACCAATATTGAAGACCATTTCCATTACAAAGGTGGCATTAAA 240
yel2            CGCCTGCTTGATAAACGCACCAATATTGAAGACCATTTCCATTACAAAGGTGGCATTAAA 240
yel3            CGCCTGCTTGATAAACGCACCAATATTGAAGACCATTTCCATTACAAAGGTGGCATTAAA 240
K122            CGCCTGCTTGATAAACGCACCAATATTGAAGACCATTTCCATTACAAAGGTGGCATTAAA 240
HF85            CGCCTGCTTGATAAACGCACCAATATTGAAGACCATTTCCATTACAAAGGTGGCATTAAA 240
HL81            CGCCTGCTTGATAAACGCACCAATATTGAAGACCATTTCCATTACAAAGGTGGCATTAAA 240
HW79            CGCCTGCTTGATAAACGCACCAATATTGAAGACCATTTCCATTACAAAGGTGGCATTAAA 240
XlLit           CGCCTGCTTGATAAACGCACCAATATTGAAGACCATTTCCATTACAAAGGTGGCATTAAA 240
XlNach          CGCCTGCTTGATAAACGCACCAATATTGAAGACCATTTCCATTACAAAGGTGGCATTAAA 240
H3016           CGCCTGCTTGATAAACTCACCAATATTGAAGACCATTTCCATTACAAAGGTGGCATTAAA 240
H3173           CGCCTGCTTGATAAACGCACCAATATTGAAGACCATTTCCATTACAAAGGTGGCATTAAA 240
H3179           CGCCTGCTTGATAAACGCACCAATATTGAAGACCATTTCCATTACAAAGGTGGCATTAAA 240
H3182           CGCCTGCTTGATAAACGCACCAATATTGAAGACCATTTCCATTACAAAGGTGGCATTAAA 240
C1              CGCCTGCTTGATAAACGCACCAATATTGAAGACCATTTCCATTATAAAGGTGGTATTAAG 240
Meg             CGCCTGCTTGATAAACGCACCAATATTGAAGACCATTTCCATTATAAAGGTGGTATTAAG 240
Habana          CGCCTGCTTGATAAACGCACCAATATTGAAGACCATTTCCATTATAAAGGTGGTATTAAG 240
NZH3            CGCTTGCTTGATAAGCGCACCAATATTGAAGATCACTTCCATTATGAAGGTGGTATTAAA 240
T327            CGCCTGCTTGATAAGCGCACCAATATTGAAGATCACTTCCATTATGAAGGTGGTATTAAA 240
USCA01          CGCTTGCTTGATAAGCGCACCAATATTGAAGATCACTTCCATTATGAAGGTGGTATTAAA 240
H3210           CGCCTGCTTGATAAACGCACCAATATTGAAGACCATTTCCATTATGAAGGTGGTATTAAG 240
CIP108426       CGCCTGCTTGATAAACGCACCAATATTGAAGACCATTTCCATTATGAAGGTGGTATTAAG 240
FR32            CGCCTGCTTGATAAACGCACCAATATTGAAGACCATTTCCATTATGAAGGTGGTATTAAG 240
H111            CGCCTGCTTGATAAACGCACCAATATTGAAGACCATTTCCATTACAAAGGTGGCATTAAA 240
H267            CGCCTGCTTGATAAACGCACCAATATTGAAGACCATTTCCATTATGAAGGTGGTATTAAG 240
H295            CGCCTGCTTGATAAACGCACCAATATTGAAGACCATTTCCATTACAAAGGTGGCATTAAA 240
H3086           CGCCTGCTTGATAAACGCACCAATATTGAAGACCATTTCCATTATGAAGGTGGTATTAAG 240
AN6             CGTTTAGTTGATAAACGCACCAATGCCGAAGATCTCTTCCACTATAAAGGCGGTATCAAA 240
                **  *  ******* * *******   ***** *  ***** **  **** ** ** **

