Competition and co-existence of *Photorhabdus temperata* subspecies *temperata* and *Photorhabdus temperata* subspecies *cinerea*, symbionts of *Heterorhabditis downesi*

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Declaration

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

Signed ----------

Contents

Abbreviations

%	percent
>	greater than
<	less than
=	equals
~	approximately
χ^2	chi-sq
°C	degree centigrade
CFU	colony forming unit
cm	centimetre
ddH2O	double distilled water
DF	degree of freedom
dH_2O	distilled water
dNTP	deoxyribonucleoside triphosphates
E. coli	*Escherichia coli*
E4	day 4
E7	day 7
E21	day 21
EPN	Entomopathogenic nematodes
et al	and other
Fig	Figure
g	gram
GLM	general linear model
hrs	hours
IJ	Infective juvenile
LB	Luria Broth
ml	milliliter
mM	millimolar
p	p value
PBS	phosphate buffered saline
PCR	polymerase chain reaction
pur	*H. downesi Photorhabdus temperata* subspecies *P. t. cinerea* isolates (purple)
RP-HPLC	reversed-phase high-performance liquid chromatography
rpm	rotation per minutes
SE	standard error
St Dev	standard deviation
subsp.	subspecies
x g	relative centrifugal force
yel	*H. downesi Photorhabdus temperata* subspecies *P. t. temperata* isolates (Yellow)
µl	microliter

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Publications

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Abstract

The aim of this project was to explore the relationship between the entomopathogenic nematode *Heterorhabditis downesi* and its two symbionts, *Photorhabdus temperata* subsp. *temperata* and *P. temperata* subsp. *cinerea*, and the relationship (co-existence and competition) between the two symbionts, which co-occur on Bull Island in Dublin Bay. There was no effect of *Photorhabdus* subspecies on the reproduction capacity of *H. downesi* in insects. Also there was no difference due to bacterial subspecies in the number of bacteria carried by *H. downesi* infective juveniles (IJs). The IJs carried around 200-300 bacteria on average. Infected insect cadavers were dried for up to 61 days and then rewetted. More IJs emerged from cadavers with *P. t. cinerea* than from cadavers with *P. t. temperata.* Clearly, *P. t. cinerea* provides an advantage to its associated nematodes under dry conditions, such as may occur at the front of a dune system, where this subspecies predominates. There was no difference between *P.t. cinerea* and *P.t. temperata* in their ability to grow at different salt concentrations, suggesting that they have similar tolerance to osmotic stress. Both symbiont subspecies grew well together when co-cultured in liquid medium, suggesting that they do not compete strongly with each other *in vitro*. When insects were co-infected with both subspecies, the majority of the IJs that emerged from the insects carried *P.t. cinerea*. This may suggest that *P.t. cinerea* competes better *in vivo*, perhaps by competing to colonise the nematodes. A small number of IJs carried both symbionts. First generation *H. downesi* hermaphrodites dissected from co-infected cadavers mostly carried only one symbiont or the other, but a small proportion (12%) carried both symbiont subspecies. In a choice experiment on agar plates, first generation *H. downesi* developing from IJ showed a preference for *P.t. cinerea*. Nine secondary metabolites were identified in culture filtrate of the Bull Island *Photorhabdus* isolates. Only one of these, isopropylstilbene, was produced by both subspecies. One (dihydro-isopropylstilebene) was produced by *P.t. cinerea* but not by *P.t. temperata*. Several other molecules were produced only by *P.t. temperata*, including anthroquinone pigments. Insects infected with *P. t. temperata* bioluminesced more intensely and emitted light at a slightly different frequency to the ones infected with *P. t. cinerea*. In conclusion, it is suggested that the main differences between the symbionts are in relation to cadaver defence.

Chapter 1

Introduction

1.1 Bacterial symbiosis in entomopathogenic nematodes

There is much research being conducted globally on symbiotic bacteria that are associated with entomopathogenic nematodes, as they are both considered amongst the best bio-control agents and also as models of symbiosis. *Photorhabdus* is a Gram-negative member of the family *Enterobacteriaceae* that lives in a mutualistic association with an entomopathogenic *Heterorhabditis* nematode (EPN) (Waterfield *et al.*, 2009).

The subject of this thesis is *Heterorhabditis downesi*, an entomopathogenic nematode associated with *Photorhabdus temperata*. These are not as well studied as the nematodes *Heterorhabditis bacteriophora* which are the vector for transmitting *Photorhabdus luminescens* between insect larvae and are both commercially produced as pesticides and are also well studied in terms of symbiosis (Aumann and Ehlers, 2001; Boemare *et al.*, 1997, Ciche *et al.*, 2001, Waterfield *et al.*, 2009). The dauer or infective juvenile nematodes retain the *Photorhabdus* in its intestine until it releases the bacteria into the hemocoel of the insect host.

1.2 Entomopathogenic Nematodes

Nematode parasites of insects have been known since the 17th century, but using nematodes to control insect pests was first tried in 1930 (Smart, 1995). Entomopathogenic nematodes mainly belong to the genera of *Steinernema* and *Heterorhabditis*. They have great potential as biological control agents. Several species are commercially produced and are used for control of insect pests in horticulture, agriculture and forestry (Ehlers; 2002, Kuske *et al.*, 2005, Dillon *et al.*, 2007). Using nematodes as insecticide is beneficial to the environment as it reduces the use of chemical pesticides (Dillon *et al.*, 2012, Kapranas *et al.*, 2017).

Steinernema and *Heterorhabditis* nematodes have strong similarities in terms of lifecycle as well as their host finding ability in the soil environment and association with symbiotic bacteria. There are differences between the two families; the first generation of *Steinernema* are amphimictic males and females and their symbiotic bacteria are carried in a specialized intestinal vesicle, whereas the first generation of *Heterorhabditis* are hermaphroditic and the infective juveniles carry the symbiotic bacteria in the midgut of the intestine (Gaugler, 2002). *Steinernema* and *Heterorhabditis* are not closely related and the entomopathogenic lifestyle is believed to have evolved independently in the two genera (Poinar, 1979).

Entomopathogenic nematodes are generally found in soil in a wide range of habitats including fields, forests, grassland, desert and even ocean beaches (Hominick, 2002, Kaya *et al.*, 2006, Maher *et al.*, 2017). EPN have a free-living infective juvenile stage (IJ) that lives in the top layers of the soil. IJs face some difficult conditions such as high temperature and desiccation, as well as diseases, predators and other biotic factors which are lethal to the nematodes (Kung *et al.*, 1991, Shapiro-Ilan *et al.*, 2006).

1.2.1 Entomopathogenic nematode lifecycle

As the entomopathogenic nematodes kill their hosts, they can be classified as parasitoids (Gaugler *et al.*, 1997). They have a direct lifecycle (single host). The free living stage which emerges from an insect cadaver and enters a new host is a specialised third stage juvenile, the infective juvenile (Downes and Griffin, 1996). Infective juveniles carry the symbiotic bacterium in the gut, *Xenorhabdus* or *Photorhabdus* in the case of *Steinernema* and *Heterorhabditis* respectively. In case of *Heterorhabditis* the bacteria colonize the entire intestine (Adams *et al.*, 2006). When a suitable host is found, the infective juvenile penetrates into the insect's body through the natural body openings (mouth, anus, spiracles) or through the cuticle. Once they reach the haemolymph they release their symbiotic bacteria through the mouth (Ciche and Ensign, 2003). The bacteria multiply rapidly and the insect dies within 24-48 hours through septicemia. The IJs develop into adults, distinct males and females in *Steinernema* and hermaphrodites in *Heterorhabditis* (Fig 1), and start to reproduce a new generation (Gaugler *et al.*, 1997). The EPN go through several generations and more IJs are produced, when the resources inside the host are depleted the IJs leave and search for new hosts (Gaugler, 2002). This usually happens within three weeks. IJs do not feed and can persist in the environment outside of the host cadaver.

When the bacteria symbionts released by the IJs start to multiply in the host, they produce toxins and exoenzymes, which result in septicemia and bioconversion of the insect cadaver into an ideal environment for nematode growth (Forst and Clarke, 2002). In the early stage of the infection, *Photorhabdus* will increase their numbers rapidly in the hemolymph and damage the immune system; the bacteria also release toxins which destroy the midgut tissue (Bowen *et al.*, 1998, Silva *et al.*, 2002). After the bacteria have multiplied the symbionts produce different types of antimicrobial material such as antibiotics that protect the cadavers

from colonization by other organisms (Akhurst, 1982, Sharma *et al.*, 2002, Duchaud *et al.*, 2003).

Ciche et al. (2008) described how *H. bacteriophora* are colonised by *P. luminescens*. The mother worms feed on the bacteria at first. Then, the bacteria infect the mother, by invading her rectal gland cells. The bacteria multiply inside the rectal gland cells and then these cells burst, releasing *P. luminescens* into the mother's body cavity. Finally, hatched juveniles feeding inside the mother (pre-IJs) take up these symbiont cells. According to Ciche *et al* (2008) usually a single symbiont cell adheres to a cell in the pre-IJ gut, between pharynx and intestine. This symbiont cell appears to invade the pre-IJs gut cell where it multiplies. Later, symbiont cells are again present in the IJ intestine where they multiply.

1.3 Infective juvenile behaviour in the entomopathogenic nematodes

Infective juveniles live in soil and seek out insect hosts. Third stage infective juveniles are also an important stage in many other parasitic nematode species (Lewis *et al.*, 2006). Entomopathogenic nematodes play a crucial role as good models for understanding the behaviour of other parasites as they are easy and cheap to culture, can be stored for long periods and their hosts are convenient to work with in the laboratory.

The infective juveniles forage and seek for new resources. Foraging behaviour can be based on behaviour response models and the ways in which searchers move through their environment (Gaugler *et al.*, 1997, Lewis *et al.*, 2006). In the first category, infective juveniles search can be divided into four stages: habitat location, host location, host acceptance and host suitability. In the second category of the model, foraging strategies are divided into two types: cruise foraging which covers a wide area of habitat compared to the ambush strategy, which is

to sit and wait (Gaugler *et al.*, 1997). Cruise foragers give more time to scanning for resources and associated cues when they are moving through the environment, whereas ambush foragers scan during long periods of stopping. Cruise foragers have higher success rate in finding non-mobile resources than ambushers, while in terms of finding resources with high movement the ambusher foragers are more successful (Lewis *et al.*, 2006). For the parasite infective stages which always search for hosts that are larger and more mobile than themselves the ambush foraging strategy is even more relevant. Active cruise foragers facilitate finding resources with high probability by use of chemotaxis and localised search patterns in patches. Different species of entomopathogenic nematodes are described as having cruise, ambush or intermediate foraging strategies (Grewal *et al.*, 1994). *Heterorhabditis* spp generally are cruise foragers. Infective juveniles use information cues such as chemicals including carbon dioxide and other host associated volatile cues for host finding (Lewis *et al.*, 2006, Hallem *et al.*, 2011).

1.4 *Photorhabdus* taxonomy

The first description of symbiotic bacteria associated with both genera of entomopathogenic nematodes was *Xenorhabdus nematophila* (*Achromobacter nematophilus*) (Poinar and Thomas, 1965). In 1993, *Xenorhabdus luminescens* was transferred to a new genus, *Photorhabdus* (Boemare *et al.*, 1993). *Photorhabdus* spp. are the only terrestrial bioluminescent bacteria, but the function of its bioluminescence is not known (Peat and Adams, 2008, Peat *et al.*, 2010). A 16S rRNA phylogeny that included two *Xenorhabdus* species, *X. bovienii* and *X. nematophila*, and two *Photorhabdus* species, *P. luminescens* subsp. *laumondii* TT01 and *P. asymbiotica* ATCC 43949 shows the close phylogenetic relationship between *Xenorhabdus* and *Photorhabdus* and their placement within the Enterobacteriaceae, relative to other bacteria in the Proteobacteria (Chaston *et al.*, 2011).

The genus *Photorhabdus* mostly consists of the bacterial symbionts of entomopathogenic nematodes of the family Heterorhabditidae (Fischer-Le Saux *et al.*, 1999a). *Photorhabdus* is a Gram- negative member of the Enterobacteriaceae family which lives in symbiotic association with the entomopathogenic *Heterorhabditis* nematodes as an influential pathogen (Waterfield *et al.*, 2009). *P. asymbiotica* causes infections in humans (Waterfield *et al.*, 2009), although some strains are also associated with nematode hosts (Gerrard *et al.*, 2006). The 16S rRNA gene was used to examine the taxonomy of *Photorhabdus* species and strains and their relatedness through the results of DNA sequencing (Fischer-Le Saux *et al.*, 1999a). According to the phenotypic characterisation and DNA relatedness *Photorhabdus* has been classified into three species *P. luminescens*, *P. temperata* and *P. asymbiotica* (Fischer-Le Saux *et al.*, 1999a). Further study was conducted on *Photorhabdus* classification of strains as shown in Figure 1.1 using a multigene sequencing approach. Three species of the genus *Photorhabdu*s, *P. asymbiotica*, *P. luminescens* and *P. temperata* were confirmed and several subspecies of each were also recognised based on sharing less than 97 % nucleotide identity (Tailliez *et al.*, 2010). Table 1.1 lists the *Photorhabdus* species and their *Heterorhabditis* hosts used in the Tailliez *et al.* (2010) study. In addition *Photorhabdus temperata* subsp. *cinerea* with type strain 3107(=DSM 19724 =NCAIM 02271) was proposed by Toth and Lakatos (2008). More recently, a fourth *Photorhabdus* species was described, *P. heterorhabditis*, from *Heterorhabditis zealandica* (Ferreira *et al.*, 2014). In that study, *P. temperata* subsp. *cinerea* DSM19274 is shown as closely related to *P. heterorhabditis*, and does not occur within the *P. temperata* clade (Ferreira *et al.*, 2014). The phylogenetic tree from Ferreira et al (2014) is shown in Figure 1.2.

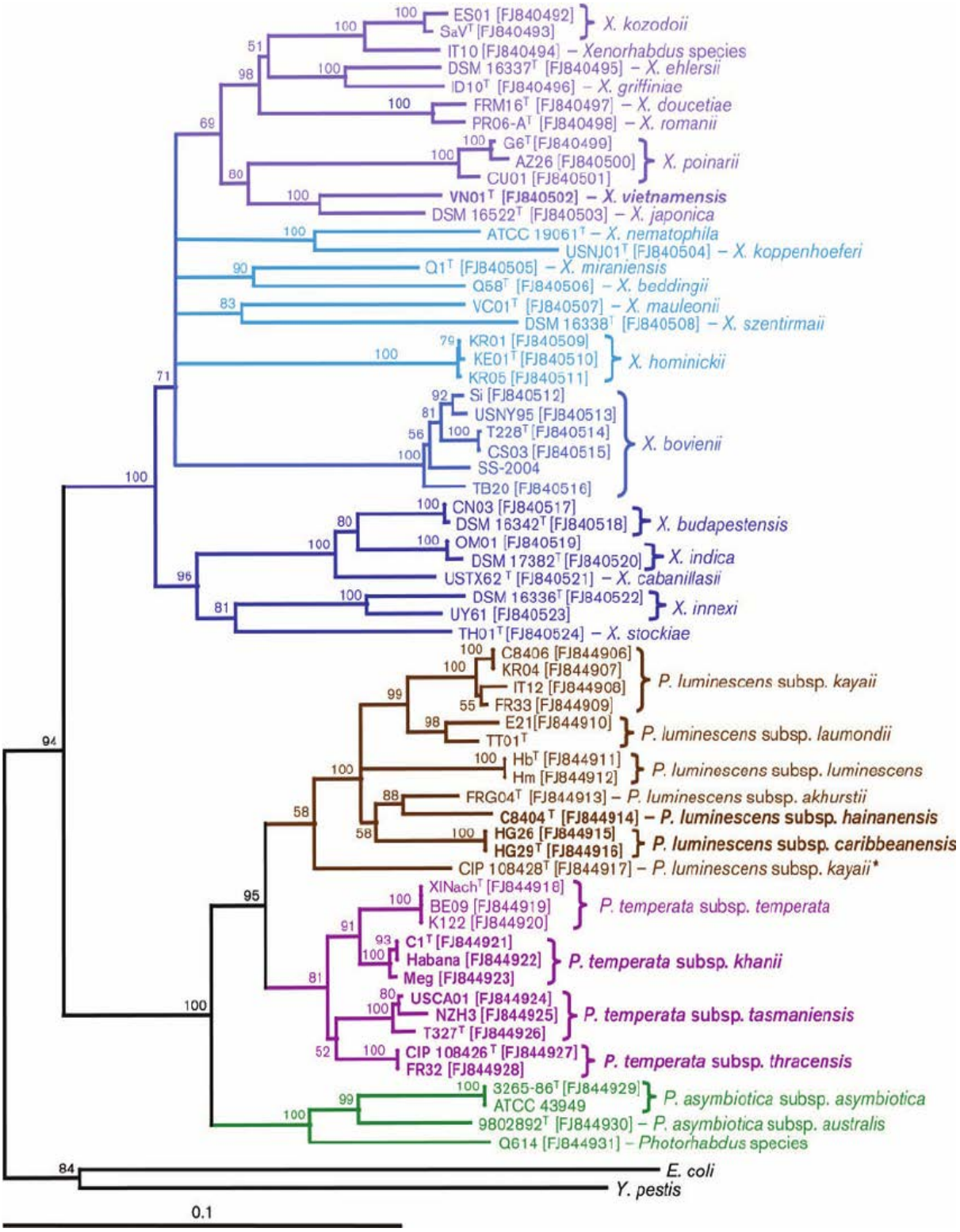


Fig. 1.1 The phylogenetic tree based on four concatenated protein-coding sequence (*recA, gyrB, dnaN, gltX*). From Tailliez (2010).