H3107           GCGTTTGTCGAATTCTTAAATAAAAACAAAACACCAATCCATCCCAATGTTTTCTATTTC 300
H3240           GCGTTTGTCGAATTCTTAAATAAAAACAAAACACCAATCCATCCCAATGTTTTCTATTTC 300
H3014           GCGTTTGTCGAATTCTTAAATAAAAACAAAACACCAATCCATCCCAATGTTTTCTATTTC 300
pur1            GCGTTTGTCGAATTCTTAAATAAAAACAAAACACCAATCCATCCCAATGTTTTCTATTTC 300
pur2            GCGTTTGTCGAATTCTTAAATAAAAACAAAACACCAATCCATCCCAATGTTTTCTATTTC 300
pur3            GCGTTTGTCGAATTCTTAAATAAAAACAAAACACCAATCCATCCCAATGTTTTCTATTTC 300
yel1            GCGTTTGTCGAATTCTTAAATAAAAACAAAACACCAATCCATCCCAATGTTTTCTATTTC 300
yel2            GCGTTTGTCGAATTCTTAAATAAAAACAAAACACCAATCCATCCCAATGTTTTCTATTTC 300
yel3            GCGTTTGTCGAATTCTTAAATAAAAACAAAACACCAATCCATCCCAATGTTTTCTATTTC 300
K122            GCGTTTGTCGAATTCTTAAATAAAAACAAAACACCAATCCATCCCAATGTTTTCTATTTC 300
HF85            GCGTTTGTCGAATTCTTAAATAAAAACAAAACACCAATCCATCCCAATGTTTTCTATTTC 300
HL81            GCGTTTGTCGAATTCTTAAATAAAAACAAAACACCAATCCATCCCAATGTTTTCTATTTC 300
HW79            GCGTTTGTCGAATTCTTAAATAAAAACAAAACACCAATCCATCCCAATGTTTTCTATTTC 300
XlLit           GCGTTTGTCGAATTCTTAAATAAAAACAAAACACCAATCCATCCCAATGTTTTCTATTTC 300
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XlNach          GCGTTTGTCGAATTCTTAAATAAAAACAAAACACCAATCCATCCCAATGTTTTCTATTTC 300
H3016           GCGTTTGTCGAATTCTTAAATAAAAACAAAACACCAATCCATCCCAATGTTTTCTATTTC 300
H3173           GCGTTTGTCGAATTCTTAAATAAAAACAAAACACCAATCCATCCCAATGTTTTCTATTTC 300
H3179           GCGTTTGTCGAATTCTTAAATAAAAACAAAACACCAATCCATCCCAATGTTTTCTATTTC 300
H3182           GCGTTTGTCGAATTCTTAAATAAAAACAAAACACCAATCCATCCCAATGTTTTCTATTTC 300
C1              GCGTTTGTTGAATTCTTAAATAAAAACAAAACACCAATCCATCCCAATGTTTTCTATTTC 300
Meg             GCGTTTGTTGAATTCTTAAATAAAAACAAAACACCAATCCATCCCAATGTTTTCTATTTC 300
Habana          GCGTTTGTTGAATTCTTAAATAAAAACAAAACACCAATCCATCCCAATGTTTTCTATTTC 300
NZH3            GCGTTTGTTGAATTCTTAAATAAAAACAAAACACCAATCCATCCCAATGTTTTCTATTTT 300
T327            GCGTTTGTTGAATTCTTAAATAAAAACAAAACACCAATCCATCCCAATGTTTTCTATTTT 300
USCA01          GCGTTTGTTGAATTCTTAAATAAAAACAAAACACCAATCCATCCCAATGTTTTCTATTTT 300
H3210           GCGTTTGTCGAATTCTTAAATAAAAACAAAACACCAATCCATCCCAATGTTTTCTATTTC 300
CIP108426       GCGTTTGTCGAATTCTTAAATAAAAACAAAACACCAATCCATCCCAATGTTTTCTATTTC 300
FR32            GCGTTTGTCGAATTCTTAAATAAAAACAAAACACCAATCCATCCCAATGTTTTCTATTTC 300
H111            GCGTTTGTCGAATTCTTAAATAAAAACAAAACACCAATCCATCCCAATGTTTTCTATTTC 300
H267            GCGTTTGTCGAATTCTTAAATAAAAACAAAACACCAATCCATCCCAATGTTTTCTATTTC 300
H295            GCGTTTGTCGAATTCTTAAATAAAAACAAAACACCAATCCATCCCAATGTTTTCTATTTC 300
H3086           GCGTTTGTCGAATTCTTAAATAAAAACAAAACACCAATCCATCCCAATGTTTTCTATTTC 300
AN6             GCGTTTGTTGAGTTCCTCAGCCGCAATAAAAACCCGATTCACCCAAATGTTTTTTATTTC 300
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H3107           TCTACAGAAAAAGATGGCATCGGTGTTGAAGCCGCATTGCAATGGAACGATGGTTTCCAG 360
H3240           TCTACAGAAAAAGATGGCATCGGTGTTGAAGCCGCATTGCAATGGAACGATGGTTTCCAG 360
H3014           TCTACAGAAAAAGATGGCATCGGTGTTGAAGCCGCATTGCAATGGAACGATGGTTTCCAG 360
pur1            TCTACAGAAAAAGATGGCATCGGTGTTGAAGCCGCATTGCAATGGAACGATGGTTTCCAG 360
pur2            TCTACAGAAAAAGATGGCATCGGTGTTGAAGCCGCATTGCAATGGAACGATGGTTTCCAG 360
pur3            TCTACAGAAAAAGATGGCATCGGTGTTGAAGCCGCATTGCAATGGAACGATGGTTTCCAG 360
yel1            TCTACAGAAAAAGATAGCATCGGTGTTGAAGTCGCATTGCAGTGGAATGATGGTTTCCAG 360
yel2            TCTACAGAAAAAGATAGCATCGGTGTTGAAGTCGCATTGCAGTGGAATGATGGTTTCCAG 360
yel3            TCTACAGAAAAAGATAGCATCGGTGTTGAAGTCGCATTGCAGTGGAATGATGGTTTCCAG 360
K122            TCTACAGAAAAAGATAGCATCGGTGTTGAAGTCGCATTGCAGTGGAATGATGGTTTCCAG 360
HF85            TCTACAGAAAAAGATAGCATCGGTGTTGAAGTCGCATTGCAATGGAATGATGGTTTCCAG 360
HL81            TCTACAGAAAAAGATAGCATCGGTGTTGAAGTCGCATTGCAATGGAATGATGGTTTCCAG 360
HW79            TCTACAGAAAAAGATAGCATCGGTGTTGAAGTCGCATTGCAATGGAATGATGGTTTCCAG 360
XlLit           TCTACAGAAAAAGATAGCATCGGTGTTGAAGTCGCATTGCAATGGAATGATGGTTTCCAG 360
XlNach          TCTACAGAAAAAGATAGCATCGGTGTTGAAGTCGCATTGCAATGGAATGATGGTTTCCAG 360
H3016           TCTACAGAAAAAGATAGCATCGGTGTTGAAGTCGCATTGCAATGGAATGATGGTTTCCAG 360
H3173           TCTACAGAAAAAGATAGCATCGGTGTTGAAGTCGCATTGCAATGGAATGATGGTTTCCAG 360
H3179           TCTACAGAAAAAGATAGCATCGGTGTTGAAGTCGCATTGCAGTGGAATGATGGTTTCCAG 360
H3182           TCTACAGAAAAAGATAGCATCGGTGTTGAAGTCGCATTGCAATGGAACGATGGTTTCCAG 360
C1              TCTACAGAAAAAGATAGCATCGGTGTTGAAGTCGCATTGCAATGGAACGATGGTTTCCAG 360
Meg             TCTACAGAAAAAGATAGCATCGGTGTTGAAGTCGCATTGCAATGGAACGATGGTTTCCAG 360
Habana          TCTACAGAAAAAGATAGCATCGGTGTTGAAGTCGCATTGCAATGGAACGATGGTTTCCAG 360
NZH3            TCAACAGAAAAAGATAGCATCGGTGTTGAAGTTGCCTTGCAGTGGAACGATGGTTTCCAG 360
T327            TCAACAGAAAAAGATAGCATCGGTGTTGAAGTTGCCTTGCAGTGGAACGATGGTTTCCAG 360
USCA01          TCAACAGAAAAAGATAGCATCGGTGTTGAAGTTGCCTTGCAGTGGAACGATGGTTTCCAG 360
H3210           TCTACAGAAAAAGATAGCATCGGTGTTGAAGTCGCATTGCAATGGAACGATGGTTTCCAG 360
CIP108426       TCTACAGAAAAAGATAGCATCGGTGTTGAAGTCGCATTGCAATGGAACGATGGTTTCCAG 360
FR32            TCTACAGAAAAAGATAGCATCGGTGTTGAAGTCGCATTGCAATGGAACGATGGTTTCCAG 360
H111            TCTACAGAAAAAGATAGCATCGGTGTTGAAGTCGCATTGCAGTGGAATGATGGTTTCCAG 360
H267            TCTACAGAAAAAGATGGCATCGGTGTTGAAGCCGCATTGCAATGGAACGATGGTTTCCAG 360
H295            TCTACAGAAAAAGATAGCATCGGTGTTGAAGTCGCATTGCAATGGAATGATGGTTTCCAG 360
H3086           TCTACAGAAAAAGATGGCATTGGTGTTGAAGCCGCATTGCAATGGAACGATGGTTTCCAG 360
AN6             TCCACAGAAAAAGACGGTATTGGTGTTGAAATTTCCATGCAATGGAATGATGGTTTTCAG 360
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H3107           GAAAATATTTACTGCTTTACCAACAACATCCCGCAACGTGACGGCGGTACTCACTTAGTG 420
H3240           GAAAATATTTACTGCTTTACCAACAACATCCCGCAACGTGACGGCGGTACTCACTTAGTG 420
H3014           GAAAATATTTACTGCTTTACCAACAACATCCCGCAACGTGACGGCGGTACTCACTTAGTG 420
pur1            GAAAATATTTACTGCTTTACCAACAACATCCCGCAACGTGACGGCGGTACTCACTTAGTG 420
pur2            GAAAATATTTACTGCTTTACCAACAACATCCCGCAACGTGACGGCGGTACTCACTTAGTG 420
pur3            GAAAATATTTACTGCTTTACCAACAACATCCCGCAACGTGACGGCGGTACTCACTTAGTG 420
yel1            GAAAATATTTACTGCTTTACCAACAACATCCCGCAACGCGACGGCGGTACTCACTTAGTT 420
yel2            GAAAATATTTACTGCTTTACCAACAACATCCCGCAACGCGACGGCGGTACTCACTTAGTT 420
yel3            GAAAATATTTACTGCTTTACCAACAACATCCCGCAACGCGACGGCGGTACTCACTTAGTT 420
K122            GAAAATATTTACTGCTTTACCAACAACATCCCGCAACGCGACGGCGGTACTCACTTAGTT 420
HF85            GAAAATATTTACTGCTTTACCAACAACATCCCGCAACGTGACGGCGGTACTCACTTAGTG 420
HL81            GAAAATATTTACTGCTTTACCAACAACATCCCGCAACGTGACGGCGGTACTCACTTAGTG 420
HW79            GAAAATATTTACTGCTTTACCAACAACATCCCGCAACGTGACGGCGGTACTCACTTAGTG 420
XlLit           GAAAATATTTACTGCTTTACCAACAACATCCCGCAACGTGACGGCGGTACTCACTTAGTG 420
XlNach          GAAAATATTTACTGCTTTACCAACAACATCCCGCAACGTGACGGCGGTACTCACTTAGTG 420
H3016           GAAAATATTTACTGCTTTACCAACAACATCCCGCAACGTGACGGCGGTACTCACTTAGTG 420
H3173           GAAAATATTTACTGCTTTACCAACAACATCCCGCAACGTGACGGCGGTACTCACTTAGTG 420
H3179           GAAAATATTTACTGCTTTACCAACAACATCCCGCAACGCGACGGCGGTACTCACTTAGTT 420
H3182           GAAAATATTTATTGCTTTACCAACAACATCCCGCAACGCGACGGCGGTACTCACTTAGTT 420
C1              GAAAATATTTACTGCTTTACCAACAACATCCCGCAACGTGATGGTGGTACTCACTTAGTT 420
Meg             GAAAATATTTACTGCTTTACCAACAACATCCCGCAACGTGATGGTGGTACTCACTTAGTT 420
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Habana          GAAAATATTTACTGCTTTACCAACAACATCCCGCAACGTGATGGTGGTACTCACTTAGTT 420
NZH3            GAAAATATTTACTGCTTTACCAACAACATCCCGCAACGTGATGGCGGTACTCACTTAGTG 420
T327            GAAAATATTTACTGCTTTACCAACAACATCCCGCAACGTGATGGCGGTACTCACTTAGTG 420
USCA01          GAAAATATTTACTGCTTTACCAACAACATCCCGCAACGTGATGGCGGTACTCACTTAGTG 420
H3210           GAAAATATTTACTGCTTTACCAACAACATCCCGCAACGTGACGGTGGTACTCACTTAGTG 420
CIP108426       GAAAATATTTACTGCTTTACCAACAACATCCCGCAACGTGACGGTGGTACTCACTTAGTG 420
FR32            GAAAATATTTACTGCTTTACCAACAACATCCCGCAACGTGACGGTGGTACTCACTTAGTG 420
H111            GAAAATATTTACTGCTTTACCAACAACATCCCGCAACGCGACGGCGGTACTCACTTAGTT 420
H267            GAAAATATTTACTGCTTTACCAACAACATCCCGCAACGTGACGGCGGTACTCACTTAGTG 420
H295            GAAAATATTTACTGCTTTACCAACAACATCCCGCAACGTGACGGCGGTACTCACTTAGTG 420
H3086           GAAAATATTTACTGCTTTACCAACAACATCCCGCAACGTGACGGCGGTACTCACTTAGTG 420
AN6             GAAAACATTTACTGCTTCACCAACAATATTCCTCAGCGCGATGGCGGAACGCACTTAGTG 420
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H3107           GGTTTCCGTACCGCGATGACTCGTACCCTTAATAGCTACATGGATAAAGAGGGGTACAAC 480
H3240           GGTTTCCGTACCGCGATGACTCGTACCCTTAATAGCTACATGGATAAAGAGGGGTACAAC 480
H3014           GGTTTCCGTACCGCGATGACTCGTACCCTTAATAGCTACATGGATAAAGAGGGGTACAAC 480
pur1            GGTTTCCGTACCGCGATGACTCGTACCCTTAATAGCTACATGGATAAAGAGGGGTACAAC 480
pur2            GGTTTCCGTACCGCGATGACTCGTACCCTTAATAGCTACATGGATAAAGAGGGGTACAAC 480
pur3            GGTTTCCGTACCGCGATGACTCGTACCCTTAATAGCTACATGGATAAAGAGGGGTACAAC 480
yel1            GGTTTCCGTACTGCGATGACCCGTACCCTCAACAGCTATATGGACAAAGAAGGGTACAAC 480
yel2            GGTTTCCGTACTGCGATGACCCGTACCCTCAACAGCTATATGGACAAAGAAGGGTACAAC 480
yel3            GGTTTCCGTACTGCGATGACCCGTACCCTCAACAGCTATATGGACAAAGAAGGGTACAAC 480
K122            GGTTTCCGTACTGCGATGACCCGTACCCTCAACAGCTATATGGACAAAGAAGGGTACAAC 480
HF85            GGTTTCCGTACCGCGATGACTCGTACCCTCAACAGCTATATGGACAAAGAAGGGTCCACC 480
HL81            GGTTTCCGTACCGCGATGACTCGTACCCTCAACAGCTATATGGACAAAGAAGGGTACAAC 480
HW79            GGTTTCCGTACCGCGATGACTCGTACCCTCAACAGCTATATGGACAAAGAAGGGTACAAC 480
XlLit           GGTTTCCGTACCGCGATGACTCGTACCCTCAACAGCTATATGGACAAAGAAGGGTACAAC 480
XlNach          GGTTTCCGTACCGCGATGACTCGTACCCTCAACAGCTATATGGACAAAGAAGGGTCCACC 480
H3016           GGTTTCCGTACCGCGATGACTCGTACCCTCAACAGCTATATGGACAAAGAAGGGTACAAC 480
H3173           GGTTTCCGTACCGCGATGACTCGTACCCTCAACAGCTATATGGACAAAGAAGGGTACAAC 480
H3179           GGTTTCCGTACTGCGATGACCCGTACCCTCAACAGCTATATGGACAAAGAAGGGTACAAC 480
H3182           GGTTTCCGTACTGCGATGACCCGTACCCTCAACAGCTATATGGACAAAGAAGGGTACAAC 480
C1              GGTTTCCGTACTGCGATGACCCGTACTCTCAATAGCTATATGGACAAAGAAGGGTACAAC 480
Meg             GGTTTCCGTACTGCGATGACCCGTACTCTCAATAGCTATATGGACAAAGAAGGGTACAAC 480
Habana          GGTTTCCGTACTGCGATGACCCGTACTCTCAATAGCTATATGGACAAAGAAGGGTACAAC 480
NZH3            GGTTTCCGTACCGCAATGACCCGTACCCTCAACAGCTATATGGACAAAGAAGGGTATACC 480
T327            GGTTTCCGTACCGCAATGACCCGTACCCTCAACAGCTATATGGACAAAGAAGGGTATAAC 480
USCA01          GGTTTCCGTACCGCAATGACCCGTACCCTCAACAGCTATATGGACAAAGAAGGGTATAAC 480
H3210           GGTTTCCGTACCGCGATGACCCGTACCCTCAACAGCTATATGGACAAAGAAGGGTACAAC 480
CIP108426       GGTTTCCGTACCGCGATGACCCGTACCCTCAACAGCTATATGGACAAAGAAGGGTACAAC 480
FR32            GGTTTCCGTACCGCGATGACCCGTACCCTCAACAGCTATATGGACAAAGAAGGGTACAAC 480
H111            GGTTTCCGTACTGCGATGACCCGTACCCTCAACAGCTATATGGACAAAGAAGGGTACAAC 480
H267            GGTTTCCGTACCGCGATGACTCGTACCCTTAATAGCTACATGGATAAAGAGGGGTACAAC 480
H295            GGTTTCCGTACCGCGATGACTCGTACCCTCAACAGCTATATGGACAAAGAAGGGTACAAC 480
H3086           GGTTTCCGTACCGCGATGACTCGTACCCTTAATAGCTACATGGATAAAGAGGGGTACAAC 480
AN6             GGTTTCCGTACCGCGATGACCCGAACCCTCAATAGCTACATGGATAAAGAGGGCTACAAC 480
                *********** ** ***** ** ** ** ** ***** ***** ***** ** *  * *

H3107           AAGAAATCCAAAGTCAGCGCTACTGGTGATGATGCCCGTGAAGGTTTAGTCGCCGTCATT 540
H3240           AAGAAATCCAAAGTCAGCGCTACTGGTGATGATGCCCGTGAAGGTTTAGTCGCCGTCATT 540
H3014           AAGAAATCCAAAGTCAGCGCTACTGGTGATGATGCCCGTGAAGGTTTAGTCGCCGTCATT 540
pur1            AAGAAATCCAAAGTCAGCGCTACTGGTGATGATGCCCGTGAAGGTTTAGTCGCCGTCATT 540
pur2            AAGAAATCCAAAGTCAGCGCTACTGGTGATGATGCCCGTGAAGGTTTAGTCGCCGTCATT 540
pur3            AAGAAATCCAAAGTCAGCGCTACTGGTGATGATGCCCGTGAAGGTTTAGTCGCCGTCATT 540
yel1            AAGAAATCCAAAGTCAGCGCCACCGGTGATGATGCCCGCGAGGGTTTAGTCGCCGTCATT 540
yel2            AAGAAATCCAAAGTCAGCGCCACCGGTGATGATGCCCGCGAGGGTTTAGTCGCCGTCATT 540
yel3            AAGAAATCCAAAGTCAGCGCCACCGGTGATGATGCCCGCGAGGGTTTAGTCGCCGTCATT 540
K122            AAGAAATCCAAAGTCAGCGCCACCGGTGATGATGCCCGCGAGGGTTTAGTCGCCGTCATT 540
HF85            AAGAAATCTAAAGTCAGCGCCACCGGTGATGATGCCCGCGAGGGTTTAGTCGCCGTCATT 540
HL81            AAGAAATCTAAAGACAGCGCCACCGGTGATGATGCCCGCGAGGGTTTAGTCGCCGTCATT 540
HW79            AAGAAATCTAAAGTCAGCGCCACCGGTGATGATGCCCGCGAGGGTTTAGTCGCCGTCATT 540
XlLit           AAGAAATCTAAAGTCAGCGCCACCGGTGATGATGCCCGCCAGGGTTTAGTCGCCGTCATT 540
XlNach          AAGAAATCTAAAGTCAGCGCCACCGGTGATGATGCCCGCCAGGGTTTAGTCGCCGTCATT 540
H3016           AAGAAATCTAAAGTCAGCGCCACCGGTGATGATGCCCGCGAGGGTTTAGTCGCCGTCATT 540
H3173           AAGAAATCTAAAGTCAGCGCCACCGGTGATGATGCCCGCGAGGGTTTAGTCGCCGTCATT 540
H3179           AAGAAATCCAAAGTCAGCGCCACCGGTGATGATGCCCGCGAGGGTTTAGTCGCCGTCATT 540
H3182           AAGAAATCCAAAGTCAGCGCCACCGGTGATGATGCCCGCGAGGGTTTAGTCGCCGTCATT 540
C1              AAGAAATCTAAAGTCAGCGCCACCGGTGATGATGCCCGTGAAGGCTTGGTCGCAGTTATT 540
Meg             AAGAAATCTAAAGTCAGCGCCACCGGTGATGATGCCCGTGAAGGCTTGGTCGCAGTTATT 540
Habana          AAGAAATCTAAAGTCAGCGCCACCGGTGATGATGCCCGTGAAGGCTTGGTCGCAGTTATT 540
NZH3            AAGAAATCTAAAGTCAGTGCCACCGGTGATGATGCCCGTGAGGGTTTGGTCGCGGTTATT 540
T327            AAGAAATCTAAAGTCAGTGCCACCGGTGATGATGCCCGTGAGGGTTTGGTCGCGGTTATT 540
USCA01          AAGAAATCTAAAGTCAGTGCCACCGGTGATGATGCCCGTGAGGGTTTGGTCGCGGTTATT 540
H3210           AAGAAATCTAAAGTCAGCGCCACCGGTGATGATGCCCGTGAAGGCTTAGTCGCCGTTATT 540
CIP108426       AAGAAATCTAAAGTCAGCGCCACCGGTGATGATGCCCGTGAAGGCTTAGTCGCCGTTATT 540
FR32            AAGAAATCTAAAGTCAGCGCCACCGGTGATGATGCCCGTGAAGGCTTAGTCGCCGTTATT 540
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H111            AAGAAATCCAAAGTCAGCGCCACCGGTGATGATGCCCGCGAGGGTTTAGTCGCCGTCATT 540
H267            AAGAAATCCAAAGTCAGCGCTACTGGTGATGATGCCCGTGAAGGTTTAGTCGCCGTCATT 540
H295            AAGAAATCTAAAGTCAGCGCCACCGGTGATGATGCCCGCGAGGGTTTAGTCGCCGTCATT 540
H3086           AAGAAATCCAAAGTCAGCGCTACTGGTGATGATGCCCGTGAAGGTTTAGTCGCCGTCATT 540
AN6             AAAAAATCCAAAGTCAGCGCCACCGGTGATGATGCCCGTGAAGGTTTGATTGCAGTTATT 540
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H3107           TCCGTTAAAGTACCTGATCCAAAATTCTCTTCTCAGACTAAAGATAAACTAGTCTCTTCC 600
H3240           TCCGTTAAAGTACCTGATCCAAAATTCTCTTCTCAGACTAAAGATAAACTAGTCTCTTCC 600
H3014           TCCGTTAAAGTACCTGATCCAAAATTCTCTTCTCAGACTAAAGATAAACTAGTCTCTTCC 600
pur1            TCCGTTAAAGTACCTGATCCAAAATTCTCTTCTCAGACTAAAGATAAACTAGTCTCTTCC 600
pur2            TCCGTTAAAGTACCTGATCCAAAATTCTCTTCTCAGACTAAAGATAAACTAGTCTCTTCC 600
pur3            TCCGTTAAAGTACCTGATCCAAAATTCTCTTCTCAGACTAAAGATAAACTAGTCTCTTCC 600
yel1            TCCGTTAAAGTACCTGATCCAAAATTCTCTTCTCAGACCAAAGACAAACTGGTTTCTTCC 600
yel2            TCCGTTAAAGTACCTGATCCAAAATTCTCTTCTCAGACCAAAGACAAACTGGTTTCTTCC 600
yel3            TCCGTTAAAGTACCTGATCCAAAATTCTCTTCTCAGACCAAAGACAAACTGGTTTCTTCC 600
K122            TCCGTTAAAGTACCTGATCCAAAATTCTCTTCTCAGACCAAAGACAAACTGGTTTCTTCC 600
HF85            TCCGTTAAAGTACCTGATCCAAAATTCTCTTCTCAGACCAAAGACAAACTGGTTTCTTCC 600
HL81            TCCGTTAAAGTACCTGATCCAAAATTCTCTTCTCAGACCAAAGACAAACTGGTTTCTTCC 600
HW79            TCCGTTAAAGTACCTGATCCAAAATTCTCTTCTCAGACCAAAGACAAACTGGTTTCTTCC 600
XlLit           TCCGTTAAAGTACCTGATCCAAAATTCTCTTCTCAGACCAAAGACAAACTGGTTTCTTCC 600
XlNach          TCCGTTAAAGTACCTGATCCAAAATTCTCTTCTCAGACCAAAGACAAACTGGTTTCTTCC 600
H3016           TCCGTTAAAGTACCTGATCCAAAATTCTCTTCTCAGACCAAAGACAAACTGGTTTCTTCC 600
H3173           TCCGTTAAAGTACCTGATCCAAAATTCTCTTCTCAGACCAAAGACAAACTGGTTTCTTCC 600
H3179           TCCGTTAAAGTACCTGATCCAAAATTCTCTTCTCAGACCAAAGACAAACTGGTTTCTTCC 600
H3182           TCCGTTAAAGTACCTGATCCAAAATTCTCTTCTCAGACCAAAGACAAACTGGTTTCTTCC 600
C1              TCCGTTAAAGTACCTGATCCAAAATTCTCTTCTCAGACCAAAGACAAACTGGTTTCTTCC 600
Meg             TCCGTTAAAGTACCTGATCCAAAATTCTCTTCTCAGACCAAAGACAAACTGGTTTCTTCC 600
Habana          TCCGTTAAAGTACCTGATCCAAAATTCTCTTCTCAGACCAAAGACAAACTGGTTTCTTCC 600
NZH3            TCCGTTAAAGTCCCTGATCCAAAATTCTCTTCTCAGACCAAAGACAAACTGGTTTCTTCC 600
T327            TCCGTTAAAGTCCCTGATCCAAAATTCTCTTCTCAGACCAAAGACAAACTGGTTTCTTCC 600
USCA01          TCCGTTAAAGTCCCTGATCCAAAATTCTCTTCTCAGACCAAAGACAAACTGGTTTCTTCC 600
H3210           TCCGTTAAAGTACCTGATCCAAAATTCTCTTCTCAGACCAAAGACAAACTGGTTTCTTCC 600
CIP108426       TCCGTTAAAGTACCTGATCCAAAATTCTCTTCTCAGACCAAAGACAAACTGGTTTCTTCC 600
FR32            TCCGTTAAAGTACCTGATCCAAAATTCTCTTCTCAGACCAAAGACAAACTGGTTTCTTCC 600
H111            TCCGTTAAAGTACCTGATCCAAAATTCTCTTCTCAGACCAAAGACAAACTGGTTTCTTCC 600
H267            TCCGTTAAAGTACCTGATCCAAAATTCTCTTCTCAGACTAAAGATAAACTAGTCTCTTCC 600
H295            TCCGTTAAAGTACCTGATCCAAAATTCTCTTCTCAGACCAAAGACAAACTGGTTTCTTCC 600
H3086           TCCGTTAAAGTACCTGATCCAAAATTCTCTTCTCAGACTAAAGATAAACTAGTCTCTTCC 600
AN6             TCCGTTAAAGTACCTGATCCTAAATTCTCTTCCCAGACGAAAGACAAACTGGTTTCTTCC 600
                *********** ******** *********** ***** ***** ***** ** ******