Table 1.1 *Photorhabdus* bacterial symbionts associated with their nematodes strains in the genus *Heterorhabditis* (Tailliez *et al.*, 2010)

Strain number	*Photorhabdus*	Nematode host species
980289^T	*P. asymbiotica* subsp. *australis*	
3265-86^T	*P. asymbiotica* subsp. *asymbiotica*	
FRG04^T	*P. luminescens* subsp. *akhurstii*	*H. indica*
CIP 108428^T	*P. luminescens* subsp. *kayaii*	*Heterorhabditis* sp.
C8406	*P. luminescens* subsp. *kayaii*	*Heterorhabditis* sp.
FR33	*P. luminescens* subsp. *kayaii*	Not yet defined
IT12	*P. luminescens* subsp. *kayaii*	*H. bacteriophora*
KR04	*P. luminescens* subsp. *kayaii*	*Heterorhabditis* sp.
E21	*P. luminescens* subsp. *laumondii*	*H. bacteriophora*
Hb^t	*P. luminescens* subsp. *luminescens*	*H. bacteriophora*
Hm	*P. luminescens* subsp. *luminescens*	*H. bacteriophora*
CIP 108426^T	*P. temperata* subsp. *thracensis*	*Heterorhabditis* sp.
FR32	*P. temperata* subsp. *thracensis*	Not yet defined
X1Nach^T	*P. temperata* subsp. *temperata*	*H. megidis*
BE09	*P. temperata* subsp. *temperata*	*H. megidis*
K122	*P. temperata* subsp. *temperata*	*H. megidis*
C1^T (NC19^T)	*P. temperata* subsp. *khanii*	*H. heliothidis*
Habana	*P. temperata* subsp. *khanii*	*Heterorhabditis* sp.
Meg	*P. temperata* subsp. *khanii*	*H. megidis*
NZH3	*P. temperata* subsp. *tasmaniensis*	*H. zealandica*
T327^T	*P. temperata* subsp. *tasmaniensis*	*H. zealandica*
USCA01	*P. temperata* subsp. *tasmaniensis*	*H. marelatus*
Q614	*Photorhabdus* sp.	*Heterorhabditis* sp.
C8404	*P. luminescens* subsp. *hainanensis*	*Heterorhabditis* sp.
HG26	*P. luminescens* subsp. *caribbeanensis*	*Heterorhabditis* sp.
HG29^T	*P. luminescens* subsp. *caribbeanensis*	*H. bacteriophora*

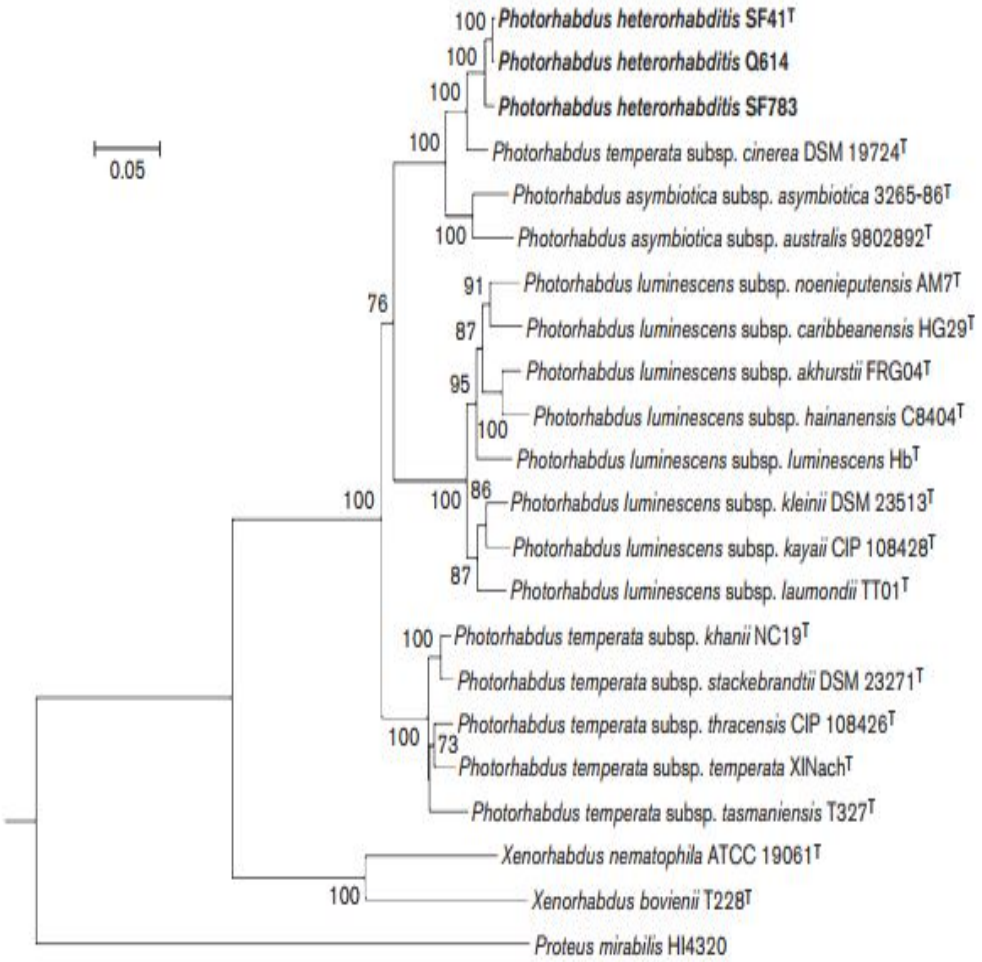


Fig. 1.2 Phylogenetic tree based on five concatenated protein-coding gene sequences (*recA, gyrB, dnaN, gltX, infB*) of known species of the genus *Photorhabdus.* From Ferreira *et al* (2014)

1.4.1 Association of different *Photorhabdus* subspecies with different *Heterorhabditis*

Some species of entomopathogenic nematodes depend more than others on the bacteria to kill insects, including *Heterorhabditis* spp. depend on the bacteria genus *Photorhabdus* with which they are associated to kill the insect (Chaston *et al.*, 2011). *Heterorhabditis* also requires *Photorhabdus* for nutrition, possibly because of a requirement for the crystalline inclusion bodies that the symbiont produces (Goodrich-Blair and Clarke, 2007).

The relationship between the *Heterorhabditis* spp. and *Photorhabdus* spp is quite sophisticated. The specificity between the nematodes and their bacterial resident is partly a result of the exclusion of bacterial competitors and specific recruitment of the symbiont before exiting the host (Adams *et al.*, 2006). Even though the entomopathogenic nematodes share a mutualistic relationship with their bacteria, other bacteria were isolated from the nematodes or their host insect post infection (Walsh and Webster, 2003, Enright and Griffin, 2004).

The specificity of the association between entomopathogenic nematodes and their bacterial symbionts is that, in general, each nematode species shares a mutualistic relationship with a single bacteria species (Adams *et al.*, 2006). The specificity is determined at two levels: firstly, each *Heterorhabditis* species can only feed and develop on a limited range of *Photorhabdus* symbionts, and secondly, only a certain range of symbionts can colonize each *Heterorhabditis* (Gerritsen and Smits, 1993, Gerritsen and Smits, 1997, Han *et al.*, 1991, Han and Ehlers, 1998a). The level of specificity seen in the bacteria-nematode interaction is dictated by events occurring at the molecular and cellular interface between the host and the bacteria during the process of colonization of the nematode by the symbiont (Goodrich-Blair and Clarke, 2007). Table 1.2 shows the associations between *Heterorhabditis* and *Photorhabdus* that have been reported to occur naturally. This shows that some species of *Heterorhabditis*, such as *H. bacteriophora*, can carry more than one species of *Photorhabdus*, and several other *Heterorhabditis* species carry more than one *Photorhabdus* subspecies.

Experiments have also been done to explore the level of specificity in the association and show that *Heterorhabditis* spp. can develop on and be colonised by some but not all non-native symbionts (Gerritsen and Smits, 1993, Gerritsen and Smits, 1997, Han and Ehlers, 2001, Kazimierczak *et al.*, 2017). In general, *Heterorhabditis* can feed on a wider range of *Photorhabdus* than they can carry as IJs (Eleftherianos *et al.*, 2010).

Table 1.2 Association of *Heterorhabditis* species with species and subspecies of *Photorhabdus*.

***Heterorhabditis* species**	***Photorhabdus* species**	Reference
H. bacteriophora	***P. luminescens* subsp. *luminescens***	(Fischer-Le Saux *et al.*, 1999a)
	P. luminescens* subsp. *laumondii	(Fischer-Le Saux *et al.*, 1999a)
	P. luminescens* subsp. *kayaii	(Hazir *et al.*, 2004)
	P. luminescens* subsp. *carabbeanesis	(Tailliez *et al.*, 2010)
	P. luminescens* subsp. *kleinii	(Maneesakorn *et al.*, 2011)
	P. temperata subsp. theracensis	(Hazir *et al.*, 2004, Tailliez *et al.*, 2010)
	P. temperata subsp. stackebrandtii	(An and Grewal, 2010)
	P. temperata subsp. cinerea	(Kazimierczak *et al.*, 2017)
H. indica	***P. luminescens* subsp. *luminescens***	(Fischer-Le Saux *et al.*, 1999a)
	P. luminescens* subsp. *akhurstii	(Fischer-Le Saux *et al.*, 1999a)
	P. luminescens* subsp. *neonieputiensis	(Ferreira *et al.*, 2013)
H. downesi	***P. temperata subsp. cinerea***	(Toth and Lakatos, 2008, Maher, 2014)
	P. temperata subsp. temperata	(Maher, 2014)
H. megidis	***P. temperata subsp cinerea***	(Toth and Lakatos, 2008)
	P. temperata subsp. temperata	(Tailliez *et al.*, 2010)
	P. temperata subsp. temperata	(Tailliez *et al.*, 2010)
H. zealandica	***P. temperata subsp. temperata***	(Tailliez *et al.*, 2010)
	P. temperata subsp. tasmaniesis	(Tailliez *et al.*, 2010)
H. georgiana	***P. luminescens* subsp. *kleinii***	(An and Grewal, 2011)
	P. temperata subsp. stackebrandtii	(Maneesakorn *et al.*, 2011)
H. marelatus	***P. temperata subsp. tasmaniesis***	(Tailliez *et al.*, 2010, Orozco *et al.*, 2013)
H. sonorensis	***P. luminescens* subsp. *sonorensis***	(Tailliez *et al.*, 2010, Orozco *et al.*, 2013)
H. gerrardi	***P. asymbiotica***	(Campos - Herrera *et al.*, 2012)
H. amazonensis	***undescribed***	(Campos - Herrera *et al.*, 2012)
H. atacamensis	***undescribed***	(Campos - Herrera *et al.*, 2012)
H. baujardi	***undescribed***	(Campos - Herrera *et al.*, 2012)
H. floridensis	***undescribed***	(Campos - Herrera *et al.*, 2012)
H. marelatus	***undescribed***	(Campos - Herrera *et al.*, 2012)
H. mexicana	***undescribed***	(Campos - Herrera *et al.*, 2012)
H. safricana	***undescribed***	(Campos - Herrera *et al.*, 2012)
H. taysarae	***undescribed***	(Campos - Herrera *et al.*, 2012)

1.4.2 *Photorhabdus* - Pathogenicity

Photorhabdus is pathogenic to a wide range of insect larvae (Poinar, 1979, Peters, 1996, Lacey *et al.*, 2001). *Photorhabdus* bacteria have two stages that are associated with hosts in their life cycle: a symbiotic stage that depends on colonising the intestine of the nematode host, which is mutualism with nematodes, and a second pathogenic stage that depends on the bacterium-nematode invading and killing insects (Goodrich-Blair and Clarke, 2007). The

pathogenicity of the combination of bacteria and nematodes depends on the bacterium pathogenicity and the interaction between them (Gerritsen *et al.*, 1998). Both *Photorhabdus* and *Xenorhabdus* produce a range of virulence factors to kill the insects, such as toxin complex (Tc) toxins that are toxic to *Manduca sexta* (Bowen *et al.*, 1998, Liu *et al.*, 2003).

Photorhabdus luminescens TT01 produces a type 2 quorum sensing molecule, AI-2, that can increase resistance to reactive oxygen species produced as part of the insect immune response (Krin *et al.*, 2006). Both the bacteria and nematodes have an effect on the immune response of the host (Adams *et al.*, 2006). The insect innate immunity consists of two components, the humoral, that recognises the invading organisms by the observer proteins, and the cellular that includes haemocytes that recognise and encapsulate the invading organism (Goodrich-Blair and Clarke, 2007). The nematodes and bacteria are living in mutualistic relation to frustrate the insect immunity as well as the antimicrobial activity of the haemolymph (Ciche *et al.*, 2006), and the detrimental effect on the enzymatic activity by the bacterium (Ciche *et al.*, 2008).

As the bacteria colonise the host they multiply rapidly and produce toxins, such as Mcf (Makes Caterpillars Floppy) which is an important factor for pathogens (Daborn *et al.*, 2002). the *mcf* gene was isolated from *P. luminescens* W14, with homologs in *P. luminescens* TT01, *P. luminescens* (*temperata*) K122 and *P. asymbiotica* ATCC43949 (Wilkinson *et al.*, 2009). There were four forms of a toxin complex produced by *P. luminescens* W14 which are Tca, Tcb, Tcc and Tcd - that are toxic to *Manduca sexta* (Waterfield *et al.*, 2001). The bacteria were also adapted to be highly virulent and resist the fast acting innate immune response in a wide range of insect prey species. In terms of the toxicity *P. luminescens* W14 bacteria produce at least two virulence factors, the gut-active toxin complex A (Tca) (Daborn *et al.*, 2001, Silva *et al.*, 2002) and a metalloprotease (PrtA) (Silva *et al.*, 2002). The *P. luminescens* TT01 genome contains genes encoding a type three secretion system (TTSS) (Brugirard-Ricaud *et al.*, 2005).

All the genomes of *Photorhabdus* that have been looked at contain secretion systems called *Photorhabdus* virulence cassettes (PVC) (Waterfield *et al.*, 2009). Another study has shown that *P. luminescens* strains produced a virulence factor, 3,5-dihydroxy-4-isopropylstilbene (ST) that inhibits the activity of phenoloxidase, that is one of the components of the insect immune response (Eleftherianos *et al.*, 2007).

1.5 Production of secondary metabolites

In general, secondary metabolites involve the interaction between the species and their host such as bacteria and the host. *Photorhabdus* spp produce antibiotics such as stilbene derivatives, anthraquinone derivatives, genistine, a furan derivative and a phenol derivative, have been identified (Hu and Webster, 2000). Anthraquinones also have antibacterial properties and also deter ants and birds from scavenging on the host cadaver (Pankewitz and Hilker, 2008). Two strains of *P. luminescens sonorensis* also produce stilbenes as well as cyclic peptides, and urea derivatives and some of these have an effect on insects, other nematodes and fungi (Orozco *et al.*, 2016).

Photorhabdus luminescens produce stilbenes and isopropylstilbene, which were proposed to protect the cadaver from other microbial organisms living in the soil. Stilbenes are found in plants and *P. luminescens* is the only known non-plant stilbene producer (Joyce *et al.*, 2008). As well as having antibacterial properties that prevent the growth of other microorganisms and inhibiting the insects host immune system (Eleftherianos *et al.*, 2007), some stilbenes are also involved in nematode growth and development (Joyce *et al.*, 2008).

Hu and Webster (2000) studied the ability of *P. luminescens* to produce antibiotics in *G. mellonella.* Twenty-four hours after nematode infection antibiotics were produced by *P. luminescens*, such as 3,5-dihydroxy-isopropylstilbene, increased rapidly from day 4 to 5 post

infection and was decreased after 21 days. This supports the assumption that the production of antibiotics by the symbiotic bacteria especially at the beginning of the infection was to prevent or minimize the competition from other microorganisms in the insect and inhibit putrefaction of the cadaver (Hu and Webster, 2000). Stilbenes play a role in the relationship between the nematode, the bacteria and the insect (Bode, 2009).

1.6 Competition and Co-existence

In Hungary, *Heterorhabditis downesi* associates with two *Photorhabdus* symbionts, *Photorhabdus temperata* subsp. *temperata* and *P. temperata* subsp. *cinerea*, which can be found within one population (Toth and Lakatos, 2009). Similarly, at a coastal dune site in Ireland (Bull Island) Maher (2014) found *H. downesi* associated with *Photorhabdus temperata* subsp. *temperata* and *Photorhabdus temperata* subsp. *cinerea*. The location of *H. downesi* associated with *Photorhabdus temperata* subsp. *cinerea* was more in front of the dune and predominantly closer to the beach than the *P. temperata subsp. temperata*. This region has less vegetation and less organic matter in the sand, and so the soil here can be drier (Verhoeven, 2002). Maher (2014) found that the two symbionts differed in certain traits, such as protection of the cadaver from desiccation and antibiotic activity. Consequently, the symbiotic bacteria can play an important role in terms of extending niche of the nematodes (Feldhaar, 2011). The co-occurrence of two different bacteria at one site leads also to a possibility of combinations of the two *Photorhabdus* subspecies developing together and competing within the one host.

1.7 Aims and objectives of this project.

The overall aim of this project was to explore the relationship between *H. downesi* and the two symbionts that it carries on Bull Island, and the relationship (co-existence and competition) between the two symbionts. For this project six isolates of *H. downesi* were used and their bacterial symbionts that were isolated on Bull Island by Maher (2014), three "pur" isolates carrying *P.t. cinerea* and three "yel" isolates carrying *P. t. temperata* were used. These are named after the colour of waxmoth larvae infected by them (purple and yellow).

Specific objectives are to

1. Assess the effect of both symbiont subspecies on the fitness of the *H. downesi* nematodes. Traits include production of infective juveniles in wax moth cadavers under normal (moist) conditions, number of bacteria carried by IJs, and protection of nematodes developing in cadavers under dry conditions (Chapter 3)
2. Test the hypothesis that if insects are co-infected by both yel and pur *H. downesi* IJs then culturing under desiccating conditions for several generations should select for more IJs in the population carrying *P. t. cinerea* than *P.t. temperata* (Chapter 4)
3. Test the preference of *H. downesi* first generation worms to move towards the two symbiont subspecies and to feed on them, in choice tests on agar (Chapter 4)
4. Assess outcome of competition between yel and pur isolates when grown together both in liquid culture and in insects (Chapter 4).
5. Develop molecular means of quantifying the two symbiont subspecies when grown together *in vivo* or *in vitro*. To do this, the genomes of *P. t. cinerea* pur1 and *P.t. temperata* yel3 was sequenced, and primers that were intended to be specific to each subspecies were designed and tested (Chapter 4).

6. Confirm that isolates of *P. t. cinerea* from sites other than Bull Island have antibiotic properties similar to those of *P.t. cinerea* from Bull Island. (Chapter 5).
7. Identify antibiotics and other secondary metabolites produced by the two symbiont subspecies (Chapter 5)
8. Compare the intensity and wavelength of bioluminescence emitted from wax moth cadavers infected by the two symbiont subspecies (Chapter 5).