H3107           GAAGTGAAAACCGCAGTTGAAACACTGATGAATGAGAAGCTGGTGGAATATCTGCTGGAA 660
H3240           GAAGTGAAAACCGCAGTTGAAACACTGATGAATGAGAAGCTGGTGGAATATCTGCTGGAA 660
H3014           GAAGTGAAAACCGCAGTTGAAACACTGATGAATGAGAAGCTGGTGGAATATCTGCTGGAA 660
pur1            GAAGTGAAAACCGCAGTTGAAACACTGATGAATGAGAAGCTGGTGGAATATCTGCTGGAA 660
pur2            GAAGTGAAAACCGCAGTTGAAACACTGATGAATGAGAAGCTGGTGGAATATCTGCTGGAA 660
pur3            GAAGTGAAAACCGCAGTTGAAACACTGATGAATGAGAAGCTGGTGGAATATCTGCTGGAA 660
yel1            GAGGTGAAAACGGCGGTTGAAACACTGATGAACGAGAAGCTGGTGGAATACCTGCTGGAA 660
yel2            GAGGTGAAAACGGCGGTTGAAACACTGATGAACGAGAAGCTGGTGGAATACCTGCTGGAA 660
yel3            GAGGTGAAAACGGCGGTTGAAACACTGATGAACGAGAAGCTGGTGGAATACCTGCTGGAA 660
K122            GAGGTGAAAACGGCGGTTGAAACACTGATGAACGAGAAGCTGGTGGAATACCTGCTGGAA 660
HF85            GAGGTGAAAACGGCGGTTGAAACACTGATGAACGAGAAGCTGGTGGAATACCTGCTGGAA 660
HL81            GAGGTGAAAACGGCGGTTGAAACACTGATGAACGAGAAGCTGGTGGAATACCTGCTGGAA 660
HW79            GAGGTGAAAACGGCGGTTGAAACACTGATGAACGAGAAGCTGGTGGAATACCTGCTGGAA 660
XlLit           GAGGTGAAAACGGCGGTTGAAACACTGATGAACGAGAAGCTGGTGGAATACCTGCTGGAA 660
XlNach          GAGGTGAAAACGGCGGTTGAAACACTGATGAACGAGAAGCTGGTGGAATACCTGCTGGAA 660
H3016           GAGGTGAAAACGGCGGTTGAAACACTGATGAACGAGAAGCTGGTGGAATACCTGCTGGAA 660
H3173           GAGGTGAAAACGGCGGTTGAAACACTGATGAACGAGAAGCTGGTGGAATACCTGCTGGAA 660
H3179           GAGGTGAAAACGGCGGTTGAAACACTGATGAACGAGAAGCTGGTGGAATACCTGCTGGAA 660
H3182           GAGGTGAAAACGGCGGTTGAAACACTGATGAACGAGAAGCTGGTGGAATACCTGCTGGAA 660
C1              GAGGTGAAAACGGCGGTTGAAACACTGATGAACGAGAAGCTGGTGGAATACCTGCTGGAA 660
Meg             GAGGTGAAAACGGCGGTTGAAACACTGATGAACGAGAAGCTGGTGGAATACCTGCTGGAA 660
Habana          GAGGTGAAAACGGCGGTTGAAACACTGATGAACGAGAAGCTGGTGGAATACCTGCTGGAA 660
NZH3            GAAGTGAAAACAGCAGTTGAAACACTGATGAACGAGAAGCTGGTGGAATACCTGCTGGAA 660
T327            GAAGTGAAAACAGCAGTTGAAACACTGATGAACGAGAAGCTGGTGGAATACCTGCTGGAA 660
USCA01          GAAGTGAAAACAGCAGTTGAAACACTGATGAACGAGAAGCTGGTGGAATACCTGCTGGAA 660
H3210           GAGGTGAAAACGGCGGTTGAAACACTGATGAACGAGAAGCTGGTGGAATACCTGCTGGAA 660
CIP108426       GAGGTGAAAACGGCGGTTGAAACACTGATGAACGAGAAGCTGGTGGAATACCTGCTGGAA 660
FR32            GAGGTGAAAACGGCGGTTGAAACACTGATGAACGAGAAGCTGGTGGAATACCTGCTGGAA 660
H111            GAGGTGAAAACGGCGGTTGAAACACTGATGAACGAGAAGCTGGTGGAATACCTGCTGGAA 660
H267            GAAGTGAAAACCGCAGTTGAAACACTGATGAATGAGAAGCTGGTGGAATATCTGCTGGAA 660
H295            GAGGTGAAAACGGCGGTTGAAACACTGATGAACGAGAAGCTGGTGGAATACCTGCTGGAA 660
H3086           GAAGTGAAAACCGCAGTTGAAACACTGATGAATGAGAAGCTGGTGGAATATCTGCTGGAA 660
AN6             GAAGTGAAAACGGCGGTCGAAACGCTGATGAATGAGAAGCTGGTGGAATACCTGCTGGAA 660
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H3107           AACCCAAATGACGCCAAAACAGTCGTCAGTAAAATTATTGATGCTGCTCGTGCCCGTGAA 720
H3240           AACCCAAATGACGCCAAAACAGTCGTCAGTAAAATTATTGATGCTGCTCGTGCCCGTGAA 720
H3014           AACCCAAATGACGCCAAAACAGTCGTCAGTAAAATTATTGATGCTGCTCGTGCCCGTGAA 720
pur1            AACCCAAATGACGCCAAAACAGTCGTCAGTAAAATTATTGATGCTGCTCGTGCCCGTGAA 720
pur2            AACCCAAATGACGCCAAAACAGTCGTCAGTAAAATTATTGATGCTGCTCGTGCCCGTGAA 720
pur3            AACCCAAATGACGCCAAAACAGTCGTCAGTAAAATTATTGATGCTGCTCGTGCCCGTGAA 720
yel1            AACCCGAATGACGCTAAAACCGTCGTCGGTAAAATTATCGACGCTGCCCGCGCCCGTGAA 720
yel2            AACCCGAATGACGCTAAAACCGTCGTCGGTAAAATTATCGACGCTGCCCGCGCCCGTGAA 720
yel3            AACCCGAATGACGCTAAAACCGTCGTCGGTAAAATTATCGACGCTGCCCGCGCCCGTGAA 720
K122            AACCCGAATGACGCTAAAACCGTCGTCGGTAAAATTATCGACGCTGCCCGCGCCCGTGAA 720
HF85            AACCCGAATGACGCTAAAACCGTCGTCGGTAAAATTATCGACGCTGCCCGCGCCCGTGAA 720
HL81            AACCCGAATGACGCTAAAACCGTCGTCGGTAAAATTATCGACGCTGCCCGCGCCCGTGAA 720
HW79            AACCCGAATGACGCTAAAACCGTCGTCGGTAAAATTATCGACGCTGCCCGCGCCCGTGAA 720
XlLit           AACCCGAATGACGCTAAAACCGTCGTCGGTAAAATTATCGACGCTGCCCGCGCCCGTGAA 720
XlNach          AACCCGAATGACGCTAAAACCGTCGTCGGTAAAATTATCGACGCTGCCCGCGCCCGTGAA 720
H3016           AACCCGAATGACGCTAAAACCGTCGTCGGTAAAATTATCGACGCTGCCCGCGCCCGTGAA 720
H3173           AACCCGAATGACGCTAAAACCGTCGTCGGTAAAATTATCGACGCTGCCCGCGCCCGTGAA 720
H3179           AACCCGAATGACGCTAAAACCGTCGTCGGTAAAATTATCGACGCTGCCCGCGCCCGTGAA 720
H3182           AACCCGAATGACGCTAAAACCGTCGTCGGTAAAATTATCGACGCTGCCCGCGCCCGTGAA 720
C1              AACCCGAATGACGCTAAAACCGTCGTCGGTAAAATTATCGACGCAGCCCGCGCCCGTGAA 720
Meg             AACCCGAATGACGCTAAAACCGTCGTCGGTAAAATTATCGACGCCGCCCGCGCCCGTGAA 720
Habana          AACCCGAATGACGCTAAAACCGTCGTCGGTAAAATTATCGACGCAGCCCGCGCCCGTGAA 720
NZH3            AATCCAAATGACGCCAAAACCGTCGTCGGCAAAATTATCGACGCGGCCCGCGCTCGTGAA 720
T327            AATCCAAATGACGCCAAAACCGTCGTCGGCAAAATTATCGACGCGGCCCGCGCTCGTGAA 720
USCA01          AATCCAAATGACGCCAAAACCGTCGTCGGCAAAATTATCGACGCGGCCCGCGCTCGTGAA 720
H3210           AACCCGAATGACGCTAAAACCGTCGTCGGTAAAATTATCGATGCTGCCCGCGCCCGCGAA 720
CIP108426       AACCCGAATGACGCTAAAACCGTCGTCGGTAAAATTATCGATGCTGCCCGCGCCCGCGAA 720
FR32            AACCCGAATGACGCTAAAACCGTCGTCGGTAAAATTATCGATGCTGCCCGCGCCCGCGAA 720
H111            AACCCGAATGACGCTAAAACCGTCGTCGGTAAAATTATCGACGCTGCCCGCGCCCGTGAA 720
H267            AACCCAAATGACGCCAAAACAGTCGTCAGTAAAATTATTGATGCTGCTCGTGCCCGTGAA 720
H295            AACCCGAATGACGCTAAAACCGTCGTCGGTAAAATTATCGACGCTGCCCGCGCCCGTGAA 720
H3086           AACCCAAATGACGCCAAAACAGTCGTCAGTAAAATTATTGATGCTGCTCGTGCCCGTGAA 720
AN6             AACCCGAACGACGCCAAAACGGTTGTCGGCAAAATCATTGATGCGGCTCGCGCCCGTGAA 720
                ** ** ** ***** ***** ** *** * ***** ** ** ** ** ** ** ** ***

H3107           GCAGCCCGTAAAGCACGCGAAATGACACGCCGCAAAGGGGCTCTTGATCTGGCTGGCTTA 780
H3240           GCAGCCCGTAAAGCACGCGAAATGACACGCCGCAAAGGGGCTCTTGATCTGGCTGGCTTA 780
H3014           GCAGCCCGTAAAGCACGCGAAATGACACGCCGCAAAGGGGCTCTTGATCTGGCTGGCTTA 780
pur1            GCAGCCCGTAAAGCACGCGAAATGACACGCCGCAAAGGGGCTCTTGATCTGGCTGGCTTA 780
pur2            GCAGCCCGTAAAGCACGCGAAATGACACGCCGCAAAGGGGCTCTTGATCTGGCTGGCTTA 780
pur3            GCAGCCCGTAAAGCACGCGAAATGACACGCCGCAAAGGGGCTCTTGATCTGGCTGGCTTA 780
yel1            GCAGCCCGTAAAGCACGTGAAATGACACGCCGCAAAGGGGCTCTTGATCTGGCTGGCCTG 780
yel2            GCAGCCCGTAAAGCACGTGAAATGACACGCCGCAAAGGGGCTCTTGATCTGGCTGGCCTG 780
yel3            GCAGCCCGTAAAGCACGTGAAATGACACGCCGCAAAGGGGCTCTTGATCTGGCTGGCCTG 780
K122            GCAGCCCGTAAAGCACGTGAAATGACACGCCGCAAAGGGGCTCTTGATCTGGCTGGCCTG 780
HF85            GCAGCCCGTAAAGCACGTGAAATGACACGCCGCAAAGGGGCTCTTGATCTGGCTGGCCTG 780
HL81            GCAGCCCGTAAAGCACGTGAAATGACACGCCGCAAAGGGGCTCTTGATCTGGCTGGCCTG 780
HW79            GCAGCCCGTAAAGCACGTGAAATGACACGCCGCAAAGGGGCTCTTGATCTGGCTGGCCTG 780
XlLit           GCAGCCCGTAAAGCACGTGAAATGACACGCCGCAAAGGGGCTCTTGATCTGGCTGGCCTG 780
XlNach          GCAGCCCGTAAAGCACGTGAAATGACACGCCGCAAAGGGGCTCTTGATCTGGCTGGCCTG 780
H3016           GCAGCCCGTAAAGCACGTGAAATGACACGCCGCAAAGGGGCTCTTGATCTGGCTGGCCTG 780
H3173           GCAGCCCGTAAAGCACGTGAAATGACACGCCGCAAAGGGGCTCTTGATCTGGCTGGCCTG 780
H3179           GCAGCCCGTAAAGCACGTGAAATGACACGCCGCAAAGGGGCTCTTGATCTGGCTGGCCTG 780
H3182           GCAGCCCGTAAAGCACGTGAAATGACACGCCGCAAAGGGGCTCTTGATCTGGCTGGCCTG 780
C1              GCAGCCCGTAAAGCACGCGAAATGACACGCCGCAAAGGGGCTCTTGATCTAGCTGGTCTG 780
Meg             GCAGCCCGTAAAGCACGCGAAATGACACGCCGCAAAGGGGCTCTTGATCTAGCTGGTCTG 780
Habana          GCAGCCCGTAAAGCACGCGAAATGACACGCCGCAAAGGGGCTCTTGATCTAGCTGGTCTG 780
NZH3            GCAGCTCGTAAAGCACGCGAAATGACACGTCGCAAAGGAGCGCTTGATCTAGCCGGTCTG 780
T327            GCAGCTCGTAAAGCACGCGAAATGACACGTCGCAAAGGAGCGCTTGATCTGGCCGGTCTG 780
USCA01          GCAGCTCGTAAAGCACGCGAAATGACACGTCGCAAAGGAGCGCTTGATCTGGCCGGTCTG 780
H3210           GCAGCCCGTAAAGCACGCGAAATGACACGCCGCAAAGGGGCTCTTGATCTGGCTGGTTTG 780
CIP108426       GCAGCCCGTAAAGCACGCGAAATGACACGCCGCAAAGGGGCTCTTGATCTGGCTGGTTTG 780
FR32            GCAGCCCGTAAAGCACGCGAAATGACACGCCGCAAAGGGGCTCTTGATCTGGCTGGTTTG 780
H111            GCAGCCCGTAAAGCACGTGAAATGACACGCCGCAAAGGGGCTCTTGATCTGGCTGGCCTG 780
H267            GCAGCCCGTAAAGCACGCGAAATGACACGCCGCAAAGGGGCTCTTGATCTGGCTGGCTTA 780
H295            GCAGCCCGTAAAGCACGTGAAATGACACGCCGCAAAGGGGCTCTTGATCTGGCTGGCCTG 780
H3086           GCAGCCCGTAAAGCACGCGAAATGACACGCCGCAAAGGGGCTCTTGATCTGGCTGGCTTA 780
AN6             GCTGCACGTAAAGCTCGTGAAATGACCCGTCGTAAAGGTGCACTGGATCTGGCCGGTTTG 780
                ** ** ******** ** ******** ** ** ***** ** ** ***** ** **  *

H3107           CCAGGCAAACTAGCTGATTGTCAGGAACGTGACCCGGCATTGTCCGAACTCTACTTAGTG 840
H3240           CCAGGCAAACTAGCTGATTGTCAGGAACGTGACCCGGCATTGTCCGAACTCTACTTAGTG 840
H3014           CCAGGCAAACTAGCTGATTGTCAGGAACGTGACCCGGCATTGTCCGAACTCTACTTAGTG 840
pur1            CCAGGCAAACTAGCTGATTGTCAGGAACGTGACCCGGCATTGTCCGAACTCTACTTAGTG 840
pur2            CCAGGCAAACTAGCTGATTGTCAGGAACGTGACCCGGCATTGTCCGAACTCTACTTAGTG 840
pur3            CCAGGCAAACTAGCTGATTGTCAGGAACGTGACCCGGCATTGTCCGAACTCTACTTAGTG 840
yel1            CCAGGCAAACTGGCGGATTGTCAGGAACGTGATCCGGCACTGTCCGAACTCTACTTAGTG 840
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yel2            CCAGGCAAACTGGCGGATTGTCAGGAACGTGATCCGGCACTGTCCGAACTCTACTTAGTG 840
yel3            CCAGGCAAACTGGCGGATTGTCAGGAACGTGATCCGGCACTGTCCGAACTCTACTTAGTG 840
K122            CCAGGCAAACTGGCGGATTGTCAGGAACGTGATCCGGCACTGTCCGAACTCTACTTAGTG 840
HF85            CCAGGCAAACTGGCGGATTGTCAGGAACGTGATCCGGCACTGTCCGAACTCTACTTAGTG 840
HL81            CCAGGCAAACTGGCGGATTGTCAGGAACGTGATCCGGCACTGTCCGAACTCTACTTAGTG 840
HW79            CCAGGCAAACTGGCGGATTGTCAGGAACGTGATCCGGCACTGTCCGAACTCTACTTAGTG 840
XlLit           CCAGGCAAACTGGCGGATTGTCAGGAACGTGATCCGGCACTGTCCGAACTCTACTTAGTG 840
XlNach          CCAGGCAAACTGGCGGATTGTCAGGAACGTGATCCGGCACTGTCCGAACTCTACTTAGTG 840
H3016           CCAGGCAAACTGGCGGATTGTCAGGAACGTGATCCGGCACTGTCCGAACTCTACTTAGTG 840
H3173           CCAGGCAAACTGGCGGATTGTCAGGAACGTGATCCGGCACTGTCCGAACTCTACTTAGTG 840
H3179           CCAGGCAAACTGGCGGATTGTCAGGAACGTGATCCGGCACTGTCCGAACTCTACTTAGTG 840
H3182           CCAGGCAAACTGGCGGATTGTCAGGAACGTGATCCGGCACTGTCCGAACTCTACTTAGTG 840
C1              CCCGGCAAACTAGCAGATTGTCAGGAGCGTGATCCGGCACTGTCCGAACTCTACTTAGTG 840
Meg             CCCGGCAAACTAGCAGATTGTCAGGAGCGTGATCCGGCACTGTCCGAACTCTACTTAGTG 840
Habana          CCCGGCAAACTAGCAGATTGTCAGGAGCGTGATCCGGCACTGTCCGAACTCTACTTAGTG 840
NZH3            CCTGGCAAACTGGCAGATTGCCAGGAGCGTGACCCTGCGCTGTCCGAACTCTACTTAGTG 840
T327            CCTGGCAAACTGGCAGATTGCCAGGAGCGTGACCCTGCGCTGTCCGAACTCTACTTAGTG 840
USCA01          CCTGGCAAACTGGCAGATTGCCAGGAGCGTGACCCTGCGCTGTCCGAACTCTACTTAGTG 840
H3210           CCCGGCAAACTAGCAGATTGCCAGGAGCGTGATCCGGCACTGTCCGAACTCTACTTAGTG 840
CIP108426       CCCGGCAAACTAGCAGATTGCCAGGAGCGTGATCCGGCACTGTCCGAACTCTACTTAGTG 840
FR32            CCCGGCAAACTAGCAGATTGCCAGGAGCGTGATCCGGCACTGTCCGAACTCTACTTAGTG 840
H111            CCAGGCAAACTGGCGGATTGTCAGGAACGTGATCCGGCACTGTCCGAACTCTACTTAGTG 840
H267            CCAGGCAAACTAGCTGATTGTCAGGAACGTGACCCGGCATTGTCCGAACTCTACTTAGTG 840
H295            CCAGGCAAACTGGCGGATTGTCAGGAACGTGATCCGGCACTGTCCGAACTCTACTTAGTG 840
H3086           CCAGGCAAACTAGCTGATTGTCAGGAACGTGACCCGGCATTGTCCGAACTCTACTTAGTG 840
AN6             CCGGGCAAACTGGCTGACTGTCAGGAGCGTGACCCGGCTTTGTCCGAACTCTACCTGGTG 840
                ** ******** ** ** ** ***** ***** ** **  ************** * ***

H3107           GAAGGGG 847
H3240           GAAGGGG 847
H3014           GAAGGGG 847
pur1            GAAGGGG 847
pur2            GAAGGGG 847
pur3            GAAGGGG 847
yel1            GAAGGGG 847
yel2            GAAGGGG 847
yel3            GAAGGGG 847
K122            GAAGGGG 847
HF85            GAAGGGG 847
HL81            GAAGGGG 847
HW79            GAAGGGG 847
XlLit           GAAGGGG 847
XlNach          GAAGGGG 847
H3016           GAAGGGG 847
H3173           GAAGGGG 847
H3179           GAAGGGG 847
H3182           GAAGGGG 847
C1              GAAGGGG 847
Meg             GAAGGGG 847
Habana          GAAGGGG 847
NZH3            GAAGGGG 847
T327            GAAGGGG 847
USCA01          GAAGGGG 847
H3210           GAAGGGG 847
CIP108426       GAAGGGG 847
FR32            GAAGGGG 847
H111            GAAGGGG 847
H267            GAAGGGG 847
H295            GAAGGGG 847
H3086           GAAGGGG 847
AN6             GAGGGTG 847
                ** ** *
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Figure A. 7: ClustalW Multiple Sequence Alignment of a portion of the gyrB gene from three *pur* and three *yel Photorhabdus temperata* isolates from North Bull Island, a selection of *P. temperata* and *P. luminescens* species and 1 outgroup (*X. nematophila* strain AN6). Asterisk (∗) denotes conserved sites

Spaces denote nucleotide polymorphism (See Section 2.3.4 Figure 2.17).

Table A 6: Time to first emergence in cross-compatibility experiment. Number of days to first emergence is calculated from the day of nematode infection (day 0) (see Section 3.3.1.3).

	Number of days to first emergence							
	Nematode infection							
	PUR IJs				YEL IJs			
Bacterial treatment	*pur*	*yel*	*pur/yel*	ctrl	*pur*	*yel*	*pur/yel*	ctrl
	16	16	16	17	17	16	16	16
	17	16	16	17	18	16	16	16
	17	16	17	18	18	16	16	16
	17	16	17	19	19	17	16	16
	18	16	17	19	19	17	18	16
	19	16	18	19	20	17	18	17
	19	17	18	20	20	17	18	17
	19	17	19	20	22	20	18	17
	19	17	19	20	23	21	21	17
	20	21	19	21	23	22	31	17
median	18.5	16.0	17.5	19.0	19.5	17.0	18.0	16.5

Table A 7: Results of pairwise comparisons of median time to first emergence of (**A**) PUR1 IJs and (**B**) YEL3 IJs from insects infected with 100 IJs. Insects had previously been injected with 1 x 10^5 cells of *P. temperata* isolates *pur1*, *yel3* or a *pur1/yel3* 1:1 mixture. Insects in the no bacteria treatment were injected with an equal volume of ¼ sterile Ringer's solution only. P values in **bold** indicate a significant difference in median time to first emergence (Mann-Whitney U-test, 95% CI Bonferroni-adjusted significance level of $\alpha = 0.008$) (See Section 3.3.1.3).

A

Bacterial treatment	*pur1*	*yel3*	*pur1/yel3*
yel3	W = 134.5, P =0.0226		
pur1/yel3	W = 116.5, P = 0.3874	W = 81.5, P = 0.0679	
no bacteria	W = 86, P = 0.1480	W = 67.5, **P = 0.0041**	W = 76.5, P = 0.0300

B

Bacterial treatment	*pur1*	*yel3*	*pur1/yel3*
yel3	W = 132.5, P = 0.0388		
pur1/yel3	W = 130.0, P = 0.0592	W = 102.5, P = 0.8762	
no bacteria	W = 152.5, **P = 0.0003**	W = 122.5, P = 0.1625	W = 125.0, P = 0.1176

Table A 8: Results of pairwise comparisons of median time to first emergence of IJs from insects following infection with 100 IJs per insect. Insects had previously been injected with 1 x 10^5 cells of *P. temperata* isolates *pur1*, *yel3*, or a *pur1/yel3* 1:1 mixture. Data was pooled for both nematode types, PUR1 and YEL3, in each bacterial treatment. P values in **bold** indicate a significant difference in median time to first emergence (Mann-Whitney U-test, 95% CI Bonferroni-adjusted significance level of $\alpha = 0.017$)(See Section 3.3.1.3)

Bacterial treatment	*pur1*	*yel3*
yel3	W = 517.5, **P = 0.0031**	
pur1/yel3	W = 489.0, P = 0.0308	W = 363.5, P = 0.1965

Table A 9: Results of Two-way ANOVA comparing the number of IJs emerging from insects by day 4 following first emergence as a proportion of the combined emergence by day 4 and at day 19. All insects were infected with 100 IJs per insect. Nematodes used were PUR1 or YEL3 isolates from North Bull Island. Insects had previously been injected with 1 x 10^5 cells of *P. temperata* isolates *pur1* or *yel3* (See Section 3.3.1.4).

Factor	Test statistic	P
Nematode type	$F_{1,12} = 0.53$	0.482
Bacteria type	$F_{1,12} = 1.79$	0.205
Interaction	$F_{1,12} = 0.00$	0.992

Table A 10: Results of Two-way ANOVA of total number of IJs emerging from *G. mellonella* larvae over a 26 day period. All insects were infected with 100 IJs per insect. Nematodes used were PUR1 or YEL3 isolates from North Bull Island. Insects had previously been injected with 1 x 10^5 cells of *P. temperata* isolates *pur1* or *yel3* (See Section 3.3.1.4).

Factor	Test statistic	P
Nematode type	$F_{1,16} = 2.39$	0.141
Bacteria type	$F_{1,16} = 0.31$	0.587
Interaction	$F_{1,16} = 0.25$	0.622

Table A 11: Two-way comparisons carried out to test whether source cadaver colour tends to predict 1 on 1 bioassay cadaver colour using IJs emerging from *pur1/yel3* 1:1 bacterial treatment. Data is pooled for PUR1 and YEL3 nematodes (See Section 3.3.2).

Source cadaver colour	1 on 1 bioassay cadaver colour	
yellow or not	yellow or not	count
P	*p*	4
P	*py/y*	6
PY/Y	*p*	0
PY/Y	*py/y*	9

Source cadaver colour	1 on 1 bioassay cadaver colour	
purple or not	purple or not	count
P/PY	*py/y*	8
P/PY	*y*	8
Y	*py/y*	1
Y	*y*	2

Source cadaver colour	1 on 1 bioassay cadaver colour	
yellow or not	purple or not	count
P	*p/py*	8
P	*y*	2
PY/Y	*p/py*	1
PY/Y	*y*	8

Source cadaver colour	1 on 1 bioassay cadaver colour	
purple or not	yellow or not	count
P/PY	*p*	4
P/PY	*py/y*	12
Y	*p*	0
Y	*py/y*	3

Table A 12: Results of Two-way ANOVA of percentage of insects dead at 44 hours for wild type and hybrid combinations of nematode and bacteria. All insects were infected with 100 IJs per insect using IJs emerging from cadavers in the *pur1* and *yel3* bacterial treatments in the cross-compatibility experiment. There were four replications of each combination with ten insects per replication (See Section 3.3.3).

Factor	Test statistic	P
Nematode type	$F_{1,12} = 4.27$	0.061
Bacteria type	$F_{1,12} = 0.37$	0.555
Interaction	$F_{1,12} = 0.37$	0.555

Table A 13: Results of pairwise comparisons of median number of insects dead at 48 hours post infection with wild type or hybrid nematode – bacteria combinations. (Mann-Whitney U test, 95% Bonferroni-adjusted significance level of $\alpha = 0.008$) (See Section 3.3.3).

Nematode/ bacteria combination	P/p	Y/p	Y/y
Y/p	W = 23.0, P = 0.1635		
Y/y	W = 25.5, P = 0.0372	W = 21.5, P = 0.3778	
P/y	W = 20.0, P = 0.6084	W = 14.0, P = 0.2881	W = 11.0, P = 0.0530

Table A 14: Number of IJs with and without detectable levels of bacteria in two experiments. Experiment 1 consisted of six isolates from North Bull Island. Experiment 2 consisted of six isolates North Bull Island and the two hybrid nematode /bacteria combinations. N = 20 for all isolates and hybrids except PUR2 (n = 10 in experiment 1) (See Section 3.3.5).

	Experiment 1		Experiment 2	
Nematode	Bacteria	No bacteria	Bacteria	No bacteria
PUR1	19	1	19	1
PUR2	10	0	18	2
PUR3	19	1	19	1
YEL1	18	2	19	1
YEL2	19	1	20	0
YEL3	19	1	20	0
YEL/*pur*	-	-	20	0
PUR/*yel*	-	-	19	1

Table A 15: Results of Two-way ANOVA of percentage of insects dead at 56 hours for wild type and hybrid combinations of nematode and bacteria in a one-on-one continuous exposure infection with *G. mellonella* larvae (See Section 4.3.1.1).

Factor	Test statistic	P
Nematode type	$F_{1,12} = 1.85$	0.198
Bacteria type	$F_{1,12} = 3.87$	0.073
Interaction	$F_{1,12} = 0.02$	0.882

Table A 16: Number of IJs emerging per cadaver from *H. abietes* larvae exposed to 50 IJs of each nematode/bacteria combination (See Section 4.3.2.1).

nematode/bacteria combination	Exp2	Exp3	Exp4
Pp	18218	27983	45933
	43700	18667	72333
	47188	22750	98667
		22960	59750
			65867
Py	50041	14100	79800
	56478	30720	48280
	37520	48167	17967
	49226	25667	79500
		28533	68000
			19000
Yy			108000
			105500
			80467
			46800
			91500
			102250
Yp			27900
			49020
			27500
			70400
			59467
			56640

Table A 17: Total number of insects dead, total number of adult EPN present and median number of IJs invading per insect at three time points. Data are from two ten-on-one bioassays with *G. mellonella*. In each experiment at each time point up to twelve insects per isolate were randomly chosen to assess the number of adults invading. n = 96 per isolate in experiment 1 and n = 48 per isolate in experiment 2 (See Section 4.3.3.1).