Chapter 2

General Methods and materials

2.1 Source of *Heterorhabditis downesi* isolates and *Photorhabdus temperata*

The research was carried out on *Heterorhabditis downesi* nematodes provided by Dr Abigail Maher (Department of Biology Maynooth University). Two subspecies of bacteria were associated with *H. downesi: Photorhabdus temperata* subspecies *cinerea* (purple) isolates (*P. t cinerea* Pur1, Pur2, Pur3) and *Photorhabdus temperata* subspecies *temperata* (Yellow) bacteria (*P. t. temperata* Yel1, Yel2, Yel3) Those nematodes were collected from North Bull Island in Dublin Bay (Maher, 2014).

In addition, *H. downesi* found in five different geographical locations in Wales (Table 2.1) were recovered from cryopreservation and the bacterial symbiont was isolated and identified.

Table 2.1 Isolates of *H. downesi* recovered from cryopreservation. All isolates were originally recovered by baiting soil samples collected in Wales in April 1991 see Griffin *et al* (1994).

Isolate	**Location**
W18	Tenby
W30/1	Freshwater West
W31	Freshwater West
W48	Pembry
W68	Swansea
W78	Kenfig

2.1.1 Culturing of nematodes

H. downesi nematodes were reared in last instar larvae of the wax moth *Galleria mellonella*. The wax moths were obtained from the Livefood Direct Ltd. (Sheffield UK), and stored at 15°C. Two 9 cm Petri dishes were lined with 9 cm (Whatman no.1) filter paper. A millilitre of a 1000 IJs/ml suspension was pipetted onto each filter paper. Ten insects (*G. mellonella*) were placed in each dish.

After infecting the *G. mellonella* with *H. downesi* nematodes dishes were stored at 20°C. After three days, the cadavers were transferred into a dish lined with moist paper tissue for three weeks, then transferred into White traps. All dead larvae were collected and placed in a White trap (Fig 2.1). The cadavers were lined around the edge of a platform formed by the lid of a 5cm diameter Petri-dish covered with a filter paper, then placed into a 15 cm diameter Petri-dish and water was poured in to a depth of 5 mm or until it reached half way up the platform. The cadavers were left in the incubator for few days to let the nematodes emerge into the water, then the nematodes were harvested, washed three times by sedimentation with tap water and stored at 9°C until required.

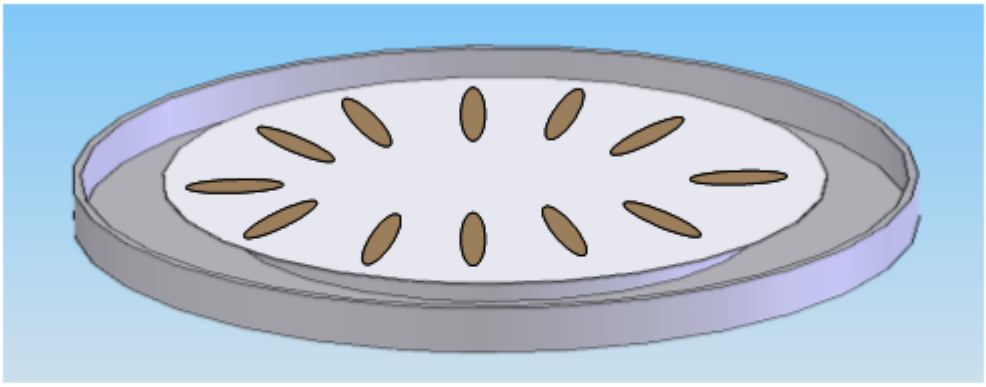


Fig. 2.1 White trap used for harvesting *H. downesi* IJs emerging from the cadavers into water.

2.1.2 Culturing nematodes from liquid nitrogen stocks

Infections in single *G. mellonella* (two for each isolate) were set up in 1. 5 ml Eppendorf tubes on filter paper moistened with 120 µl sterile dH_2O with +/- 10 IJs per insect picked up in 20 µl dH_2O. Insects stored at 20°C and checked daily for mortality from day 4 – 8 after infection.

All insects positive for *Photorhabdus* infection (checked for bioluminescence) were transferred to moist paper in individual 3.5 cm Petri dishes and stored at 20°C prior to placing on White traps at 10 days after mortality.

2.2 Source of symbiotic bacteria

2.2.1 Isolation of Bacteria from infective juveniles

Symbiotic bacteria were isolated from surface sterilised IJs (1 ml Hyamine 1622 solution (0.16 mM) (Sigma-Aldrich) to nine ml of nematode suspension (1000 IJs/ml), followed by three rinses with sterile phosphate buffered saline (PBS). Single or pooled IJs were transferred to 100 µl PBS and crushed with a sterile micro pestle for one minute then streaked onto MacConkey agar and incubated in the dark for 48 hours at 27°C.

2.2.2 Isolation of Bacteria from haemolymph of infected insects (*G. mellonella*)

Insects were infected with IJs and checked for bioluminescence after 72 hrs, the cadavers were surface sterilised by washing the cadaver in 70% ethanol, flaming and extinguishing by plunging into sterile distilled water. Cadavers were opened and a loopful of haemolymph was streaked onto MacConkey agar plates and incubated in the dark at 27 °C for 36 - 48 hours.

2.2.3 Culturing bacteria

Individual colonies were picked from a plate using a sterile 200 µl pipette tip and transferred to 5 ml of LB broth in a 50 ml tube (Sarstedt) and incubated for 16-24 hrs at 27°C with shaking at 200 rpm.

2.3 Media preparation

2.3.1 MacConkey agar

One litre of MacConkey agar was prepared using 52g MacConkey agar powder (Oxoid) / 1000 ml of distilled water (dH_2O). The agar was autoclaved at 121ºC for 15 minutes, cooled to 65ºC and poured in 9cm Petri dishes and allowed to set over night. The next day the dishes were wrapped in cling film and tinfoil and stored inverted at 4ºC.

2.3.2 Nutrient agar

One litre of nutrient agar was prepared using 28g nutrient agar powder (Oxoid) / 1000 ml of distilled water (dH_2O). The same methods were used as for the preparation of the MacConkey agar.

2.3.3 LB agar, Miller/ Pyruvate:

One litre of LB agar Miller was prepared using 40g LB agar, Miller (BD) / 1000 ml of distilled water (dH_2O), supplemented with 1g of sodium pyruvate, (Sigma Aldrich). The same methods were used as for the preparation of the MacConkey agar.

2.3.4 Lipid Agar

One litre of lipid agar (Lacey, 2012) was prepared using the following ingredients.

Nutrient Broth	8 g			
Agar	15 g			
Yeast extract	5 g			
ddH_2O	890 ml			
0.2 g/ ml $MgCl_2.6H_2O$	10 ml			
Corn oil	4 ml			
Corn syrup mix	96 ml	Corn syrup mix:	corn syrup	7 ml
			ddH_2O	89 ml

The corn oil and corn syrup were autoclaved separately and were added after autoclaving. The medium was mixed thoroughly to ensure that the oil was evenly dispersed throughout the medium in tiny droplets.

2.3.5 Phosphate Buffered Saline (PBS):

One litre of phosphate buffered saline (PBS) was prepared using 10 tablets PBS (Oxoid) / 1000 ml of distilled water (dH_2O), autoclaved at 121ºC for 15 minutes and stored at 4ºC.

2.4 Identification of bacterial symbionts

2.4.1 Extraction of DNA

Individual colonies were picked using a sterile 200 µl pipette tip and transferred to 5 ml of LB broth in a 50 ml tube (Sarstedt) and incubated for 16-24 hrs at 27°C with shaking at 200 rpm. Absorbance at 660 nm was checked and cell suspension were adjusted to give approximately $1X10^{9}$ cells/ml.

DNA was extracted using the DNeasy Blood and Tissue Mini Spin Column Kit (Qiagen, Crawley, UK). All buffers (AT1, AL, AW1, AW2, AE), proteinase K, spin column and the collection tubes were supplied with the kit. DNA was extracted according to the manufacturer's protocol with the following variations:

Cultured cells were centrifuged using a maximum of $1X10^9$ cells for 10 minutes at 5000g in micro centrifuge (Sigma 1-15 Micro centrifuge), the supernatant was discarded and the cells were resuspended in 200 µl PBS. Then 20 µl proteinase K and 200 µl buffer AL were added and mixed thoroughly by vortexing. The samples were incubated at 56°C for 1.5 hour at 100 rpm with vortexing every 15 minutes. Then 200 µl of ethanol was added and mixed thoroughly by vortexing.

The mixture was transferred to a DNeasy mini spin column in a 2 ml collection tube and centrifuged at ≥ 6000 X g for 1 minute and the flow-through and the collection tube were discarded. The spin column was placed in a new 2 ml collection tube; Buffer AW1 (500 µl) was added, and samples were centrifuged at 8000 X g for 1 minute. 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 samples were centrifuged at 20,000 X g for 3 minutes.

The collection tube and flow-through were discarded and spin column was transferred to 1.5 ml Eppendorf or 2 ml micro-centrifuge tube. Elution Buffer AE (2X 50 µl) was added and the samples were incubated for 1 minute at room temperature then centrifuged at 6000 X g for 1 minute. This step was repeated once.

The spin column was discarded and concentration of DNA in the eluate was quantified using a Nano Drop (ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, USA). A portion of the eluate was adjusted using ultrafilter water to a concentration of 20 ng/µl and stored at – 20°C.

2.4.2 PCR amplification

The PCR was carried out using primers for the 16S rRNA gene (Fischer-Le Saux *et al.*, 1999a) (Table 2.2). The reactions were carried out using an Eppendorf Mastercycler

Gradient machine (Eppendorf, Cambridge, UK). The reagents used were kept at -20°C, Materials were obtained from Qiagen.

Table 2.2 the 16S gene primer sequences (Fischer-Le Saux et al, 1999)

16S-F	**16S-R**
GAAGAGTTTGATCATGGCTC	**AAGGAGGTGATCCAGCCGCA**

An appropriate master mix was prepared in a 1.5 ml micro centrifuge tube **(**Table 2.3**)**. Individual reactions were carried out in 0.5µl PCR tubes (Fisher Scientific, Ireland) using 22.5µl of master mix and 2.5µl of DNA extract (20 ng/µl). Water (ddH2O) was used as a negative control that was 2.5 µl and 22.5 µl of master mix reaction. The cycle condition for amplification of 16S gene see (Table 2.4)

Table 2.3 PCR mixture for amplification of 16S gene from *P. temperata* (Fischer-Le Saux *et al.*, 1999a)

Reagents	**Volume (µl)**	**Volume (µl)**	**Final concentration**	**Supplier**
5X myTaq reaction buffer	5	40	2	Bioline
Forward primer (10 µM)	0.5	4	0.2	Eurofins MWG Operon
Reverse primer (10 µM)	0.5	4	0.2	Eurofins MWG Operon
Water (ddH2O)	16.375	131		
DNA template (20 ng/µl)	2.5	as required		
Taq Polymerase (5 units/µl)	0.125	1	0.025	Sigma Aldrich
Total	25			

Table 2.4 PCR cycle conditions for amplification of 16S gene from symbiotic bacteria isolated from *H.downesi* nematodes.

Stage	cycles	Temperature	Duration
Initial denaturation	1	95°C	5min
Denaturation		95°C	1min
Annealing	34	52°C	1 min
Extension		72°C	2 min
Final extension	1	72°C	3min
Hold	1	4°C	Indefinite

The PCR product 5µl , mixed with 1 µl loading buffer (Bioline, UK) was analysed on a 1% agarose gel: 3g agarose (Life Technologies & Sigma- Aldrich) dissolved in 150 ml 1x TAE buffer (Sigma-aldrich), SYBR safe DNA gel 3g stain 20µl (1 x SYBR Safe) (Invitrogen, Thermo Fisher Scientific). Gels were run at 120V for an hour in 1 x TAE buffer and were visualised using the G: Box with GeneSnap software (Syngene).

2.4.3 DNA sequencing

The samples of DNA were purified (Isolate II PCR and Gel Kit (Bioline), for volume <30 µl the volume was adjusted to 50 µl with 20 µl of water, then 1 volume of sample was mixed with 2 volumes of binding buffer BC. The sample was transferred to a spin column in a 2 ml collection tube, centrifuged for 30 sec at 11,000 X g and the flow-through was discarded. Wash Buffer CW (700 µl) was added, the sample was centrifuged for 30s at 11,000 x g and the flow through was discarded. This step was repeated. The sampe was centrifuged for 1 minute at 11,000 X g to remove residual ethanol. The spin column was then placed in a 1.5ml micro centrifuge tube. Then the DNA was eluted by adding 15µl Elution Buffer C, incubated at room temperature for 1 minute and centrifuged for 1 min at 11 000 X g. This step was repeated once. Samples were sent for sequencing to GATC Biotech, Konstanz, Germany www.gatc.biotech.com

Chapter 3

Effects of *P. temperata* subspecies on *H. downesi* Fitness

3.1 Introduction

Heterorhabditis nematodes are dependent on their *Photorhabdus* symbiont. Without them they cannot kill insects and cannot develop (Ciche *et al.*, 2006). *Photorhabdus* also produces metabolites that protect the cadaver from competing microbes and scavengers. There is a degree of species and strain-specificity, with each *Heterorhabditis* species carrying and developing on a limited range of *Photorhabdus* species or subspecies (Tailliez *et al.*, 2010), though the specificity is not as strict as in *Steinernema* where each *Steinernema* species is associated with only one species of *Xenorhabdus* (Campos - Herrera *et al.*, 2012).

While it has been shown that *Heterorhabditis* can develop on and carry *Photorhabdus* strains or even species other than its native strain, the quality of the services provided by different strains for the *Heterorhabditis* may vary, but this is less often quantified (Gerritsen *et al.*, 1998). In this chapter, the effects of two *P. t. temperata* subspecies on fitness traits of *H. downesi* were compared using six natural isolates from Bull Island. The most direct measure of fitness is reproductive output, and this was measured as numbers of IJs produced from wax moth larvae. Since *Heterorhabditis* cannot kill insects without *Photorhabdus*, IJs carrying low numbers of bacteria may fail to kill a host, and therefore fail to reproduce. Numbers of bacteria retained by IJs emerging from hosts was the second fitness trait measured in this chapter. The third fitness trait measured was desiccation survival within a host cadaver. Maher (2014) found

that when cadavers were placed in desiccating conditions, more IJs emerged from cadavers infected with *H. downesi* pur1 (carrying *P. t. cinerea*) than with *H. downesi* yel3 (carrying *P. t. temperata*). All six Bull Island isolates were used to test whether this effect was due to differences between bacterial subspecies rather than just a difference between the two isolates pur1 and yel3. The hypothesis that *P. t. cinerea* slows the rate at which water is lost from the cadaver was tested, by measuring the rate of weight loss from cadavers under dry conditions.

As an insect cadaver dries, the concentration of the haemolymph will increase, including the salt concentration. The hypothesize was that the *P.t. cinerea* isolates from Bull Island will tolerate high salt concentrations better than *P.t. temperata*, as they occur in a drier part of the dunes. The hypothesis was tested by culturing the six Bull Island *P. temperata* isolates in different concentrations of sodium chloride (NaCl) and recording optical density of the cultures. Sodium chloride is one of the most widely used osmotic stressors (Jallouli *et al.*, 2011).

3.2 Methods

3.2.1 Production Capacity of *Heterorhabditis downesi* Bull Island isolates (experiment 1)

In this experiment *Galleria mellonella* were infected with each of the six Bull Island isolates, to assess the production capacity of nematodes carrying each subspecies of bacteria. Furthermore, it will assess the penetration of the nematodes carrying each variant and the number of bacteria carried by IJs emerging from the cadavers. In addition, the number of bacteria carried by IJs used to infect the insects, and the number of bacteria per insect were also assessed.

The experiment was performed three times, with one Yel (carrying *P. temperata temperata*) and one Pur (carrying *P. temperata cinerea*) isolate in each experiment. The Pur

and Yel isolate were chosen randomly. The first trial used pur1and yel1, second trial pur3 and yel 2, and the third trial pur 2 and yel3. Pur 1, Yel 1, Pur 2, Yel 2, were one week old and Pur 3, Yel 3 were two weeks old.

Two 15mm filter papers were placed in each well of 24 well multi-well plates. All wells received 100µl of nematode suspension with 100 IJs. One *Galleria mellonella* larva was placed in each well. The tray was sealed with parafilm and stored at 20ºC. The mortality was checked after three days of the infection.

Bacterial quantification from individual infective juveniles

Infective juveniles of *H. downesi* were surface sterilised. Hyamine (1 ml) was poured into a 15ml tube (Sarstedt) and 9 ml of nematode suspension was added and inverted 5 times then allowed to settle for 15 minutes.

Then the supernatant was discarded and 9 ml of PBS was added to rinse the suspension of nematodes, and again the suspension was inverted 3 times and allowed to settle down, then the PBS rinse was repeated two more times.

PBS (90µl) was put in a 1.5 ml Eppendorf tube, then a single IJ was placed in 10µl volume into the Eppendorf tube, and the IJ was crushed for one minute using a micro-pestle motor (Fisher Scientific).

Homogenate of (50 µl) of IJ was spread on LB pyruvate plates on each of three replicate plates using a sterilised spreader. The Petri dishes were incubated in 27ºC for 48 hours to allow the colony of bacteria to grow. After the incubation period, the *Photorhabdus* colonies were verified by luminescence and then colonies were counted.

Invasion rate of IJs into each *Galleria mellonella* and numbers of IJs emerging

Five cadavers were selected randomly for bacteria quantification, and then the remaining cadavers were stored in individual 3.5cm Petri dishes lined with filter paper. At day 4 or 5 after the infection 5 (exp1) or 10 (exp 2, 3) cadavers were chosen randomly from each treatment, dissected and the number of nematodes was counted to determine the number of IJs invading the host. The dissection was carried out in sterile PBS solution with the aid of dissection microscope.

After 14 days of infection the remaining cadavers were placed in individual White traps as described in Chapter 2. The White trap was checked daily for emergence of IJs and the date of first emergence was noted for each White trap (E0).

From each individual White trap the suspension of IJs was collected in 50ml Falcon tube (Sarstedt) on day 4 emergence (E4), the IJs were stored at 9ºC until counted.

The White traps were harvested weekly until day 21 (E21) then all the harvests of IJs were counted, to allow to comparison of numbers emerging early from each treatment as proportion of total emergence in that treatment. (Modified from Boff *et al*. 2000).

Bacterial quantification from infected *G. mellonella*

Three days after infection 5 cadavers were chosen randomly for bacterial quantification. The cadavers were surface sterilised by rinsing in 70% ethanol, igniting and extinguishing by plunging into sterile PBS solution and placed in a 3.5 cm Petri dish. The cadaver was partially macerated with forceps and then transferred to a plastic vial (15ml Falcon tube). Then the dish was rinsed into the vial with 2ml PBS solution. The body contents were allowed to soften for 4 hours with gentle agitation on a Grant Bio PS-3d platform rotator at 30 rpm. Then the body contents were vortexed for two minutes and allowed to stand for 20 minutes. A 20µl aliquot of

the homogenate was added to 0.98ml sterile PBS solution and 4 10-fold serial dilutions were prepared. Then three replicate MacConkey agar plates were spread with 50µl aliquots of each of 2 dilutions and incubated at 27ºC for 48-72 hour to assess the bacteria load in the insects at day 3 post infection.

Statistical analysis

Insect mortality at day 3 was compared using Yates test (comparing all six isolates) and the exact chi-square test (data pooled by bacterial subspecies). To test whether bacterial subspecies had an effect on any of the parameters measured, data were analysed by GLM (general linear methods), with bacterial subspecies as fixed factor and isolate as random factor nested within bacterial subspecies. Where subspecies was not significant but isolate was, the six isolates were subjected to one-way ANOVA followed by Tukey's post-hoc test to detect where differences lay. Where data were not normal (numbers of bacteria per IJ used to infect, and numbers of bacteria per insect) they were analysed by Kruskal-Wallis (comparing all six isolates) and Mann-Whitney (comparing bacterial species).

3.2.2 Production Capacity of *Heterorhabditis downesi* Bull Island isolates (experiment 2)

The experiment was repeated with measurements only of total yield and time of emergence for the six Bull Island *H. downesi* (Yel and Pur) isolates. 10 *Galleria mellonella* were infected with 2000 IJs or 200 IJs per insect, then after three days of infection the cadavers were put individually in White traps and checked daily to record the emergence of IJs from each cadaver.

For each isolate 5 cadavers were randomly chosen, then the IJs were harvested after four (E7) and twenty-one days (E21) of emergence and counted.

3.2.3 Effect of *P. temperata* subspecies on production of IJs from cadavers under dry conditions in sand

In a previous project (Maher, 2014) *Galleria mellonella* larvae were infected with IJs carrying either *P. temperata temperata* (yel) or *P. temperata cinerea* (pur) bacteria. Some of the cadavers were dried, either by putting them in dry sand or in a desiccator at 0% relative humidity (RH). In general, more IJs emerged from dry cadavers that contained pur bacteria than those that contained yel bacteria. This indicates that pur bacteria protect cadavers and the developing nematodes from desiccation, and may explain why pur bacteria are more likely than yel to be found at the front of the sand dunes on Bull Island, where the sand is drier. However, Maher worked with only one strain of *P. t. cinerea* (pur1) and one strain of *P. t. temperata* (yel3).

The aims of this experiment are firstly to test whether the difference between pur1 and yel3 reflects a difference between *P. temperata* subspecies, by testing all three Bull island strains of each subspecies and secondly to see the effect of the time that the cadavers are in dry sand on the numbers of IJs emerging.

Assay

For each isolate 60 insects of *Galleria mellonella* were infected. Nematode suspensions were made of about 1000 IJs per ml, and 1 ml of nematode suspension was added to filter paper in a 9cm-Petri dish with 10 insects per dish, which is 100 IJs per insect

Three days after infection, live or contaminated insects were discarded. (i) One or two cadavers were chosen randomly from each dish and placed on moist paper (10 insects per isolate), they were incubated at 20°C and placed individually in White traps at the usual time

after infection. (ii) Seven or eight insects were taken from each Petri dish (total: 40 insects) and placed individually in tubs of dry sand.

Tubs (polypropylene, 4 cm x 4.4 cm, H x D; 60 cm^3) were filled half-way with dry sand and an insect was added, then covered with more dry sand (Play-Pit sand, B and Q which had been over-dried overnight and then allowed to cool) and the lid was added. The 40 tubs were randomised for each isolate and 8 of them were assigned to each of 5 blocks. Each block was contained in one box.

After 28, 42, 54 and 61 days (after infection), 10 tubs of sand were removed per isolate (2 from each box). The insects were placed in individual in White-traps. For the long time exposure such as 54 days cadavers were first rehydrated for 24 hours with 1ml of water on a filter paper then placed in White-traps. The time of first emergence was observed, as well as number of cadavers with emergence. After 21 days the IJs were harvested and counted.

Statistical analysis

Data for the number of cadavers from which nematodes emerged were analysed using chi-square. Numbers of nematodes emerging from cadavers where IJs were produced were analysed by GLM with bacterial subspecies as the fixed factor and isolate as random factor nested within it.

A further analysis on the total number of IJs that emerged (including the zero values where no IJs emerged) was carried out by Dr Caroline Brophy of the Mathematics and Statistics Department, Maynooth University. A zero inflated Poisson regression model was fitted to the total number of IJs that emerged. A difference in the total number of emerging IJs between *P. t. cinerea* and *P. t. temperata* was estimated and tested for at each time point.

3.2.4 Desiccation of cadavers infected with *H. downesi* - *P. temperata* hybrids at 0% relative humidity

Hybrid nematodes were produced by exchanging the bacterial symbiont between two of Bull Island *H. downesi* isolates, pur1 and yel3 (Maher, 2014). There are four strains and combinations. (Table 3.1). The aim of the experiment was to assess the desiccation tolerance of these four strains of the *H. downesi- P. temperata* hybrids and to record weight loss of cadavers infected with them.

Desiccant cartridges containing silica beads (197 mm, DES 850-050G, Fisher Scientific) were placed in the oven for 24h at 100°C. Then the cartridges were placed in glass desiccators for three days at 20°C to allow the desiccators to equilibrate (silica gel beads absorb the humidity). Thirty *G. mellonella* weighing between 230-320 g were infected for each hybrid strain (Pp, Py, Yy, Yp) and incubated at 20°C for 72 hours.

Table 3.1 Wild-type and hybrid strains of *H. downesi* made by transferring *P. temperata* symbiont between isolates.

	Nematode	**Bacteria**
Strain	*H. downesi*	*P. temperata*
Pp (wild type)	**Pur1**	**Pur1 (*P.t. cinerea*)**
Py (hybrid)	**Pur1**	**Yel3 (*P.t. temperata*)**
Yy (wild type)	**Yel3**	**Yel3 *P.t. temperata*)**
Yp (hybrid)	**Yel3**	**Pur1 (*P.t. cinerea*)**

4-well plates were prepared by drilling the base of 4-well plates with 3mm diam holes to allow air circulating inside the desicators. After three days of infection insects were transferred into the 4-well plates. Each 4-well plate held one cadaver of each of the 4 strains (Pp, Py, Yy, Yp). For each strain 10 insects were left in the desiccators for 28 days at 20°C, and then weighed and placed in individual White traps. For the moist condition which was used as control, 10 insects for each strain were put on moist tissues individually for 14 days and then were transferred into individual White traps. After the incubation period all the individual

White traps were observed for IJs emerging. The IJs were harvested at intervals, and harvests were pooled and IJs were counted.

The experiment was performed twice. In the first run of the experiment an additional 5 insects for each strain were weighed at 3-days intervals throughout the experiment, and then the percentage weight loss of each insect was calculated at each third day interval.

Statistical analysis

Numbers of nematodes emerging from cadavers infected with *P.t. temperata* and *P.t. cinerea* were compared using Mann-Whitney tests.

Cadaver weight loss: data for both nematode isolates were combined and weight loss differences between bacterial subspecies were analysed by Dr Caroline Brophy, who fitted a repeated measures linear mixed model to the logit of the weight loss percentage values. The explanatory variables were bacterial subspecies, time and the interaction between bacterial subspecies and time.

3.2.5 Effect of salt concentration on *P. temperata* growth

Luria Broth was prepared with different salt concentrations (0, 0.5, 1.0, 2.0, 2.5 and 5% NaCl). Aliquots (500 µl) were pipetted into wells of 24-well plates. To each well was added 5 µl of a 24-hour liquid culture of *P. temperata* adjusted to a concentration of 1 X 10^6 cells per 5 µl. Plates were sealed with Parafilm to minimise evaporation. These were cultured at 27ºC with shaking at 200 rpm overnight (16 hours) and absorbance was read at 600 nm on a Clariostar plate reader. All six Bull Island isolates were tested. There were three replicate wells per isolate and salt concentration. Result were analysed by GLM and salt concentration and bacterial subspecies as main factor and isolate nested within subspecies.

3.3 Results

3.3.1 Production capacity of *Heterorhabditis downesi* Bull Island isolates (experiment 1)

Bacterial quantification from individual infective juveniles used for infection

Bacteria quantification was carried out using IJs from stock *Heterorhabditis downesi* nematodes carrying *Photorhabdus temperata temperata* (yellow) and *P.temperata cinerea* (purple), and then the same stock was used in the rest of the experiment. The mean number of bacteria carried by IJs ranged from about 20 in pur1 and yel3 to over 150 in yel2 (Fig 3.1). According to a Kruskal- Wallis test there was no significant difference among isolates (H = 10.43, DF = 5, *P* = 0.064). Also according to the Mann- Whitney test there was no significant difference between *P. temperata* purple and yellow subspecies (Mann-Whitney W= 245.5, *P* = 0.604).

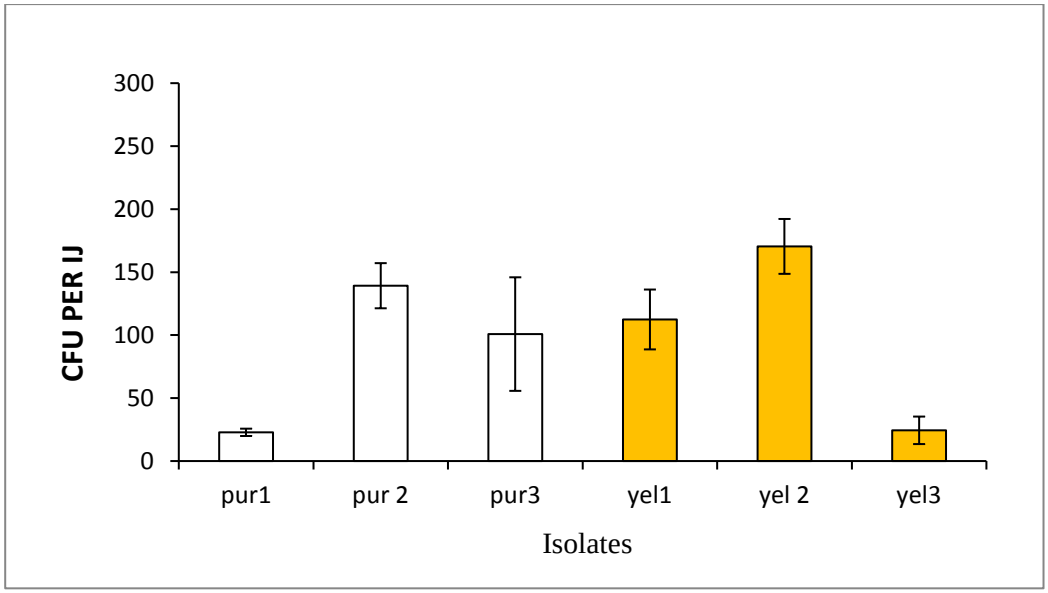


Fig 3.1 Mean (±SE) number of bacteria per IJ for six isolates of *H. downesi* (IJs used for infection). There was no significant different between the isolates. When the data were pooled by bacterial subspecies, there was no difference between pur and yel *P. temperata* subspecies (Mann-Whitney W= 245.5, *P* = 0.604) (N=10/ isolate).

Insect mortality

Mortality of insects was recorded (based on luminescence) three days after exposure to IJs (Table 3.2). There was no significant difference in mortality among the six isolates on day 3 (Yates chi-sq = 7.822, DF = 5, $P = 0.1660$). However, according to the pooling of data by *P. temperata* subspecies, there was a significant difference between *P. t. cinerea* and *P. t. temperata* (Fishers exact test, $P = 0.033$). Although they were exposed to IJs at the same time, 99% of the insects which were infected by the IJs that carry *P. temperata* subspecies were dead by day 3 compared to 89% of insect that were infected by the IJs which carry purple bacteria. The remaining insects were dead on day four.

Table. 3.2 Number and percentage of insects (showing luminescence) three days after exposure to six isolates of *H. downesi* (Yates chi-sq $\chi^2 = 7.822$, $P = 0.1660$).

P.temperata subspecies		number dead at day 3 (/24)	% dead
P. t. cinerea	pur1	19	79.17
	pur2	23	95.83
	pur3	22	91.67
P. t. temperata	yel1	24	100.00
	yel2	23	95.83
	yel3	24	100.00

Number of bacteria per insect

The mean number of bacteria per insect ranged from 2.31×10^8 million to 3.8×10^9 billion (Fig3.2), There was no significant difference due to bacterial subspecies ($F_{1, 24} = 0.81$, $P = 0.419$) but there was a difference between isolates ($F_{4, 24} = 8.21$, $P < 0.001$). Pur1 nematodes carried fewer bacteria than each of the Yel isolates (Fig 3.2).

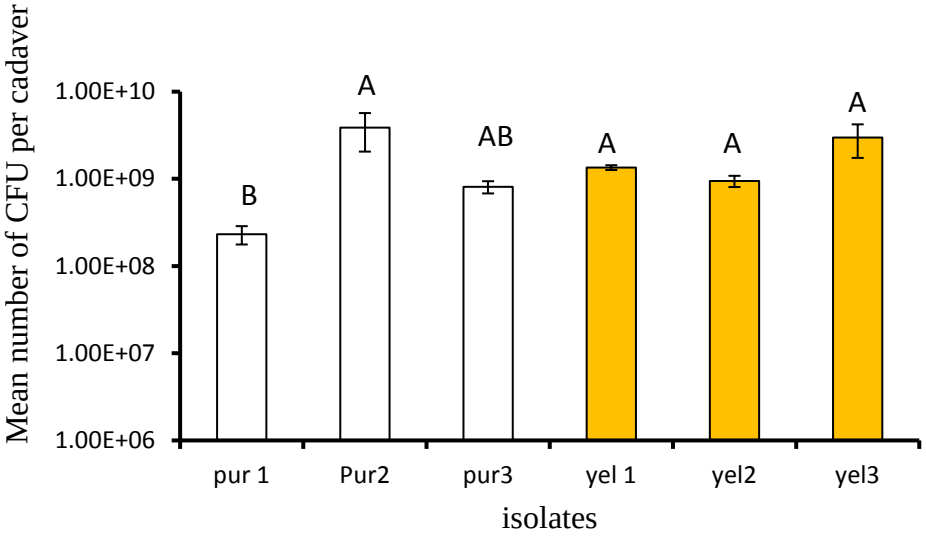


Fig. 3.2 Mean (±SE) number of bacteria per insect cadaver three days after exposure to *H. downesi* of six isolates. Bacteria were assessed by colony forming units on agar plates- Bars accompanied by the same letter are not significantly different (ANOVA $F_{5, 24} = 7.90$, $P < 0.00$, Tukey's test) (N = 5 / isolate).

The invasion rate of IJs in each *Galleria mellonella*

The mean number of nematodes invading was ranged from 31.2 (Pur 1) to 54.1 nematodes (Yel 2) (Fig 3.3). The invasion rate of the IJs in each cadaver is one of the factors which might have an influence on rate of the production of new IJs. There was no significant difference in terms of the numbers invading due to bacterial subspecies ($F_{1, 44} = 0.89$, $P = 0.399$), but there was a difference between isolates ($F_{4, 44} = 6.74$, $P < 0.001$), with Pur3 invading at a lower rate than two of the Yel isolates (Yel1 and Yel2).

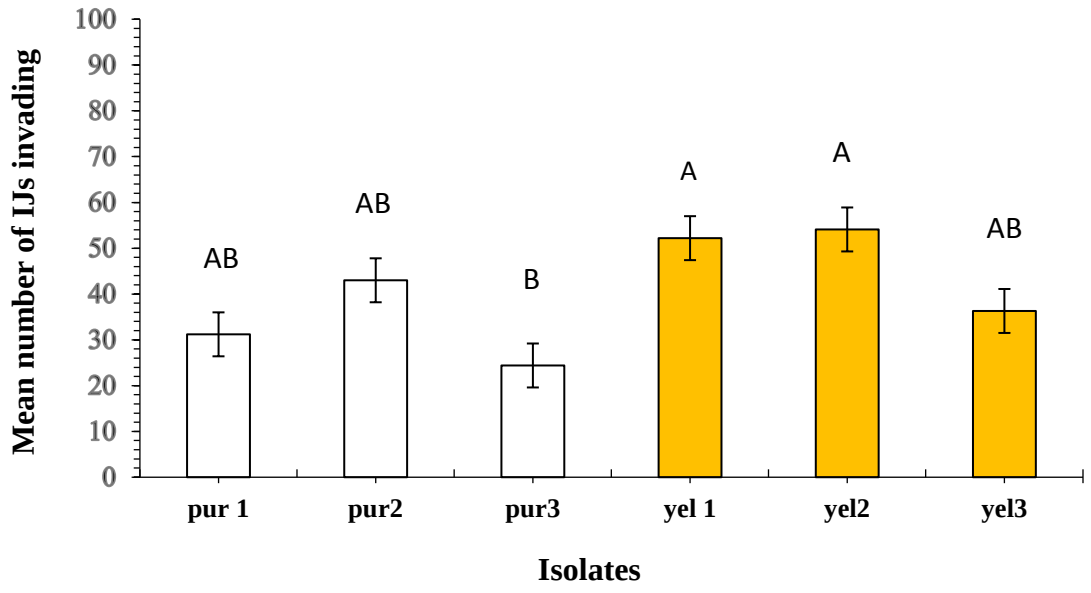


Fig. 3.3 Mean (±SE) number of IJs invading among six isolates of *H.downesi*. There was no significant difference due to the Bacterial subspecies, Bars accompanied by the same letter are not significantly different (ANOVA test $F_{5, 44} = 6.23$, $P < 0.001$).