Experiment	Day	Isolate	Number of insects dead	Number of insects dissected	Total number of adult EPN present	Median number (/10) of IJs invading per insect
1	2	PUR1	18	10	39	3.5
		PUR2	19	9	29	3
		PUR3	18	12	30	2
		YEL1	8	7	22	3
		YEL2	6	6	26	3.5
		YEL3	13	11	36	3
	3	PUR1	50	12	43	3
		PUR2	49	12	29	2
		PUR3	39	12	36	3
		YEL1	53	12	52	4
		YEL2	57	12	44	3.5
		YEL3	55	12	46	3.5
	4	PUR1	19	11	35	3
		PUR2	11	10	29	3
		PUR3	28	12	36	3
		YEL1	19	12	38	3
		YEL2	17	11	39	4
		YEL3	13	11	33	3
2	2	PUR1	5	3	12	3
		PUR2	10	5	20	3
		PUR3	5	3	11	3
		YEL1	0	0	0	0
		YEL2	1	1	3	3
		YEL3	7	2	7	3.5
	3	PUR1	18	9	24	3
		PUR2	20	9	35	4
		PUR3	17	8	21	2.5
		YEL1	21	11	34	3
		YEL2	35	12	39	3
		YEL3	21	10	39	4
	4	PUR1	9	5	15	3
		PUR2	11	6	21	3.5
		PUR3	10	5	12	2
		YEL1	15	7	21	3
		YEL2	6	3	7	2
		YEL3	11	6	21	3

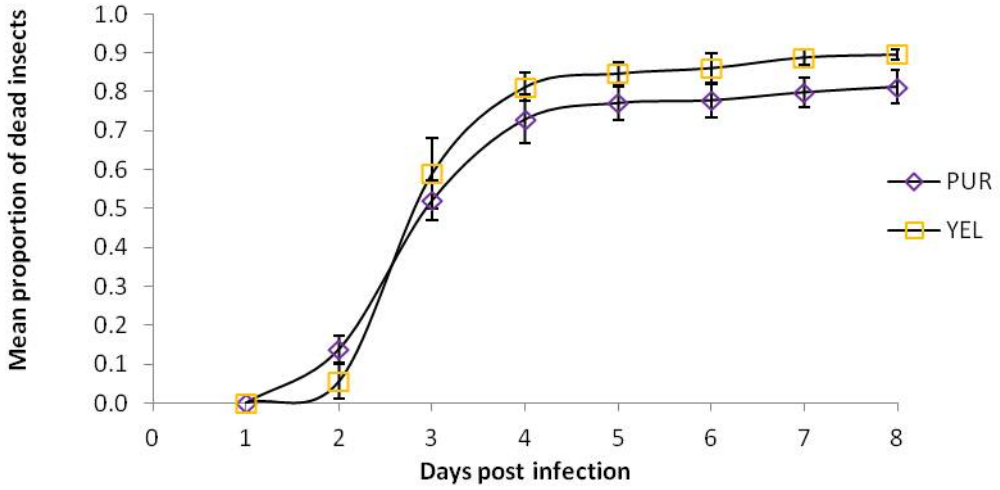

Figure A. 8: Mean proportion (± SE) of *G. mellonella* killed by PUR and YEL IJs over eight days. Data shown is from experiment 2 with three isolates of each colour phenotype, n per colour phenotype = 144. Data were arcsine square root transformed for analysis (See Section 4.3.3.1)

Table A 18: Median number of IJs invading per insect at three time points. Data are from two experiments with *G. mellonella.* Nematodes were applied at a rate of ten IJs per insect. Up to twelve insects were randomly chosen per isolate in each experiment to assess the number of adults invading. n = 96 per isolate in experiment 1 and n = 48 per isolate in experiment 2 (See Section 4.3.3.2).

Experiment	Day	Isolate	Median number (/10) of IJs invading per insect
1	2	PUR1	3.5
		PUR2	3
		PUR3	2
		YEL1	3
		YEL2	3.5
		YEL3	3
	3	PUR1	3
		PUR2	2
		PUR3	3
		YEL1	4
		YEL2	3.5
		YEL3	3.5
	4	PUR1	3
		PUR2	3
		PUR3	3
		YEL1	3
		YEL2	4
		YEL3	3
2	2	PUR1	3
		PUR2	3
		PUR3	3
		YEL1	0
		YEL2	3
		YEL3	3.5
	3	PUR1	3
		PUR2	4
		PUR3	2.5
		YEL1	3
		YEL2	3
		YEL3	4
	4	PUR1	3
		PUR2	3.5
		PUR3	2
		YEL1	3
		YEL2	2
		YEL3	3

Table A 19: Results of pairwise comparisons of median number of adult nematodes per cadaver in insects which were dead on days 2, 3 and 4 post infection for each of 3 PUR and 3 YEL isolates. Data are from experiment 1 of a ten-on-one bioassay with *G. mellonella.* P values in bold indicate a significant difference in median number of adults at the 5% level (Mann-Whitney U-test, 95% Bonferroni-adjusted significance level of $\alpha = 0.003$) (See Section 4.3.3.2, Table 4.7).

	PUR Day 2	PUR Day 3	PUR Day 4	YEL Day 2	YEL Day 3
PUR Day 3	W = 1052.5, P = 0.9894				
PUR Day 4	W = 997.0, P = 0.8877	W = 1242.5, P = 0.8282			
YEL Day 2	W = 794.0, P = 0.1914	W = 1001.5, P = 0.1215	W = 884.0, P = 0.2171		
YEL Day 3	W = 839.5, P = 0.0054	**W = 1031.5, P = 0.0009**	**W = 916.5, P = 0.0030**	W = 633.5, P = 0.1219	
YEL Day 4	W = 952.5, P = 0.3336	W = 1184.5, P = 0.2418	W = 1055.5, P = 0.3818	W = 738.0, P = 0.6197	W = 1466.5, P = 0.0205

Table A 20: Results of pairwise comparisons of median number of adult nematodes per cadaver in insects which were dead on days 2, 3 and 4 post infection for each of 3 PUR and 3 YEL isolates in two ten-on-one bioassays with *G. mellonella.*. Data were pooled by colour phenotype for both experiments. P values in bold indicate a significant difference in median number of adults at the 5% level (Mann-Whitney U-test, 95% Bonferroni-adjusted significance level of $\alpha = 0.003$) (See Section 4.3.3.2, Table 4.8).

	PUR Day 2	PUR Day 3	PUR Day 4	YEL Day 2	YEL Day 3
PUR Day 3	W = 2248.0, P = 0.7665				
PUR Day 4	W = 1970.0, P = 0.7539	W = 3471.0, P = 0.9975			
YEL Day 2	W = 1385.5, P = 0.2800	W = 2619.5, P = 0.1062	W = 1755.5, P = 0.1341		
YEL Day 3	W =1998.5, P = 0.0259	**W = 3382.0, P = 0.0006**	**W = 2363.0, P = 0.0016**	W = 1176.5, P = 0.2557	
YEL Day 4	W = 1922.5, P = 0.8051	W = 3387.2, P = 0.4746	W = 2359.0, P = 0.5032	W = 1138.5, P = 0.3350	W = 4580.0, P = 0.0131

Table A 21: Median time to death, number of cadavers placed on White traps (WT), percentage (%) of cadavers with emergence, and median time to first emergence of infective juveniles for insects infected with one of six isolates of *H. downesi* nematodes from North Bull Island in experiment 2 of a ten-on-one bioassay using *G. mellonella* (See Section 4.3.3.3).

Isolate	median time to death (days)	number of cadavers placed on WT	% cadavers with emergence	median time to emergence (days post infection)
PUR1	3	18	100	17
PUR2	3	22	100	17
PUR3	3	20	95	19
YEL1	3	23	100	19
YEL2	3	22	100	17
YEL3	2.5	22	100	17

While a Kruskal-Wallis test indicated there was a highly significant difference in time to first emergence between isolates, (5 df, H = 18.10, P = 0.003), a series of pairwise comparisons failed to show any differences (Mann-Whitney U-test, 95% Bonferroni-adjusted significance level of $\alpha = 0.003$) (See Table A 22).

Table A 22: Results of pairwise comparisons of median time to first emergence of infective juveniles for six isolates of *H. downesi* nematodes from North Bull Island in experiment 2 of a ten-on-one bioassay using *G. mellonella*. P values in bold indicate a significant difference in median number of adults at the 5% level (Mann-Whitney U-test, 95% Bonferroni-adjusted significance level of $\alpha = 0.003$) (See Section 4.3.3.3).

	PUR1	PUR2	PUR3	YEL1	YEL2
PUR2	W = 414.5 P = 0.2057				
PUR3	W = 268 P = 0.0233	W = 356.5 P = 0.0051			
YEL1	W = 310.5 P = 0.0724	W = 390 P = 0.0074	W = 404.0 P = 0.9186		
YEL2	W = 395.0 P = 0.4735	W = 470.5 P = 0.5570	W = 631.5 P = 0.0182	W = 631.5 P = 0.0182	
YEL3	W = 405.5 P = 0.3124	W = 493.5 P = 0.9801	W = 641.5 P = 0.0094	W = 641.5 P = 0.0094	W = 514.0 P = 0.6506

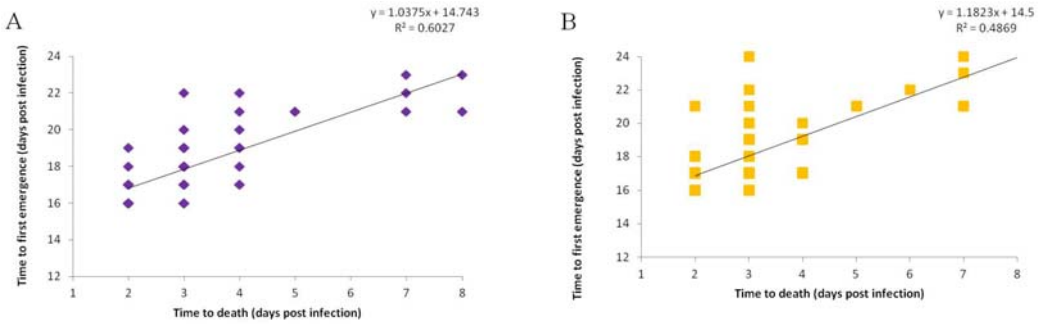

Figure A. 9: Correlation between time to death and time to first emergence of IJs for (A) PUR isolates and (B) YEL isolates in a ten-on-one bioassay with *G. mellonella* (See Section 4.3.3.3).

Table A 23: Total and mean number of IJs emerging over 17 days from *G. mellonella* in experiment 2 of a ten-on-one bioassay. There were two pooled harvests per isolate and three isolates per colour phenotype, with 7 - 12 cadavers per pooled harvest (See Section 4.3.3.4).

Isolate	total harvest	number of cadavers	mean emergence per cadaver
PUR1	892440	7	127491
PUR1	1494670	11	135879
PUR2	1366400	11	124218
PUR2	611800	11	55618
PUR3	1055600	10	105560
PUR3	1515900	9	168433
YEL1	1158720	12	96560
YEL1	1316117	11	119647
YEL2	1362900	11	123900
YEL2	1159667	11	105424
YEL3	1371040	10	137104
YEL3	1744640	12	145387

Table A 24: Results of nested ANOVA of mean number of IJs per cadaver in pooled harvests over a 17 day period from *G. mellonella* in a ten-on-one bioassay. There were two pooled harvest per isolate and three isolates per colour phenotype. See Table A.23 for total counts and n per isolate (See Section 4.3.3.4).