Number of IJs emerging

The mean number of IJs emerging from the infected cadavers ranged from 72,786 (pur2) to 106,665 (yel3) (Fig 3.4). There was no significant difference in the number of emerging IJs due to either bacterial subspecies (GLM, $F_{1, 24} = 3.254$, $P = 0.146$) or isolate ($F_{4, 24} = 0.66$, $P = 0.628$)

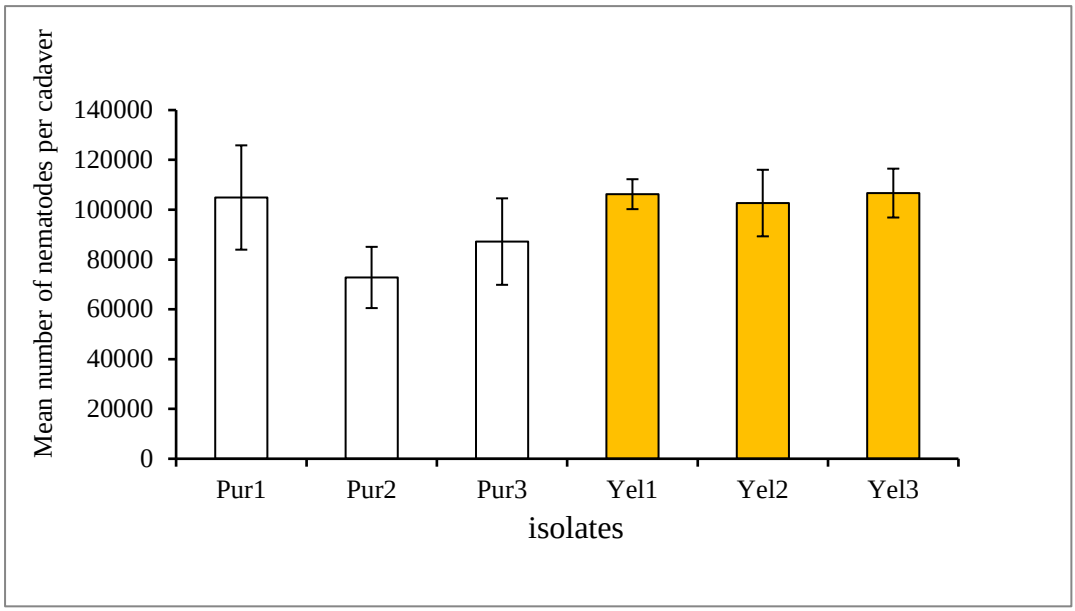


Fig. 3.4 The mean (± SE) of the total emergence among the six isolates of *H. downesi*. There were no significant differences between bacterial subspecies ($F_{1, 24}$ = 3.254, *P* = 0.146) or isolate ($F_{4, 24}$ = 0.66, *P* = 0.628) (N = 5 / isolate).

The average proportion in the early emergence (E4)

The percentage of IJs emerging early at day 4 (E4) appeared to differ between yel and pur isolates. The proportion of IJs emerging early in isolates carrying *P. t. cinerea* bacteria appeared higher (the mean percentage of IJs emerging early for pur isolates ranged from 18.7 to 33.5%) compared with the *P. t. temperata* isolates (mean percentage for yel isolates ranged from 11.9 to 17.5%) (Fig3.5). However, there was no significant difference due to either bacterial subspecies or isolate in a nested GLM (bacterial subspecies $F_{1, 24}$ = 4.09, *P* = 0.113 , isolate $F_{4, 24}$ = 1.05, P = 0.402)

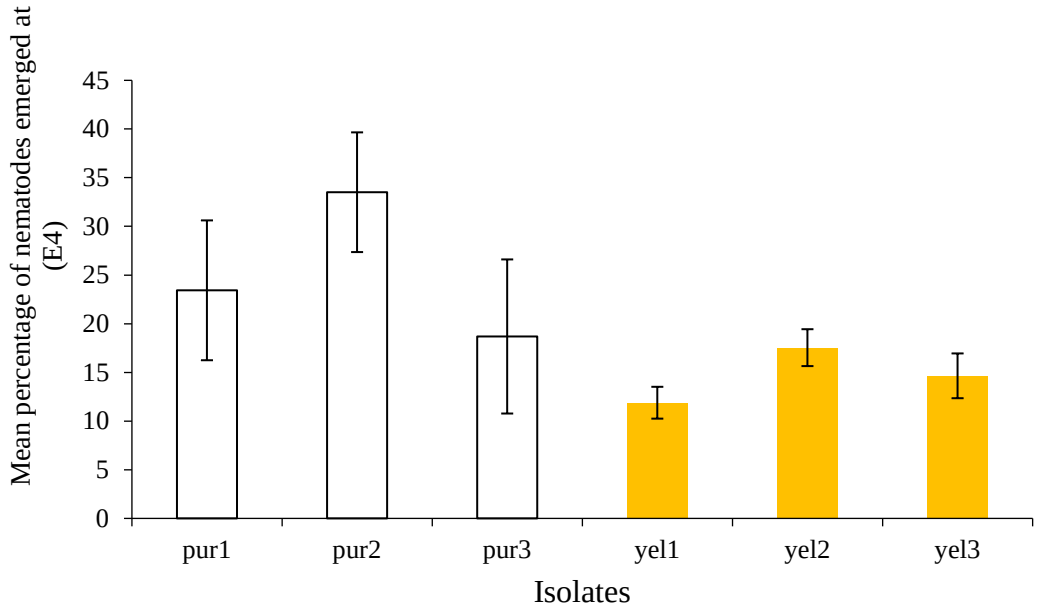


Fig. 3.5 Mean (±SE) percentage of IJs emerging early day 4 (E4) in each isolate There was no significant difference due to bacterial subspecies (GLM, $F_{1, 24} = 4.09$, $P = 0.113$) or isolate (GLM, $F_{4, 24} = 1.05$, $P = 0.402$).

Number of bacteria per emerging IJ

The bacteria were extracted from infective juveniles of *H. downesi* emerging from infected cadavers (Fig. 3. 6). There was a significant difference due to the isolates (GLM $F_{4, 294} = 7.39$, $P < 0.001$). However, there was no significant difference due to the bacterial subspecies (GLM $F_{1, 294} = 0.77$, $P > 0.05$).

The number of bacteria per IJ for early and late emerging IJs were assessed in each of two isolates (Pur1 & Yel1) within five cadavers each. GLM analysis show that there was a significant difference in the number of bacteria colony between early and late emergence (Fig3.7A), there was no significant difference between of the isolates. but there was a significant interaction between isolate and time of emergence. The interaction is shown more clearly by pooling the result for all of the cadavers for each isolate, there was a bigger difference between early and late emergence for pur1 than for yel1 (Fig 3.7B).

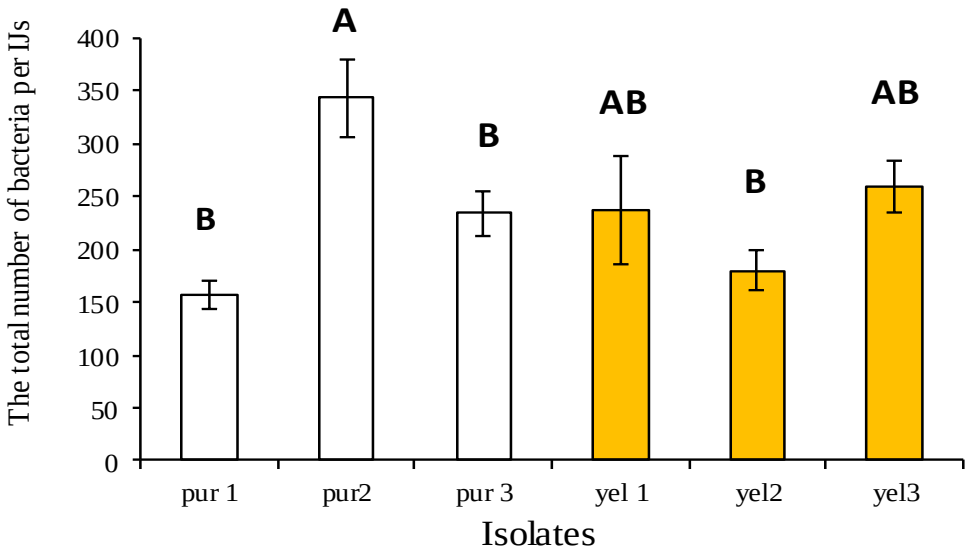
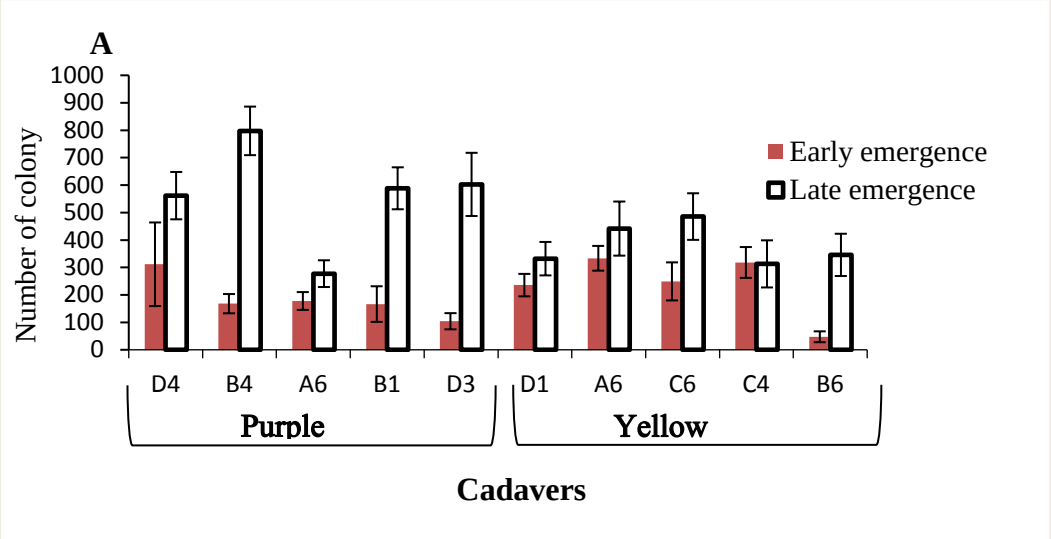


Fig. 3.6 Mean (±SE) number of bacteria per IJ in each of six isolates. Bacteria were assessed by colony forming units on agar plates. GLM analysis show that there were not significant different in terms of the bacterial subspecies (GLM $F_{1, 294} = 0.77$, $P > 0.05$), however there were significant differences in terms of the isolate. (GLM $DF_{4,294}$, $F = 7.39$, $P < 0.001$). Bars accompanied by the same letter are not significantly different (Tukey's, Test) (N= 50 IJs / isolate).

There were difference between the replicate cadavers (Table 3.3, Fig 3.7A), for example in yel1 cadaver C4, there were approximately 300 bacteria per IJ in both early and late emergence, while in yel1 cadaver B6 there were very few bacteria per IJ in early emerging IJs (Fig. 3.7A)

Table. 3.3 Analysis of variance

Source	DF	F-Value	P-Value
colour	1	0.95	0.359
harvest	1	77.62	< 0.001
colour*harvest	1	17.2	< 0.001
Cadaver (colour)	8	2.82	0.006
Error	188		



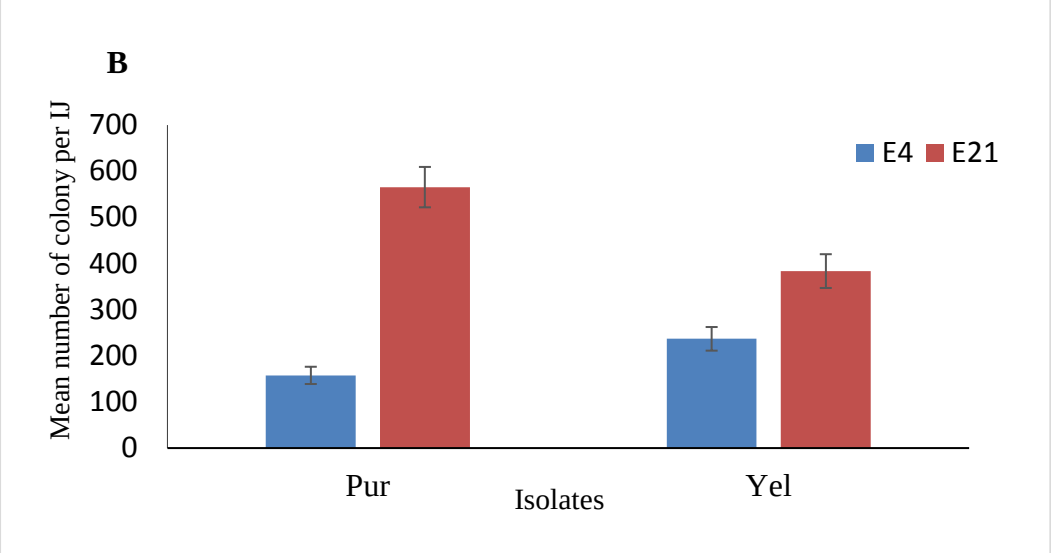


Fig. 3.7 Mean (±SE) number of bacteria colony per IJ in each of two isolates (Pur1 & Yel1). Data are shown for IJs from each of 10 cadavers. Bacteria subspecies were assessed by colony forming units on agar plates for early and late emergence.

3.3.2 Production Capacity of *Heterorhabditis downesi* Bull Island isolates (experiment 2)

The total number of juveniles emerging

The average of total emergence shows that nematodes carrying *P. t. cinerea* (pur isolates) have slightly higher number than the nematodes carrying *P. t. temperata* (yel isolates) (Fig3.8). However, there was no significant difference due to either bacterial subspecies ($F_{1, 24} = 0.62$, $P = 0.475$) or isolate ($F_{4, 24} = 0.83$, $P = 0.519$).

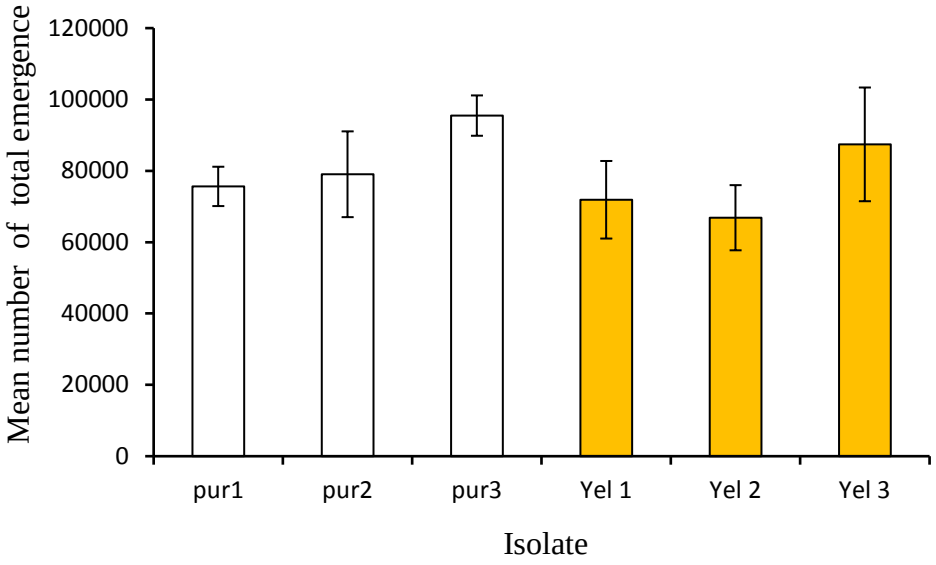


Fig. 3.8 Mean (± SE) of the total emergence among the isolates. There was no significant difference between bacterial subspecies ($F_{1, 24} = 0.62$, $P = 0.475$) or isolate ($F_{4, 24} = 0.83$, $P = 0.519$).

The average proportion in the early emergence (E7)

In terms of the proportion of IJs in the early emergence (E7) in each isolate there was no significant difference due to the bacterial subspecies (Fig 3.9) (GLM, $F_{1, 24} = 0.01$, $P = 0.929$) or isolate (GLM, $F_{4, 24} = 0.99$, $P = 0.433$).

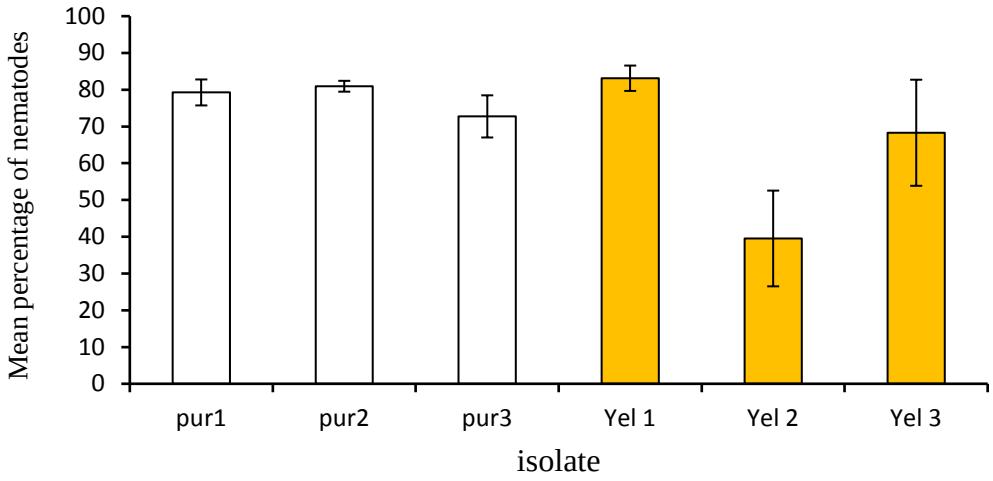


Fig. 3.9 Mean (±SE) percentage of IJs emerging early (by day 4 or 7) in each isolate. There was no significant difference due to bacterial subspecies (GLM, $F_{1, 24} = 0.01$, $P = 0.929$) or isolate (GLM, $F_{4, 24} = 0.99$, $P = 0.433$).

3.3.3 Effect of *P. temperata* subspecies on production of IJs from cadavers under dry conditions in sand

Mortality of infected insects

At 64 hrs after infection, insects that were infected with pur isolates carrying *P. t. cinerea* had slightly higher number dead than the insects infected with the *P. t. temperata* (yel) isolates (Fig 3.10). Mortality data for all three isolates of each type were pooled for analysis, showing a significant difference due to bacterial species (Mann Whitney test pur vs yel W = 435, $P = 0.004$). However, after 72 hrs the mortality was 100% for both isolates (Fig 3.11).

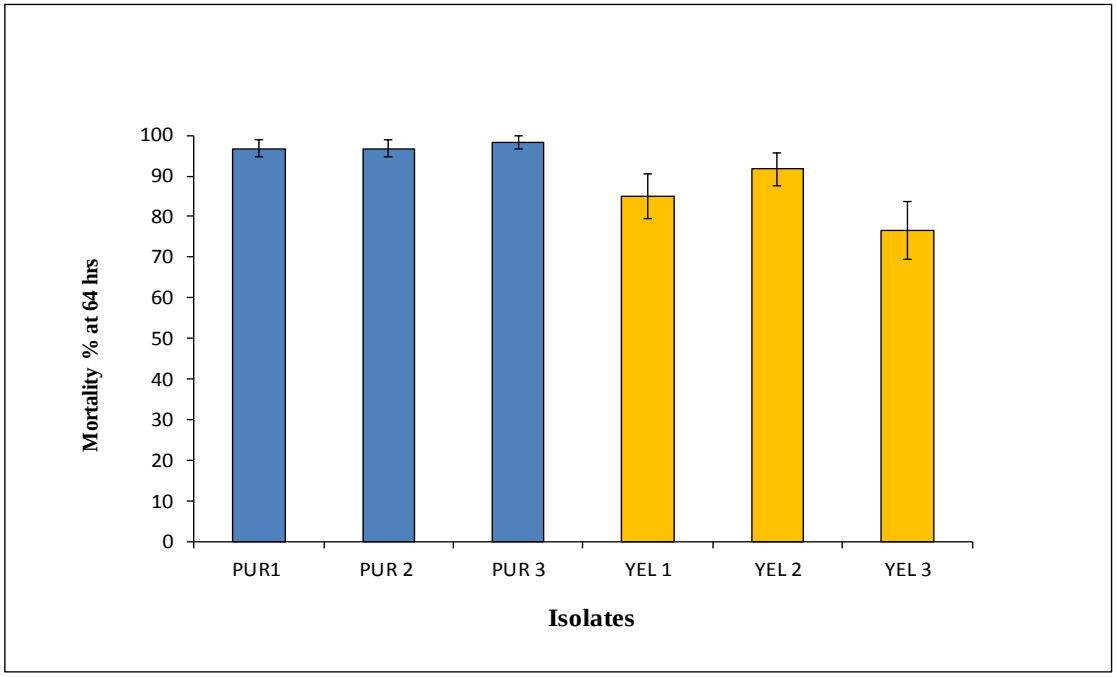


Fig. 3.10: Mean (±SE) percentage mortality at 64 hrs in each isolate, **(**Mann Whitney test pur vs yel *P* = 0.435) (N = 50 insect / isolate).

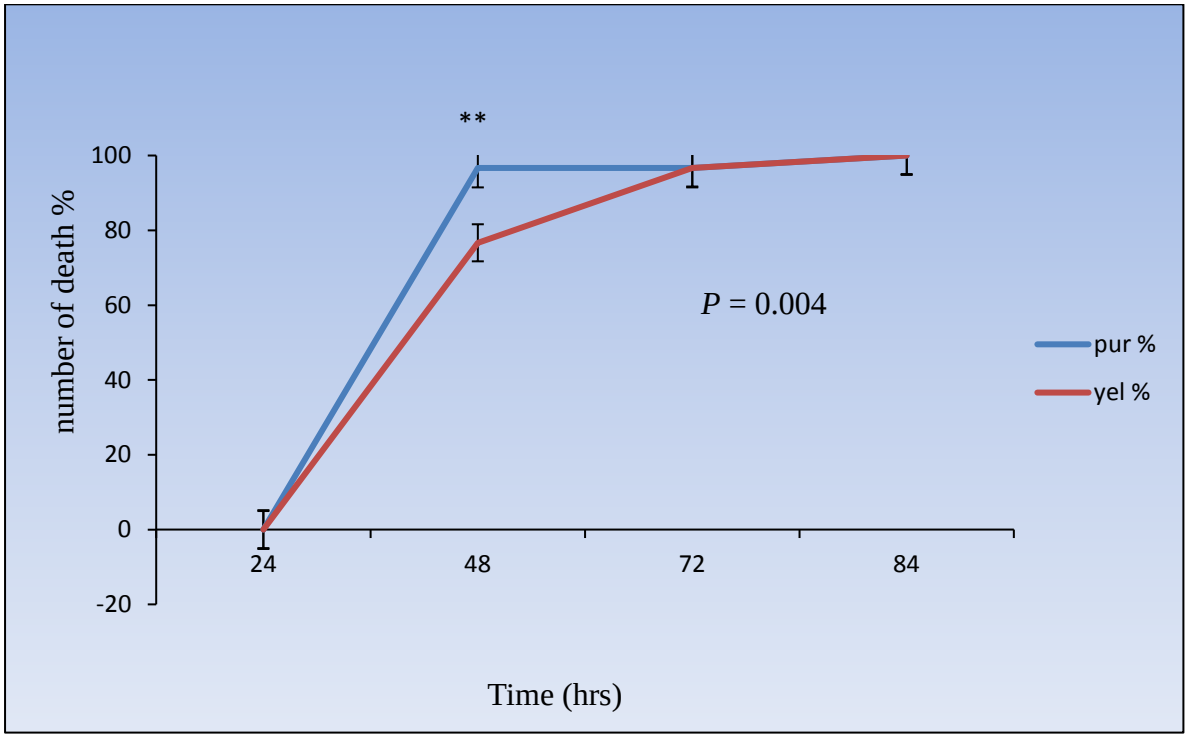


Fig. 3.11 The graph show the percentage of mortality of *G. mellonella* infected with pur or yel nematodes carrying *P. t. cinerea* or *P. t. temperata*, respectively, at various times post infection. Mean of three isolates for each type (N = 60 insects /isolate).

Emergence of IJs - moist conditions (control)

The average total number of IJs emerging for both early and late emerging (E4 and E21) from cadavers kept under moist conditions ranged from least (87666 IJs yel2) to maximum (129809 IJs pur2) **(**Fig3.12**)**. There was no difference in numbers emerging due to either bacterial subspecies or isolate.

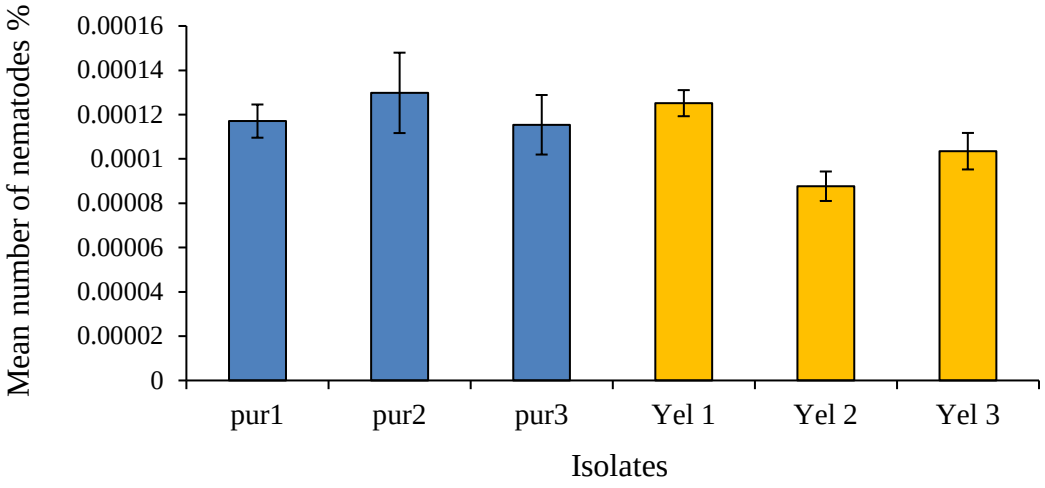


Fig. 3.12 Mean (±SE) number IJs of six *H. downesi* isolates emerging when kept under moist conditions. GLM analysis for bacterial subspecies ($F_{1,24}$ = 1.69, P = 0.063, Isolates $F_{4,24}$ = 1.75, P = 0.171) (N = 5 insects / isolates).

For the cadavers maintained in moist conditions the proportion of IJs emerging early was low (less than 12% on average for each isolate) (Fig.3 13). The average proportion of IJs emerging early (E4 as percentage of total E4 +E21) differed by bacterial subspecies: a lower proportion of IJs emerged early for the pur isolates carrying *P. t. cinerea* than for the yel isolates carrying *P. t. temperata* (Fig.3. 13).

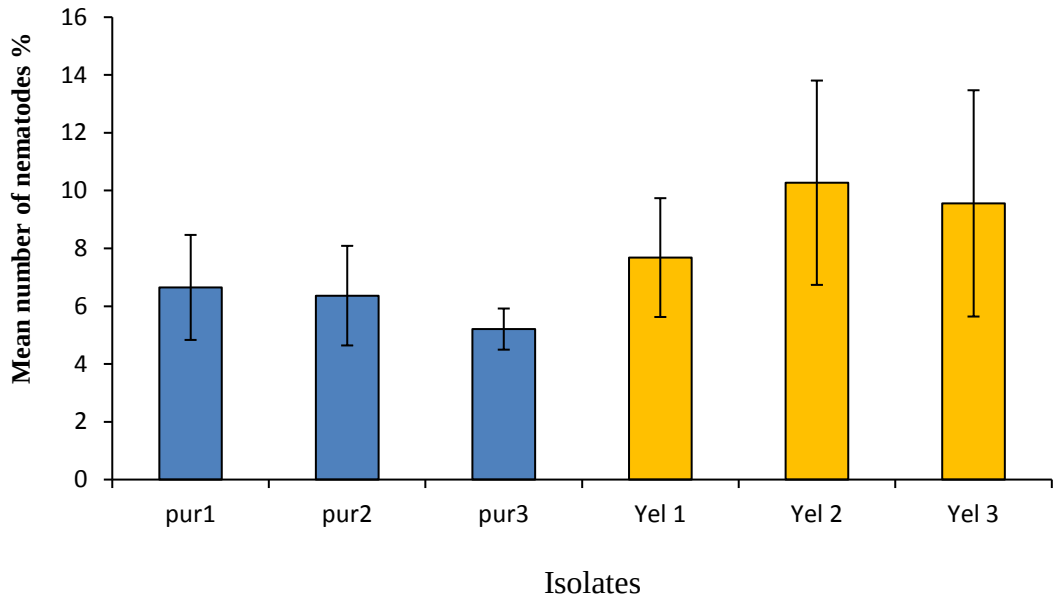


Fig. 3.13 Mean (±SE) percentage of IJs emerging early for six isolates of *H. downesi* maintained in moist conditions (GLM, bacterial subspecies $F_{1.24,} = 12.13$, $P = 0.025$, Isolate $F_{4, 24,} = 0.18$, $P = 0.945$) (N = 5 insects / isolates).

Production of IJs from cadavers under dry conditions

When cadavers were wetted after 28 days in dry sand, all of them produced IJs (Table 3.3), but after longer exposure times fewer cadavers produced IJs (Table 3.3 and Fig 3.14). After 42, 54 and 61 days in dry sand, more pur isolates (carrying *P. t. cinerea*) than yel isolates (carrying *P. t. temperata*) produced IJs, and the difference between bacterial subspecies was significant after 42 and 54 days (Fig3.15).

The emergence of IJs following 28 and 48 days in dry sand is shown for each of the six *H. downesi* isolates in Figures 3.14 and 3.15. These figures include data only for those cadavers that produced IJs. Cadavers infected with pur isolates (carrying *P. t. cinerea*) tended to produce more IJs than those infected with yel isolates (carrying *P. t. temperata*) (Fig. 3. 16). The difference was significant at 28 days and approached significance ($P = 0.058$) of 42 days.

Table. 3. 3 Number of *G. mellonella* cadavers (out of 10) from which IJs emerged after the cadavers had been in dry sand for 28-61 days. Cadavers were infected with *H. downesi* isolates carrying either *P. t. cinerea* (pur) *or P. t. temperata* (yel).

Number of cadaver with IJs emerging in a white – trap after the exposure time (days)				
Isolate	28days	42days	54days	61days
pur1	10	8	2	0
pur2	10	7	0	1
pur3	10	10	6	0
yel1	10	5	0	0
yel2	10	8	1	0
yel3	10	3	1	0
Total	60	41	10	1

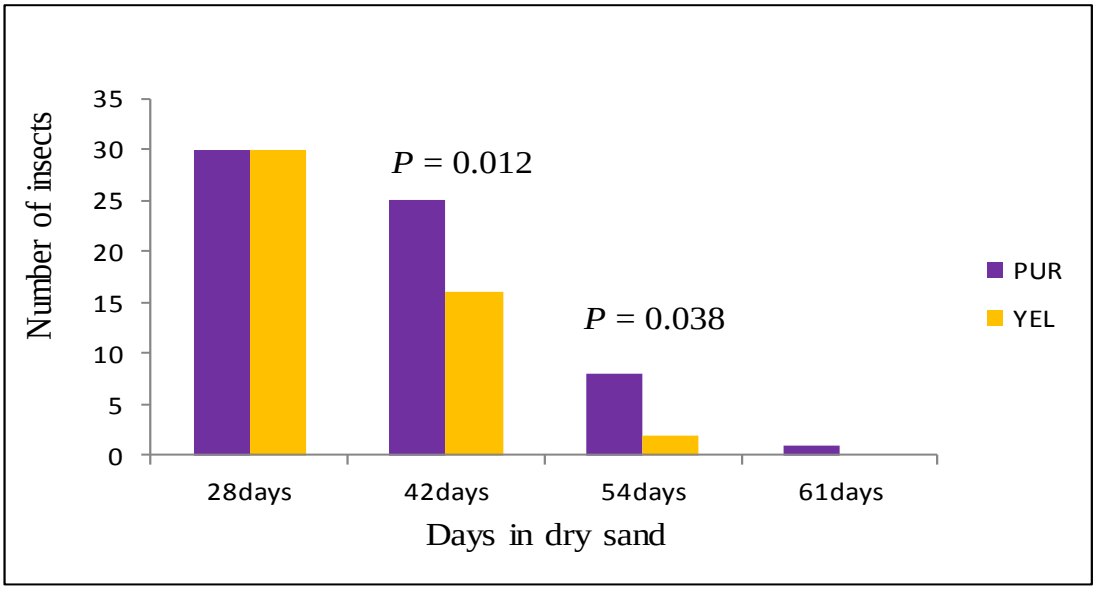


Fig. 3.14 Number of *G. mellonella* cadavers (out of 30) from which *H. downesi* IJs emerged when wetted following 28-61 days in dry sand (total for all three isolates of each type). *P* values show difference between *H. downesi* pur isolates carrying *P. t. cinerea* and yel isolates carrying *P. t. temperata* (Chi square test).

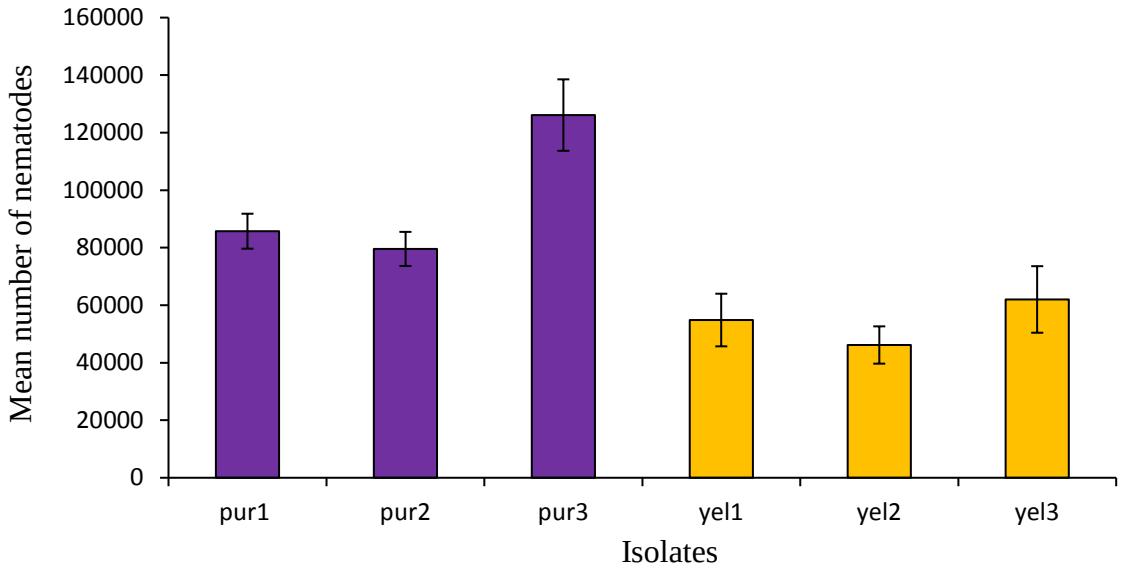


Fig. 3.15 Mean (±SE) number of *H. downesi* IJs emerging from cadavers following 28 days in dry sand. There was a significant difference between bacterial subspecies (Nested GLM analysis, ($F_{1,54}$ = 7.83, *P* = 0.049).There was also a significant difference between isolates (GLM $F_{4,54}$, = 4.33, *P* = 0.004).

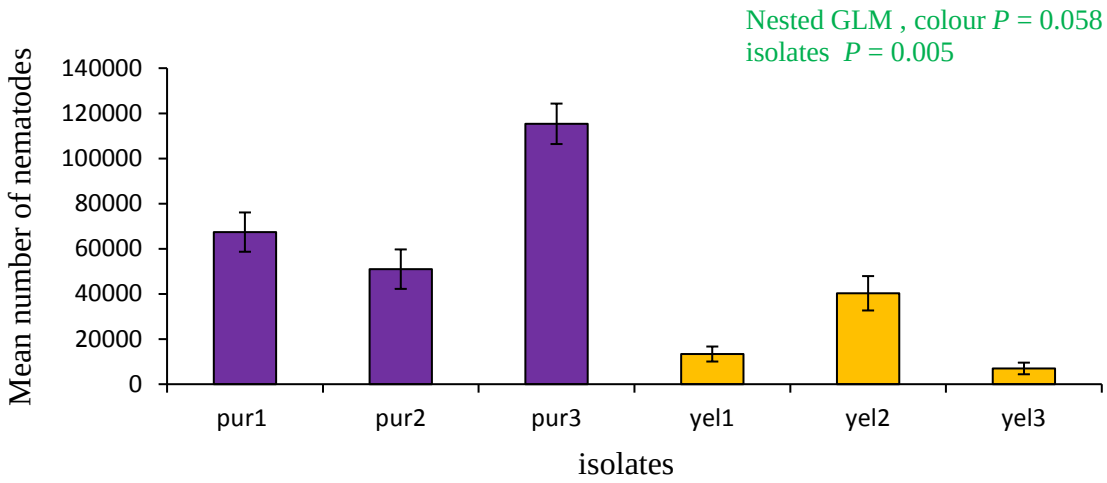


Fig. 3.16 Mean (±SE) number of *H. downesi* IJs emerging from cadavers flowing exposure in dry sand for 42 days. There was no significant difference between bacterial subspecies (Nested GLM analysis ($F_{1, 54}$, = 6.97, *P* = 0.058), but there was a significant difference between isolates (GLM $F_{4,54}$ = 4.22, *P* = 0.005).