Factor	df		P
Colour phenotype	1	$F_{1,6} = 0.10$	0.925
Isolate	4	$F_{4,6} = 1.207$	0.398

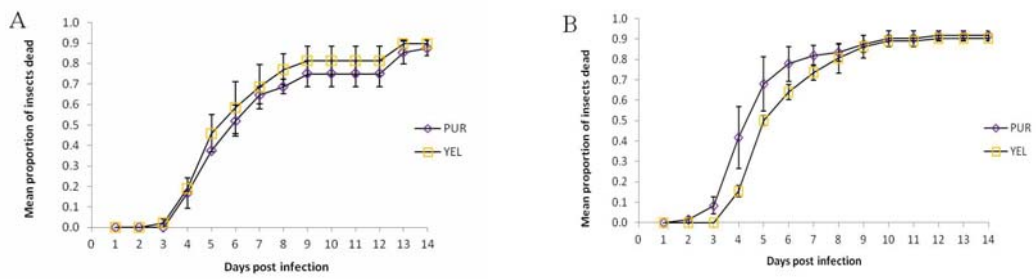

Figure A. 10: Mean (± SE) proportion of *H. abietis* larvae killed by PUR and YEL IJs over fourteen days in two experiments. Data shown is mean of three isolates of each colour type in each experiment. (A) n = 16 and (B) n = 24 per colour isolate (See Section 4.3.4.1).

Table A 25: Cumulative percentage mortality of *H. abietis* larvae due to EPN infection day 4 - 14 post infection in two experiments with 3 PUR and 3 YEL *H. downesi* isolates from North Bull Island. n = 16 per isolate in experiment 1 and n = 24 per isolate in experiment 2. IJs were applied at a rate of fifty to one. Data from both experiments were combined for each isolate and analysed using a one-way ANOVA. Data were arcsine square root transformed for analysis (See Section 4.3.4.1, Figure 4.5).

Experiment	Isolate	Day										
		4	5	6	7	8	9	10	11	12	13	14
1	PUR1	0.31	0.38	0.56	0.69	0.69	0.69	0.69	0.69	0.69	0.94	0.94
	PUR2	0.06	0.38	0.63	0.69	0.75	0.88	0.88	0.88	0.88	0.88	0.88
	PUR3	0.13	0.38	0.38	0.56	0.63	0.69	0.69	0.69	0.69	0.75	0.81
	YEL1	0.19	0.63	0.81	0.88	0.88	0.94	0.94	0.94	0.94	0.94	0.94
	YEL2	0.19	0.31	0.38	0.50	0.63	0.69	0.69	0.69	0.69	0.88	0.88
	YEL3	0.19	0.44	0.56	0.69	0.81	0.81	0.81	0.81	0.81	0.88	0.88
2	PUR1	0.71	0.92	0.92	0.92	0.92	0.92	0.96	0.96	0.96	0.96	0.96
	PUR2	0.21	0.46	0.63	0.75	0.79	0.92	0.92	0.92	0.92	0.92	0.92
	PUR3	0.33	0.67	0.79	0.79	0.79	0.79	0.83	0.83	0.88	0.88	0.88
	YEL1	0.21	0.54	0.71	0.79	0.92	0.92	0.92	0.92	0.92	0.92	0.92
	YEL2	0.13	0.46	0.58	0.75	0.83	0.92	0.92	0.92	0.92	0.92	0.92
	YEL3	0.13	0.50	0.63	0.67	0.67	0.75	0.83	0.83	0.88	0.88	0.88

Table A 26: Mean (± SE) or median time to death, number of cadavers placed on White traps (WT), percentage (%) of cadavers with emergence of IJs, mean (± SE) or median time to first emergence of infective juveniles and mean emergence per cadaver for insects infected with one of six isolates of *H. downesi* nematodes from North Bull Island in two fifty-on-one bioassays using *H. abietis* (See Section 4.3.4.2 and Section 4.3.4.3).

Experiment	Isolate	median time to death (days)	number of cadavers placed on WT	% cadavers with emergence	mean/median time to emergence (days post infection)	Mean emergence per cadaver†
1	PUR1	6	15	80.0	26 (1.25)	47356
	PUR2	6	14	85.7	25.8 (0.70)	49977
	PUR3	7	13	84.6	25.3 (3.30)	45730
	YEL1	5	14	92.9	25.5 (0.99)	57755
	YEL2	7	14	85.7	25.2 (1.37)	45164
	YEL3	5.5	14	100.0	25.2 (0.95)	29528
2	PUR1	4	23	91.3	18	50305
	PUR2	5.5	22	95.5	23	45167
	PUR3	5	21	100.0	20	64285
	YEL1	5	22	95.5	22	50791
	YEL2	6	22	95.5	21	46545
	YEL3	5	21	95.2	21.5	46751

† A single count was carried out on the pooled harvest for each isolate in each experiment.

Table A 27: Results of 2-Sample T-test on percentage emergence for insects infected with one of six isolates of *H. downesi* nematodes from North Bull Island in two fifty-on-one bioassays using *H. abietis* (See Section 4.3.4.3).

			percentage of cadavers with IJ emergence	
Experiment			PUR	YEL
1			83.44	92.86
	P value	P = 0.170		
	test statistic	T =-2.10		
2			95.59	95.38
	P value	P = 0.943		
	test statistic	T = 0.08		
Experiment 1 & 2 combined			89.51	94.12
	P value	P = 0.237		
	test statistic	T = -1.28		

Table A 28: Results of pairwise comparisons of time to first emergence of IJs in experiment 2 of a fifty-on-one bioassay with *H. abietis* larvae. (Mann-Whitney U-test with a 95% Bonferroni confidence interval, α = 0.003) (See Section 4.3.4.2, Table 4.10).

	PUR1	PUR2	PUR3	YEL1	YEL2
PUR2	**W = 320.0, P = 0.0006**				
PUR3	W = 356.0, P = 0.0125	W = 530.0, P = 0.0479			
YEL1	W = 350.0, P = 0.0076	W = 503.5, P = 0.1909	W = 416.5, P = 0.3792		
YEL2	W = 367.0, P = 0.0251	W = 523.0, P = 0.0708	W = 445.0, P = 0.8780	W = 478.5, P = 0.4971	
YEL3	**W = 321.5, P = 0.0013**	W = 471.5, P 0.4315	W = 397.0, P = 0.2526	W = 424.0, P = 0.6642	W = 402.0, P = 0.3102

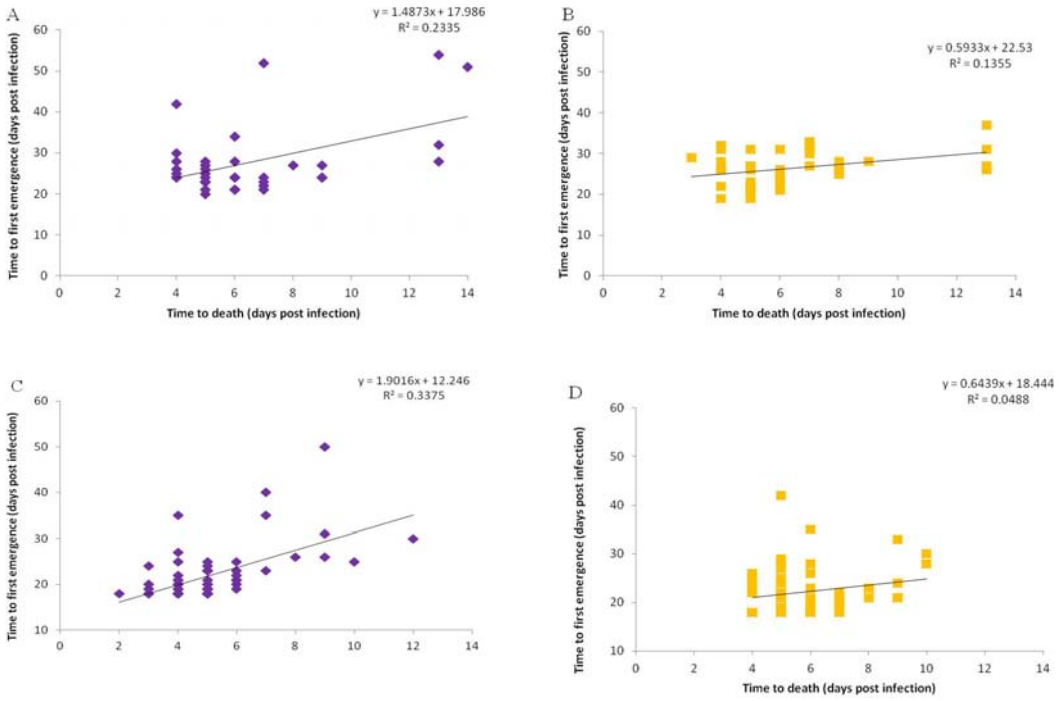

Figure A. 11: Correlation between time to death and time to first emergence of IJs for (A) PUR isolates and (B) YEL isolates in experiment 1 and (C) PUR isolates and (D) YEL isolates in experiment 2 of a one-on-fifty bioassay with *H. abietis* (See Section 4.3.4.2).

Table A 29: Mean (±SE) proportion mortality of kelp fly larvae in a single 48 hour exposure experiment with 3 PUR and 3 YEL *H. downesi* isolates at two concentrations, 1000 or 5000 IJs per 10 larvae. n = 5 for each isolate in each treatment (See Section 4.3.6.1 Figure 4.8A, 4.8B).

	Concentration	
Isolate	1000	5000
PUR1	$0.84_{(0.06)}$	$0.80_{(0.05)}$
PUR2	$0.86_{(0.04)}$	$0.62_{(0.08)}$
PUR3	$0.90_{(0.04)}$	$0.70_{(0.06)}$
YEL1	$0.86_{(0.06)}$	$0.68_{(0.05)}$
YEL2	$0.86_{(0.05)}$	$0.66_{(0.12)}$
YEL3	$0.90_{(0.03)}$	$0.80_{(0.06)}$

Table A 30: Median percentage cadavers (A) per isolate and (B) per colour phenotype with IJ emergence in tests with kelp fly larvae in a single 48 hour exposure experiment with 3 PUR and 3 YEL *H. downesi* isolates at two concentrations, 1000 or 5000 IJs per 10 larva. n= 5 for each isolate in each treatment (See Section 4.3.6.3).

A	Concentration	
Isolate	1000	5000
PUR1	100.00	100.00
PUR2	100.00	100.00
PUR3	100.00	77.78
YEL1	100.00	100.00
YEL2	100.00	100.00
YEL3	77.78	71.43

B	Concentration	
Colour phenotype	1000	5000
PUR	100.00	100.00
YEL	100.00	90.00

Table A 31: Mean (±SE) emergence per cadaver from Kelp fly larvae infected with 1000 IJs per 10 larvae. Data is from individual harvests with n of 3 - 7 per isolate (See Section 4.3.6.3i).

Isolate	Mean (±SE) number of IJs per cadaver
PUR1	8862 (626)
PUR2	8904 (3032)
PUR3	10384 (2807)
YEL1	10075 (860)
YEL2	3981 (1032)
YEL3	7271 (1655)

Table A 32: Mean (±SE) emergence per cadaver from Kelp fly infected with 5000 IJs per 10 larvae. Data is from individual harvests with n of 4 - 9 per isolate (See Section 4.3.6.3i).

Isolate	Mean (±SE) number of IJs per cadaver
PUR1	4258 (1072)
PUR2	11016 (2106)
PUR3	10301 (2348)
YEL1	11168 (1098)
YEL2	5026 (1231)
YEL3	6135 (1104)

Table A 33: Individual (I) or mean (P) emergence per cadaver from Kelp fly larvae in a single 48 hour exposure experiment with 3 PUR and 3 YEL *H. downesi* isolates at two concentrations, 1000 or 5000 IJs per 10 larvae. Emergence from each White trap was counted (I) individually or (P) pooled with up to seven cadavers in pooled counts. There was no difference between individual or mean emergence per cadaver at the isolate or colour phenotype (PUR or YEL) level. (nested GLM with isolate nested within colour and count method (I or P) included as a factor, $P > 0.05$ for all factors) (See Section 4.3.6.3ii).

Harvest	Isolate	Mean emergence per cadaver (1000 IJ concentration)	Mean emergence per cadaver (5000 IJ concentration)
I	PUR1	8862	4258
	PUR2	8904	11016
	PUR3	10384	10301
	YEL1	10075	11168
	YEL2	3981	5026
	YEL3	7271	6135
P	PUR1	4667	7514
	PUR2	3335	4566
	PUR3	5500	7769
	YEL1	2417	8540
	YEL2	1502	4644
	YEL3	4433	8308
P	PUR1	5224	10613
	PUR2	4630	10168
	PUR3	5033	4528
	YEL1	3367	7856
	YEL2	3486	6050
	YEL3	2827	6047
P	PUR1	11939	6976
	PUR2	6333	8094
	PUR3	3146	7904
	YEL1	2665	7060
	YEL2	5313	10941
	YEL3	22647	12767
P	PUR1		7079
	PUR2		9291
	PUR3		11046
	YEL1		11109
	YEL2		3609
	YEL3		9423

Table A 34: Nematode abundance*, survival and life stages‡ present in *G. mellonella* cadavers stored for six days in sand at a range of different moisture levels. n = 3 for each nematode/bacteria combination at each moisture level.