Both the number of cadavers producing IJs and the number of IJs produced per cadaver that yielded IJs were higher for pur than for yel isolates. The number of IJs emerging from all cadavers (including those that produced no IJs) is shown in Fig 3.16. A further analysis was done (by Dr Caroline Brophy of MU Mathematics and Statistics Department) accounting for both of these parameters together and including the data for the cadavers that were kept moist (controls- 0 days desiccation). This again showed evidence of a difference in the average numbers of IJs per cadaver between *P. t. cinerea* and *P. t. temperata* (3 isolates of each) after 28, 42 and 53 days in dry sand (P = 0.031, <0.001 and 0.009 respectively) but not after 0 (controls) days (Fig.3.17). Thus, in cadavers that were maintained in moist conditions throughout (controls), symbiont subspecies did not affect the number of IJs emerging. All of these cadavers produced IJs, with an average of over a hundred thousand IJs per insect (mean $\pm$ SE, 113,099 $\pm$ 4816, N = 30).

However, when cadavers were subjected to desiccation, generally fewer IJs emerged from those cadavers harbouring *P. t. temperata* than from those with *P. t. cinerea,* and after two months in dry sand the only nematodes to emerge were from cadavers with *P. t. cinerea* (Fig 3.17).

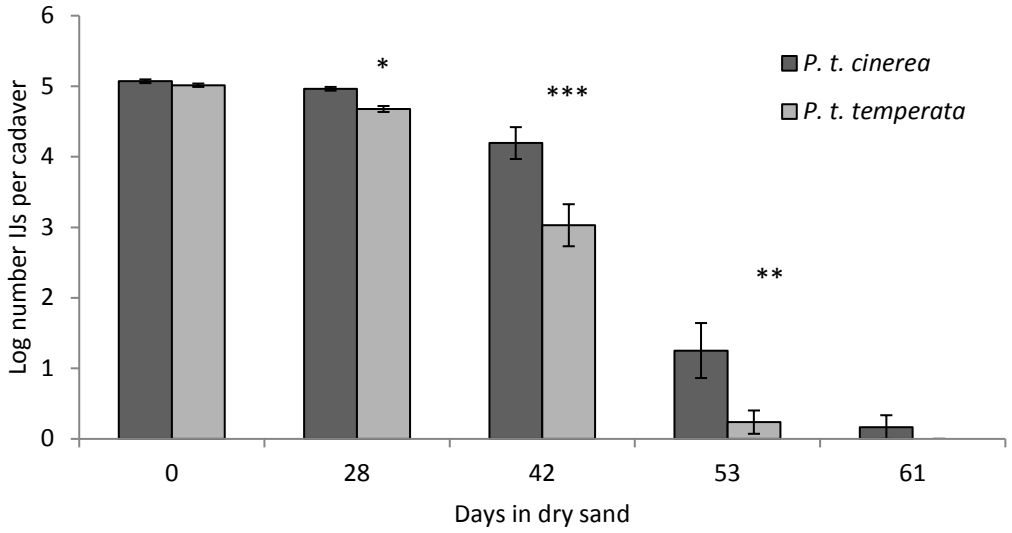


Fig. 3.17 Number of *H. downesi* IJs emerging from wax moth cadavers after various periods in dry sand (log_{10} mean $\pm$ SE IJs per cadaver). The nematodes used to infect the wax moths carried either *P.t. cinerea* or *P. t. temperata.* Asterisks indicate a significant difference between subspecies at a given time interval (* $P < 0.05$, ** $P < 0.01$; *** $P < 0.001$).

3.3.4 Desiccation of cadavers infected with *H. downesi- P. temperata* hybrids at 0% relative humidity

There was no difference between bacterial subspecies for either strain of nematode (Pur1 or Yel3) in number of cadavers from which IJs emerged after 28 days in 0% RH (Table3.4) or in the median number of IJs emerging per cadaver (Fig. 3.18).

Cadavers infected with either Pur or Yel nematodes carrying *P. t. cinerea* lost weight more slowly than cadavers infected with the same nematodes carrying *P. t. temperata* (Fig3.19). The data for both nematode strains were combined for analysis, which showed strong evidence of a difference in cadaver weight loss between symbiont subspecies after 12, 15 and 18 days (Fig. 3.20) at % RH ($P < 0.01$ in each test), weaker evidence of differences after 3, 6 and 9 days ($P = 0.028$, 0.042 and 0.058 respectively), and no evidence of differences at the four later time points. Cadavers harbouring *P. t. cinerea* lost weight more slowly than

those harbouring *P. t. temperata*, though both sets of insects eventually lost about 70% of their original weight during desiccation (Fig. 3.20).

Table. 3.4 Number of *G. mellonella* cadavers (out of 20) from which *H. downesi* IJs emerged after 28 days at 0% RH. Cadavers were infected with two wild-type strains and two strains where the symbionts had been swopped

	Nematode	**Bacteria**	**Number of cadavers with IJ**
Strain	*H. downesi*	*P. temperata*	
Pp (wild type)	Pur1	Pur1 (*P.t. cinerea*)	8
Py (hybrid)	Pur1	Yel3 (*P.t. temperata*)	8
Yy (wild type)	Yel3	Yel3 *P.t. temperata*)	5
Yp (hybrid)	Yel3	Pur1 (*P.t. cinerea*)	4

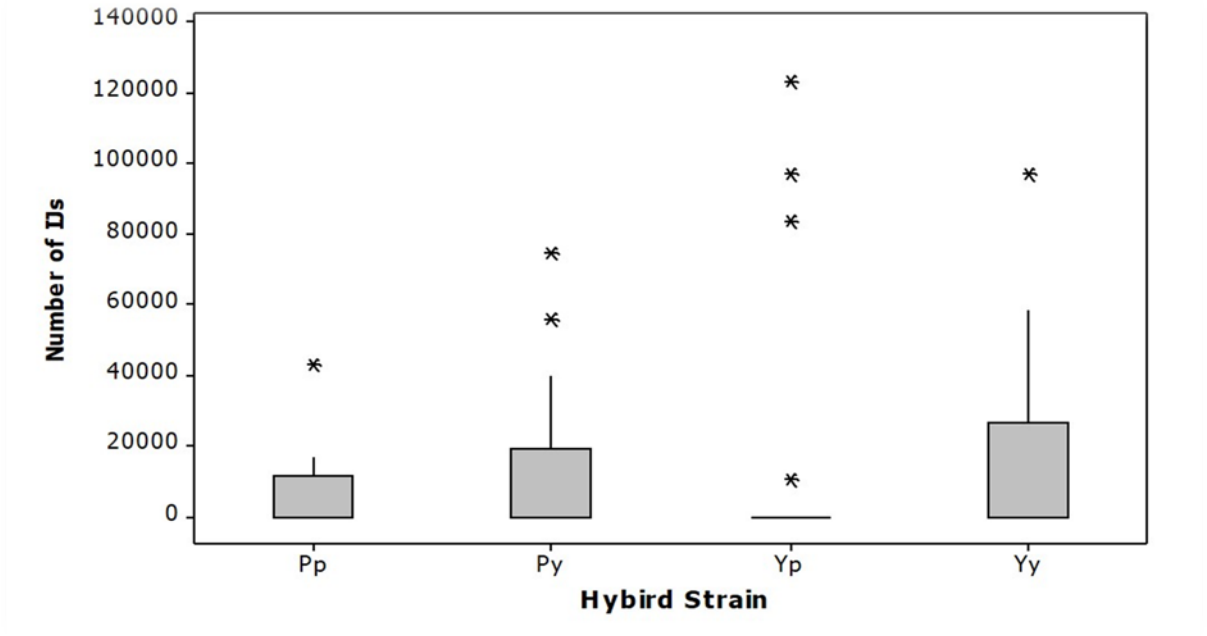


Fig. 3.18 Number of *H. downesi* IJs emerging from wax moth cadavers after 28 days at 0% relative humidity. The nematodes (pur1 and yel3) used to infect the wax moths each carried either *P. t. cinerea* or *P. t. temperate.* The box represents the inter-quartile range with the median shown as a line within it. There was no difference due to bacterial species either for Pur1 nematodes (Pp versus Py: Mann Whitney W = 295, *P* =0.695) or Yel3 nematodes (Yy versus Yp: Mann Whitney W = 416, *P* = 0.882).

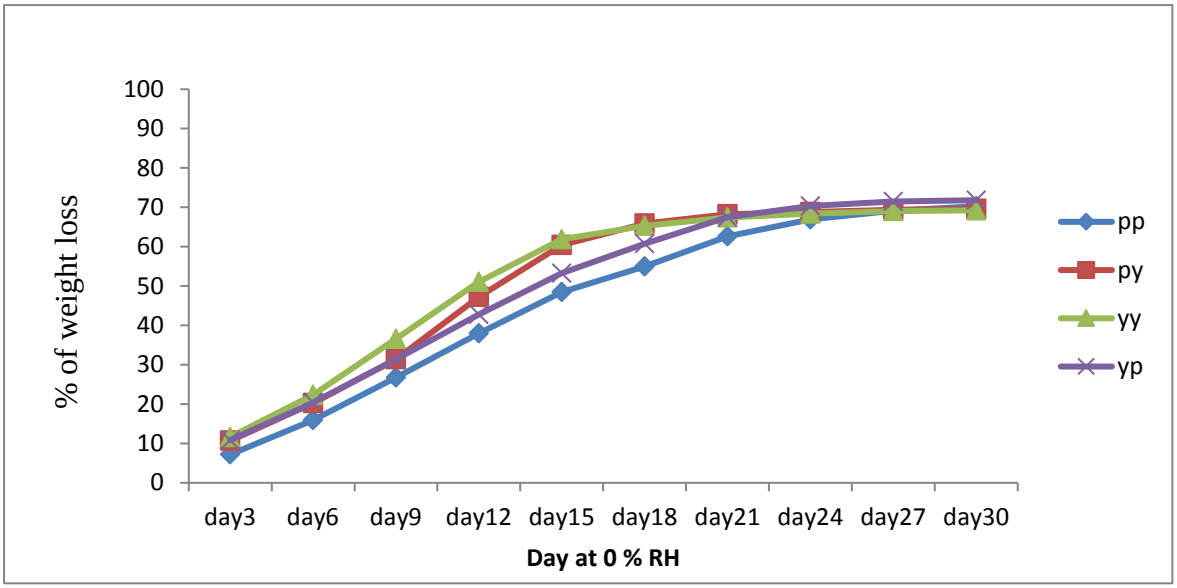


Fig. 3.19 Percentage weight loss of *G. mellonella* cadavers infected with *H. downesi* – *P. temperata* hybrid and wild type (see Table 3.4 for explanation).

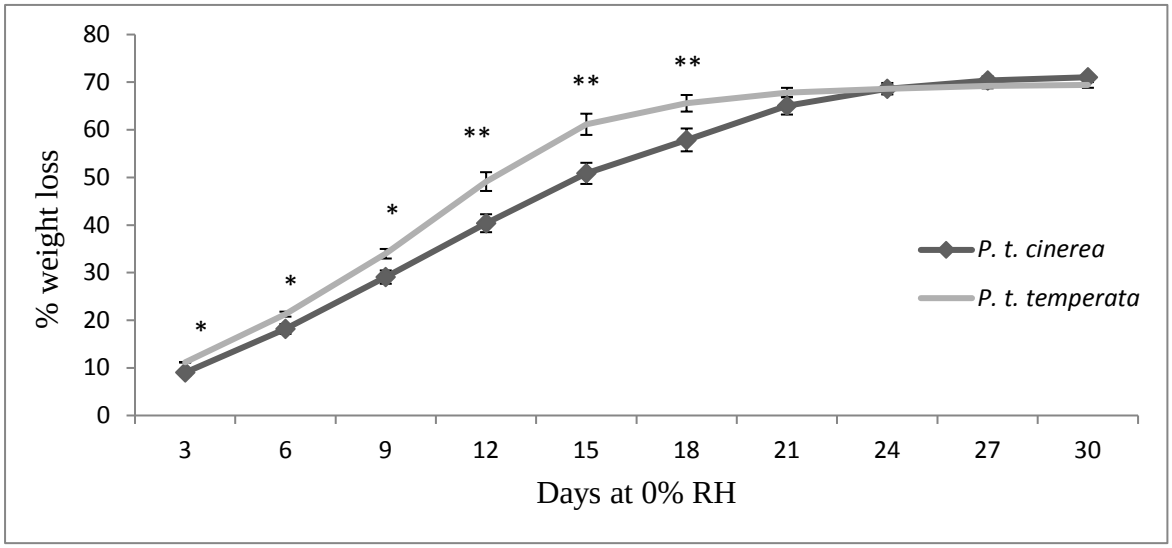


Fig. 3.20 Percentage of weight loss of *G. mellonella* cadavers infected with *H. downesi* carrying either *P. t. cinerea* or *P. t. temperata* and incubated at 20°C over silica gel at 0% relative humidity. Asterisks indicate a significant difference between subspecies at a given time interval (* P <0.05, ** P < 0.01).

3.3.5 Effect of salt concentration on *P. temperata* growth

Salt concentration had a significant effect on growth of *P. temperata* (ANOVA, $F_{6, 90}$ = 98.53, P < 0.001), but there was no difference due to subspecies (ANOVA, $F_{1, 90}$ = 0.05, P = 0.834) or isolate (nested within subspecies: ANOVA, $F_{4, 90}$ = 2.30, P = 0.088) and no

interaction between isolate and salt concentration (ANOVA, $F_{24, 90} = 0.66$, $P = 0.877$). Results for all isolates are shown in Fig 3.21.

Overall, the optimum salt concentration for *P. temperata* was 0.5% NaCl, though 1.0 % and 1.5% did not differ significantly from 0.5% (Fig 3.22).

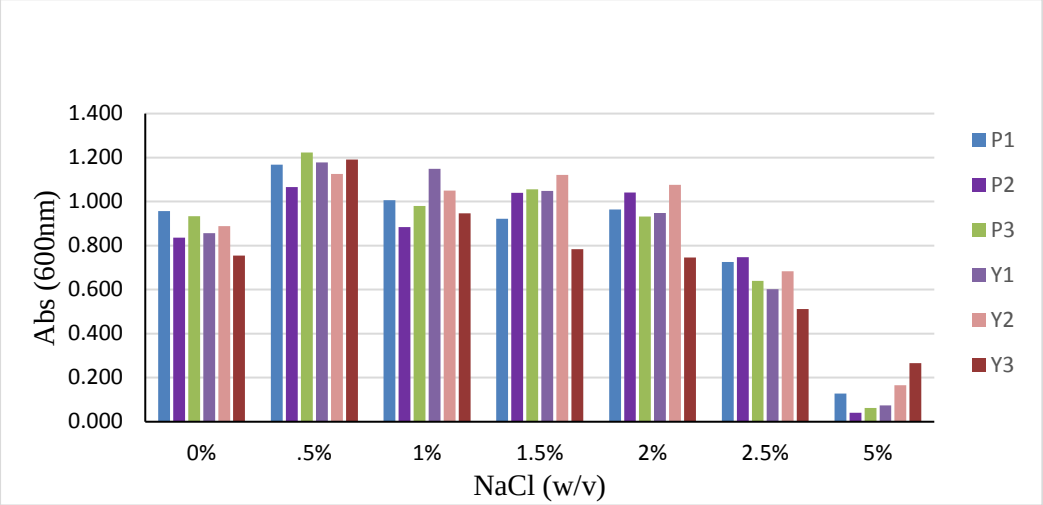


Fig. 3.21 Absorbance at 600 nm of six isolates of *P. temperata* grown overnight in Luria broth with different concentrations of NaCl (0-5%). P isolates are *P. t. cinerea* and Y isolates are *P. t. temperata*. Mean of three replicates per treatment.

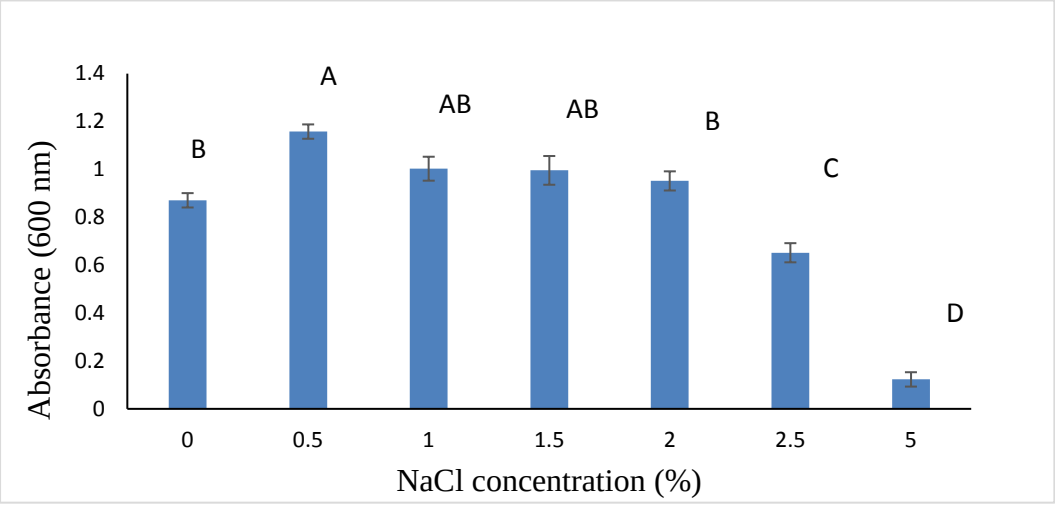


Fig. 3.22 Mean (+/- SE) absorbance at 600 nm of *P. temperata* grown overnight in Luria broth with different concentrations of NaCl (0-5%). N= 18 for each NaCl concentration. Bars accompanied by the same letter do not differ significantly (Tukey's test).

3.4 Discussion

There was no effect of *Photorhabdus* subspecies on reproductive capacity of *H. downesi*, measured by the numbers of IJs produced in wax moth larvae. This experiment was repeated three times (including the control insects from the desiccation experiment), and in no case was there a significant difference in IJ yield due to the bacterial symbiont. This suggests that the two subspecies of bacteria are equally valuable for the nematodes as a food source. However, there may be differences in the quality of the IJs produced on these two bacterial types. The IJ quality was not measured in the experiments, but Maher (2014) found no difference in IJ survival or virulence for various hosts due to these two subspecies, using the same Bull Island isolates.