			Day 6				
			Life stages				
			Juveniles		Adults		Bagged worms†
Nematode/bacteria combination	Moisture content	Nematode abundance	Live	Dead	Live	Dead	
Pp	0%	1	1	0	1	0	0
		2	1	0	1	0	0
		1	1	0	1	0	0
	1%	2	1	0	1	0	0
		3	1	0	1	0	0
		2	1	0	1	0	0
	2%	2	1	0	1	0	0
		3	1	0	1	0	0
		3	1	0	1	0	0
	3%	2	1	0	1	0	0
		2	1	0	1	0	0
		2	1	0	1	0	0
	8%	2	1	0	1	0	0
		3	1	0	1	0	0
		3	1	0	1	0	0
Py	0%	1	1	0	1	0	0
		2	1	0	1	0	0
		1	1	0	1	0	0
	1%	1	1	0	1	0	0
		2	1	0	1	0	0
		1	1	0	1	0	0
	2%	2	1	0	1	0	0
		1	1	0	1	0	0
		2	1	0	1	0	0
	3%	2	1	0	1	0	0
		2	1	0	1	0	0
		2	1	0	1	0	0
	8%	1	1	0	1	0	0
		1	1	0	1	0	0
		2	1	0	1	0	0

* Nematode abundance was assessed semi-quantitatively: 1: < 500 nematodes present; 2: 500 - 1000 nematodes present; 3: 1000 - 10000 nematodes present.

‡ Life stages: 1 = present; 0 = absent for each category.

† In a process known as *endotokia matricida*, in each generation some eggs hatch within the maternal body and consume it. These are referred to as bagged worms.

Table A 35: Nematode abundance*, survival and life stages‡ present in *G. mellonella* cadavers stored for nine days in sand at a range of different moisture levels. n = 3 for each nematode/bacteria combination at each moisture level.

		Day 9					
			Life stages				
			Juveniles		Adults		Bagged worms†
Nematode/bacteria combination	Moisture content	Nematode abundance	Live	Dead	Live	Dead	
Pp	0%	3	1	0	1	0	0
		3	1	0	1	0	0
		3	1	0	1	0	0
	1%	3	1	0	1	0	0
		3	1	0	1	0	0
		3	1	0	1	0	0
	2%	3	1	0	1	0	0
		3	1	0	1	0	0
		3	1	0	1	0	0
	3%	3	1	0	1	0	0
		3	1	0	1	0	0
		3	1	0	1	0	0
	8%	3	1	0	1	0	0
		3	1	0	1	0	0
		3	1	0	1	0	0
Py	0%	2	1	0	1	0	0
		2	1	0	1	0	0
		3	1	0	1	0	0
	1%	3	1	0	1	0	0
		2	1	0	1	0	0
		3	1	0	1	0	0
	2%	3	1	0	1	0	0
		3	1	0	1	0	0
		2	1	0	1	0	0
	3%	3	1	0	1	0	0
		3	1	0	1	0	0
		3	1	0	1	0	0
	8%	2	1	0	1	0	0
		3	1	0	1	0	0
		3	1	0	1	0	0

* Nematode abundance was assessed semi-quantitatively: 1: < 500 nematodes present; 2: 500 - 1000 nematodes present; 3: 1000 - 10000 nematodes present.

‡ Life stages: 1 = present; 0 = absent for each category.

† In a process known as *endotokia matricida*, in each generation some eggs hatch within the maternal body and consume it. These are referred to as bagged worms.

Table A 36: Nematode abundance*, survival and life stages‡ present in *G. mellonella* cadavers stored for 12 days in sand at a range of different moisture levels. n = 3 for each nematode/bacteria combination at each moisture level.

		Day 12					
			Life stages				
			Juveniles		Adults		Bagged worms†
Nematode/bacteria combination	Moisture content	Nematode abundance	Live	Dead	Live	Dead	
Pp	0%	3	1	0	1	0	1
		2	1	1	1	1	1
		3	1	0	1	0	1
	1%	3	1	0	1	0	1
		3	1	0	1	0	1
		3	1	0	1	0	1
	2%	3	1	0	1	0	1
		3	1	0	1	0	1
		3	1	0	1	0	1
	3%	3	1	0	1	0	1
		3	1	0	1	0	1
		3	1	0	1	0	1
	8%	3	1	0	1	0	1
		3	1	0	1	0	1
		3	1	0	1	0	1
Py	0%	3	1	0	1	0	1
		3	1	0	1	0	1
		1	1	0	1	0	1
	1%	3	1	0	1	0	1
		3	1	0	1	0	1
		3	1	0	1	0	1
	2%	3	1	0	1	0	1
		3	1	0	1	0	1
		2	1	0	1	0	1
	3%	3	1	0	1	0	1
		3	1	0	1	0	1
		3	1	0	1	0	1
	8%	3	1	0	1	0	1
		3	1	0	1	0	1
		3	1	0	1	0	1

* Nematode abundance was assessed semi-quantitatively: 1: < 500 nematodes present; 2: 500 - 1000 nematodes present; 3: 1000 - 10000 nematodes present.

‡ Life stages: 1 = present; 0 = absent for each category.

† In a process known as *endotokia matricida*, in each generation some eggs hatch within the maternal body and consume it. These are referred to as bagged worms.

Table A 37: Nematode abundance*, survival and life stages‡ present in *G. mellonella* cadavers stored for 15 days in sand at a range of different moisture levels. n = 3 for each nematode/bacteria combination at each moisture level.

			Day 15				
			Life stages				
			Juveniles		Adults		Bagged worms†
Nematode/bacteria combination	Moisture content	Nematode abundance	Live	Dead	Live	Dead	
Pp	0%	3	1	1	1	1	1
		3	1	1	1	1	1
		3	1	1	1	1	1
	1%	3	1	0	1	0	1
		3	1	0	1	0	1
		3	1	0	1	0	1
	2%	3	1	0	1	0	1
		3	1	0	1	0	1
		3	1	0	1	0	1
	3%	3	1	0	1	0	1
		3	1	0	1	0	1
		3	1	0	1	0	1
	8%	3	1	0	1	0	1
		3	1	0	1	0	1
		3	1	0	1	0	1
Py	0%	2	1	0	1	0	1
		1	1	0	1	0	1
		3	1	0	1	0	1
	1%	3	1	0	1	0	1
		3	1	0	1	0	1
		3	1	0	1	0	1
	2%	3	1	0	1	0	1
		2	1	0	1	0	1
		3	1	0	1	0	1
	3%	3	1	0	1	0	1
		3	1	0	1	0	1
		3	1	0	1	0	1
	8%	3	1	0	1	0	1
		3	1	0	1	0	1
		3	1	0	1	0	1

* Nematode abundance was assessed semi-quantitatively: 1: < 500 nematodes present; 2: 500 - 1000 nematodes present; 3: 1000 - 10000 nematodes present.

‡ Life stages: 1 = present; 0 = absent for each category.

† In a process known as *endotokia matricida*, in each generation some eggs hatch within the maternal body and consume it. These are referred to as bagged worms.

Table A 38: Nematode abundance*, survival and life stages‡ present in *G. mellonella* cadavers stored for 18 days in sand at a range of different moisture levels. n = 3 for each nematode/bacteria combination at each moisture level.

			Day 18				
			Life stages				
			Juveniles		Adults		Bagged worms†
Nematode/bacteria combination	Moisture content	Nematode abundance	Live	Dead	Live	Dead	
Pp	0%	2	1	0	1	0	1
		3	1	0	1	0	1
		3	1	0	1	0	1
	1%	3	1	0	1	0	1
		3	1	0	1	0	1
		3	1	0	1	0	1
	2%	3	1	0	1	0	1
		3	1	0	1	0	1
		3	1	0	1	0	1
	3%	3	1	0	1	0	1
		3	1	0	1	0	1
		3	1	0	1	0	1
	8%	3	1	0	1	0	1
		3	1	0	1	0	1
		3	1	0	1	0	1
Py	0%	3	1	0	1	0	1
		3	1	0	1	0	1
		3	1	0	1	0	1
	1%	3	1	0	1	0	1
		3	1	0	1	0	1
		3	1	0	1	0	1
	2%	2	1	0	1	0	1
		3	1	0	1	0	1
		3	1	0	1	0	1
	3%	3	1	0	1	0	1
		2	1	0	1	0	1
		3	1	0	1	0	1
	8%	2	1	0	1	0	1
		2	1	0	1	0	1
		3	1	0	1	0	1

* Nematode abundance was assessed qualitatively: 1 < 500 nematodes; 2 > 1000 nematodes present; 3 > 10,000 nematodes present.

‡ Life stages: 1 = present; 0 = absent for each category.

† In a process known as *endotokia matricida*, in each generation some eggs hatch within the maternal body and consume it. These are referred to as bagged worms.

Table A 39: Numbers of IJs emerging from *G. mellonella* cadavers following desiccation for 28 days at 0% RH in three experiments. n = 8 per nematode/bacteria combination in experiment 2, n = 5 per nematode/bacteria combination in experiment 3 and n = 25 per nematode/bacteria combination in experiment 4 (See Section 5.3.4).

Experiment	Nematode	Bacteria	Numbers of IJs emerging†
2	P	p	5306
	P	p	87500
	P	p	79661
	P	p	65904
	P	p	62248
	Y	p	117491
	Y	p	99329
	Y	p	87800
	Y	p	77971
	Y	y	24666
	Y	y	1227
	Y	y	18
3	P	p	44920
	P	p	3099
	Y	p	1045
	Y	y	39784
	Y	y	23049
	P	y	129
4	P	p	76667
	P	p	61667
	P	p	15000
	P	p	10667
	Y	p	71667
	Y	p	70800
	Y	p	26667
	Y	p	12333
	Y	p	11667
	Y	y	18667

† Overall percentage of cadavers with emergence and total number of IJs emerging by nematode type and bacteria type are shown in Table A 40.

Table A 40: Percentage of cadavers with emergence and total numbers of IJs emerging from *G. mellonella* cadavers following desiccation for 28 days at 0% RH in experiments 2, 3 and 4. The data were pooled by nematode type and by bacteria type and were combined for the three experiments with n = 16 per nematode type or bacteria type in experiment 2, n = 10 per nematode type or bacteria type in experiment 3 and n = 50 per nematode type or bacteria type in experiment 4 (See Section 5.3.4)

	Percentage of cadavers with emergence (n = 76)	Total number of IJs emerging
Nematode type		
PUR	15.8	512767
YEL	21.1	665513
Bacteria type		
pur	27.6	1089408
yel	7.0	107540

Table A 41: Mean (± SE) percentage change in weight of *G. mellonella* cadavers desiccated in sand at varying moisture content for up to 18 days. Data is from experiment 1, chapter 5 (See Section 5.3.5.1).

Duration (days)	Percentage moisture	Nematode/bacteria combination	
		Pp	Py
6	0%	-19.80 (2.505)	-17.92 (1.324)
	1%	-1.03 (0.453)	-1.69 (0.147)
	2%	0.90 (0.139)	0.76 (0.441)
	3%	0.67 (0.670)	0.63 (0.327)
	8%	0.45 (0.367)	0.04 (0.228)
9	0%	-23.62 (1.231)	-25.09 (1.892)
	1%	-1.23 (0.489)	-2.70 (1.909)
	2%	3.07 (0.707)	1.48 (0.208)
	3%	2.06 (0.419)	1.43 (0.382)
	8%	2.91 (1.035)	3.08 (1.018)
12	0%	-29.65 (5.630)	-32.56 (4.759)
	1%	-1.45 (0.662)	-1.42 (0.152)
	2%	5.90 (4.641)	1.16 (0.228)
	3%	4.15 (1.299)	3.17 (0.707)
	8%	6.65 (0.589)	2.75 (0.907)
15	0%	-38.28 (2.789)	-38.74 (4.311)
	1%	-4.48 (1.333)	-2.31 (1.043)
	2%	0.97 (0.161)	3.42 (2.507)
	3%	1.71 (0.393)	4.28 (1.396)
	8%	3.95 (0.810)	6.69 (1.213)
18	0%	-32.19 (1.453)	-37.68 (3.379)
	1%	-4.76 (1.638)	-1.97 (1.197)
	2%	-11.02 (2.120)	-5.92 (2.967)
	3%	1.75 (3.074)	1.91 (3.533)
	8%	-3.50 (4.451)	13.00 (10.028)