There was not a consistent difference between the two subspecies in the speed of kill. In the first experiment, *P. t. temperata* killed the insects slightly faster, while in the controls for the desiccation experiment, *P. t. cinerea* killed slightly faster. In these experiments, insects were infected by IJs carrying the symbiont, and so the number of bacteria in the inoculum may have varied. For comparison of virulence between subspecies, it would be better to inject a known dose of bacteria. In the first experiment, there were differences between isolates – but not between bacterial subspecies – in the number of IJs invading, and in the average number of bacteria carried per IJ. In experiment 1, Pur1 had both a relatively low number of IJs invading (Fig.3.3) and a low number of bacteria per IJ (Fig. 3.1) which may explain why it had the lowest proportion of insects dead by day three in the same experiment (Table 3.2). Pur1 also had the lowest number of bacteria per cadaver on day 3. This shows the importance of the bacterial inoculum on virulence, and thus the importance of the number of bacteria carried by the IJ, then fitness trait was measured.

There was no difference due to bacterial subspecies in the number of bacteria carried by *H. downesi* IJs. The IJs carried around 200-300 bacteria on average, which is slightly higher than the numbers carried by *S.carpocapsae* and *H. bacteriophora* (Flores-Lara *et al.*, 2007, Ciche *et al.*, 2008). Interestingly, for both pur1 and yel1, IJs emerging later carried more bacteria than those emerging early, on day 4. The early emerging IJs probably are those that were produced in the first generation hermaphrodites, while the later emerging ones may include IJs produced by later generations (Ryder and Griffin, 2002). Whether this is due to a difference between the nematodes or the bacteria present at different times is unclear. An effect of time of emergence on number of bacteria carried has not been previously reported for any EPN species, though behavioural differences between early and later emerging *H. megidis* IJs have been described (O'Leary *et al.*, 1998, Ryder and Griffin, 2003).

The experiment in dry sand provides evidence of differing ability of the symbiont subspecies to protect nematodes within insect cadavers under dry conditions. Under moist conditions, both symbiont subspecies gave a similar yield of IJs, whereas following desiccation, cadavers with *P. t. cinerea* produced more IJs than those with *P. t.* temperata. Better protection against desiccation correlates with the observed distribution of the subspecies on Bull Island, where *P. t. cinerea* predominates in the drier seaward region of the dunes (Rolston *et al.*, 2005, Maher, 2014). While the protective effect of the insect cadaver on entomopathogenic nematodes under desiccating conditions has previously been demonstrated for *Heterorhabditis bacteriophora* and for several species of *Steinernema* (Koppenhöfer *et al.*, 1997, Spence *et al.*, 2011, Serwe-Rodriguez *et al.*, 2004), the differential effect of symbiont subspecies has not been reported, and raises significant ecological questions about niche extension and survival strategies of entomopathogenic nematode species.

Since emergence from desiccated cadavers usually began within a few days of being wetted, it is likely that IJ formation had already been completed and IJs emerged once they had recovered sufficiently, adequate moisture was available for movement, and/or the insect cuticle had become sufficiently permissive for them to breach. IJs can persist for considerable lengths of time in cadavers under conditions that would be rapidly lethal to the same IJs in soil: slowing the rate at which water is lost is important in allowing nematode survival (Perry, 1999, Glazer, 2002). (Koppenhöfer *et al.*, 1997) suggested that the drying of the insect cuticle may act as a buffer against further moisture loss within the cadaver, and the host contents (partially digested tissues and bacterial biomass) may afford further protection. In this experiment, the rate at which cadavers lost weight differed between symbiont subspecies, with evidence that *P. t. cinerea* slows the rate of drying relative to *P. t. temperata*. Thus, the protective difference between subspecies may be at least partially explained by an effect on the drying rate of cadavers. Bacterial species and strains differ in their ability to survive drying, and amongst the mechanisms responsible for desiccation resistance is the production of extracellular polymeric substances, particularly exopolysaccharides (Ferreira *et al.*, 2010, Espinal *et al.*, 2012, Greene *et al.*, 2016). Exopolysaccharides not only protect the bacterial cells, but also modify the local environment, for example by altering soil water retention (Rossi *et al.*, 2012). A novel polysaccharide has been identified in *P. t. cinerea* (isolated from *H. downesi* in Hungary), which is unique among bacterial polysaccharide structures and differs significantly from those of *P. t. temperata* and two other *Photorhabdus* species (Kondakova *et al.*, 2015). Since polysaccharides with different structure have different physical properties (Rinaudo, 2004), production of disparate exopolysaccharides is a plausible mechanism by which bacterial strains might differentially affect the survival of organisms with which they associate.

Apart from differentially modifying the cadaver environment, it is possible that differences between the symbiont subspecies also affect the nematodes' stress tolerance

directly. Under dry conditions, nematodes undergo various biochemical changes which allow them to survive desiccation, including the accumulation of polyols and sugars, alterations in fatty acid composition, and synthesis of several low molecular weight proteins (Glazer, 2002, Grewal *et al.*, 2006), any of which might be influenced by diet. In insects also, dietary factors can influence resistance to desiccation and other physical stresses (Colinet and Renault, 2014, Sisodia and Singh, 2012, Andersen *et al.*, 2010). For example, dietary live yeast promoted thermal tolerance in *Drosophila*, as well as altering total lipids and proteins and metabolic profiles (Colinet and Renault, 2014). *Heterorhabditis* species have nutritional requirements for specific *Photorhabdus* strains in order to reproduce (Han *et al.*, 1991, Han and Ehlers, 1998b, Gerritsen and Smits, 1993, Gerritsen and Smits, 1997), indicating that *Photorhabdus* taxa provide different nutritional factors, though the nature of these differences are largely unknown (Clarke, 2008). Additionally, potential protectant molecules may be taken up by nematodes across their cuticles (Qiu and Bedding, 2002, Qiu *et al.*, 2000). All this suggests that *P. t. cinerea* and *P. t. temperata* are likely to convert the interior of their insect hosts to quite different media, upon which the multiplying nematodes feed or from which they may absorb different materials across their cuticles.

Whatever the reason for the differences between the *P. temperata* subspecies in the protection they confer on the nematodes within the cadaver, *P. t. cinerea* clearly provides an advantage to its associated nematodes under dry conditions, such as may occur at the front of a dune system, where this subspecies predominates. While it is unlikely that such extreme drought would persist for weeks in Ireland, the effects of drying on organisms may be exacerbated by other adverse conditions in the dune sands, such as high temperatures and fluctuating salinity. Our experiments were conducted at 20^{o}C (within the optimum temperature range for this species), while the sand near the surface may reach 40^{o}C in summer (Huiskes, 1979), which would both increase the rate of drying and add additional thermal stress.

Furthermore, the constant conditions of the experiment are not representative of those likely in the natural habitat, where bare sands are even more prone than normal soils to cycles of drought and re-wetting and extreme diurnal fluctuations of temperature (Willis *et al.*, 1959). Finally, the wax moth larvae used here are large relative to the typical hosts of entomopathogenic nematodes (Puza, 2005); smaller hosts would be expected to lose moisture more rapidly.

There was no difference between *P.t. cinerea* and *P.t. temperata* in their ability to grow at different salt concentrations, suggesting that they have similar tolerance to osmotic stress. Therefore the hypothesis of better desiccation tolerance of *P.t. cinerea* was not supported. Both subspecies grew best at 0.5% NaCl, and also grew well at concentrations up to 2.0 % NaCl. The standard concentration of NaCl in bacterial growth media including nutrient broth and McConkey broth is 0.5%, and so such media are suitable for those strains. Similarly, *P. t. temperata* strain K122 grew better in medium with 0.3 - 0.6 % NaCl than without NaCl (Jallouli *et al.*, 2011). When *Photorhabdus* strains were grown without NaCl in the medium they were unstable, undergoing phase shift from primary to secondary forms (Krasomil-Osterfel, 1995). Thus, some salt in the medium is important both for growth and stability of *Photorhabdus*.

Chapter 4

Competition and coexistence of *P. temperata* subspecies in vivo and in vitro

4.1 Introduction

On Bull Island *Heterorhabditis downesi* can carry either *Photorhabdus temperata temperata* (yel) or *Photorhabdus temperata cinerea* (pur) as a symbiont. The distributions of these two bacterial subspecies overlap (Rolston *et al.*, 2005, Maher, 2014) and therefore it is possible that IJs, some carrying one subspecies and some carrying the other, might enter the same insect host. Maher (2014) infected insects with a 50:50 mixture of both symbiont subspecies and then infected the insects with *H. downesi* IJs. She identified the symbiont carried by IJs emerging from these cadavers and found that some cadavers produced only one type (either yel or pur) and others produced a mixture of IJs, some carrying yel and some carrying pur. This showed that the two subspecies can co-exist in one cadaver, but sometimes either one or the other predominates.

The aim of this chapter was to further explore the co-existence and competition of these two subspecies. Different bacteria can compete with each other either indirectly by competing for resources (exploitative competition), or directly, by interference competition where cells damage each other e.g. by producing antagonistic substances to inhibit the growth of the other types present (Hibbing *et al.*, 2010, Ghoul and Mitri, 2016). Antagonistic substances include

small antibiotics, proteins such as colicins, and bacteriocins (Ciezki *et al.*, 2017). Bacteriocins are produced to inhibit the growth of closely related strains; for example, *Xenorhabdus* spp produce bacteriocins (xenorhabdicins) that are selectively active against different *Xenorhabdus* species (Ciezki *et al.*, 2017) and *Photorhabdus* produce lumincins active against other species and subspecies of *Photorhabdus* (Sharma *et al.*, 2002). In this chapter, the growth of the two symbionts when cultured either alone or together (co-culture) was investigated. In the case of *Photorhabdus*, the bacteria also have to be carried by the IJs, which involves infecting both the *Heterorhabditis* mother and then the developing IJs (Ciche *et al.*, 2008), and so there could also be competition between the bacteria at this stage. Therefore, also the colonisation of IJs in the presence of a mixture of symbionts was investigated, both *in vivo* and *in vitro*. Since the mother worms must feed on the bacteria to be colonised, their choice of food may also influence which bacteria colonise them and then the IJs developing inside them.

The outcome of competition may depend on the environmental conditions (Kraft *et al.*, 2015, Chesson and Huntly, 1997, Chesson, 2000, Whitman *et al.*, 2014). In chapter 3 (Fig 3.14), was shown that the insect cadavers infected with *P. t. cinerea* produced more IJs than insects infected with *P. t. temperata*, but only when the cadavers were dried. So the hypothesise was that in dry conditions, cadavers where *P. t. cinerea* predominates would produce more IJs than cadavers where *P. t. temperata* predominates, and this would result in more IJs carrying *P. t. cinerea* in a population, but only under dry conditions. This hypothesis is tested here.

Maher (2014) used colour phenotype to distinguish between yel and pur bacteria, and the PCR was used to validate this method here. However, this method requires a lot of effort for plating and counting colonies. It would be better to use quantitative PCR to measure the relative amounts of each symbiont present in a mixture (for example in an infected insect or a group of IJs). To do this, primers needed to distinguish between the two subspecies. Therefore,

the genomes were sequenced for the two subspecies in order to identify DNA sequences that differed between the two subspecies.

The overall aim of this chapter was to explore the co-existence and competition of the two symbiont subspecies *P. t. cinerea* and *P. t. temperata*. Specific objectives are

- To test the hypothesis that *P. t. cinerea* will predominate in a population when *H. downesi* develops in insect cadavers with both symbionts present - but only when the cadavers are desiccated, and to validate the use of colony phenotype for recognising *P. t. cinerea* and *P. t. temperata* in a mixture.
- To investigate whether one symbiont sub-species predominates over the other when grown together in liquid culture.
- To investigate colonisation of mother-worms and IJs by symbiont when both symbionts are present together, both *in vivo* and *in vitro*
- To test whether *H. downesi* have a preference for either *P. t. cinerea* and *P.t. temperata* when offered a choice
- To develop molecular methods of distinguishing between *P. t. cinerea* and P. *t temperata*, by sequencing genomes of pur1 and yel3, identifying regions that differ, designing primers to amplify sequences within these regions, and test whether the resulting primers reliably amplify only the DNA from one but not the other subspecies

4.2 Materials and methods

4.2.1 Effect of environmental conditions on competition between subspecies of *Photorhabdus temperata* co-infecting the same insects

The aim of the experiment was to test the hypothesis that under ideal moist conditions, yellow bacteria (*Photorhabdus temperata temperata*) will be favoured, while desiccating conditions (for infected cadavers) will favour purple bacteria (*Photorhabdus temperata cinerea).*

Mixtures of nematodes carrying *P. t. temperata* and *P. t. cinerea* were allowed to infect insects which were subjected to moist or dry conditions for repeated infection cycles. The relative proportion of each symbiont in newly emerging nematodes was assessed after first and third infection cycles.

Two lines were set up – "Dry" and "Moist" by infecting *Galleria* larvae with three mixes of isolates: pur1 and yel3, pur2 and yel1, and pur3 and yel2 (pur (15 mm) of *H. downesi* carry *P. t. cinerea* and yel carry *P. t. temperata*). Filter paper isolates were put in 24 multi-well plates and 100 µl of a mix containing 50 IJs of each isolate was poured in each well of the multi-well plates, and one *Galleria* larva was added. After the infection multi-well plates were incubated at 20^{o}C, the mortality was recorded and the cadavers were checked for luminescence.

For the dry condition, after 3 days of infection 10 *Galleria* were placed individually in vials of oven-dried sand, they were left at 20^{o}C for 42 days then removed from sand and placed in White traps individually. For the moist condition 10 *Galleria* were placed on moist paper and transferred to White traps as usual 14 days after infection. IJs were harvested from all six lines. 50 IJs from each line were crushed individually and spread on agar plates, then incubated at 27^{o}C to assess the type of bacteria that was carried. The assessment was based on phenotype (colour of bacterial colonies).

IJs emerging from the ten *Galleria* for each line (wet or dry, for each mix) were pooled and used to infect for a second round under the same conditions. This was repeated again to give three rounds under wet or dry conditions for each of the three mixes. The identity of the bacteria carried was assessed for rounds 1 and 3, by crushing and plating 50 IJs for each treatment and recording the phenotype of the colonies.

The reliability of using colony colour phenotype for routine identification where there was a mix of the two subspecies was confirmed by PCR of the 16S rRNA gene of DNA isolated from yellow and purple colonies on plates from mixtures (Pur1 Yel3, pur2 Yel1 and Pur3 Yel2)(see Section 2.5). There were 18 colonies were tested in total. As a result of the DNA sequencing was confirmed that the purple luminescent colonies were *P. t. cinerea* and yellow luminescent colonies were *P. t. temperata.*

4.2.2 Colonisation of IJs in cadavers with mixed bacterial infection (in vivo)

Galleria mellonella larvae were infected with a 50:50 mix of *P. t. cinerea* Pur1 and *P. t. temperata* Yel3 IJs. Larvae were infected individually in multiwell plates with 100 IJs per insect. After 24 hours post infection, the *Galleria* were rinsed with water to remove any nematodes that had not entered the *Galleria* (24 hrs exposure) and incubated at 20^{o}C individually on moist tissue.

At 3-4 days post infection, cadavers were observed for colour and bioluminescence. After 9-11 days post infection, six *G. mellonella* infected with the mix of nematodes were dissected and ten first generation mother nematodes (hermaphrodites) were collected from each of them. *G. mellonella* were prioritised which showed yellow-purple mixed colour. Mothers were collected at the “bagged worm” stage, when the juveniles were moving around inside and before they emerged. Unused cadavers were incubated at 20^{o}C and put on White traps at 14

days post infection. The time of first emergence of IJs from cadavers in White trap was used to time the collection of IJs from mother worms.

The mother worms were rinsed thoroughly in full-strength sterile PBS; each mother was removed from the cadaver and placed individually in a Petri dish of PBS, then transferred to a second dish of PBS, and finally to a third dish of PBS. Worms were transferred either using a glass hook or in a small volume of fluid (10-20 µl) using a pipette. Each mother was placed in a separate well of a sterile 24-well plate with 1 ml of full-strength PBS. The mothers were observed daily for IJ emergence because the IJs may need more than 24 hrs to emerge before collecting IJs.

When the IJs were emerged in the White trap of the control insects, IJs were harvested and rinsed three times with PBS, then surface sterilised using hyamine, rinsed three times and crushed with a micropestle. The suspension was diluted ten-fold by adding 900 µl sterile PBS. Then 50µl of diluted suspension was spread on each of three LB/ pyruvate plates. Plates were incubated at 27^{O}C for 48-72 hours and then examined to check for colony colour and number.

4.2.3 Preference of *P. temperata* subspecies by *H. downesi* in vitro

Lipid agar plates were marked into six equal segments, with a central application zone. Overnight culture of *P. temperata* (Section 2.2.3) was used to create a lawn in each segment. On each plate, three segments were streaked with a loopful of *P. t. cinerea* (pur isolates) and three were streaked with *P.t. temperata* (yel isolates) (Fig. 4.1), taking care to avoid overlap. The plates were incubated at 27^{o}C for three days. Then 100 *H. downesi* IJs in 10 µl water were added to the central zone in which there was no bacterial lawn. The plates were incubated at 20^{o}C and checked daily. From day 5, the number of first generation nematodes

(hermaphrodites) in each segment was counted. The numbers from the three segments each bacterial type were summed.

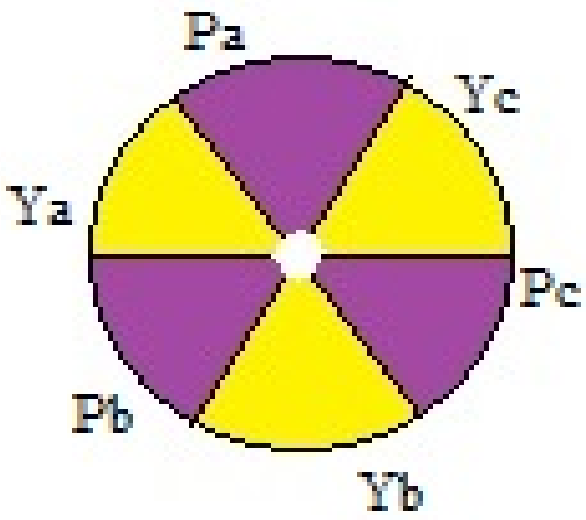


Fig. 4.1 Layout of the plates used in the nematode preference experiment. Segments Pa, Pb and Pc were streaked with a pur isolate of *P. t. cinerea*. Segments Ya, Yb and Yc were streaked with a yel isolate of *P. t. temperata*. After 3 days, *H. downesi* IJs (100 IJs in 10 µl water) were placed in the central clear zone.

Three combinations of pur/yel isolates were tested: pur1/yel3, pur2/yel1 and pur3/yel2. There were 10 plates for each bacterial combination. For each combination of bacteria, five plates received the nematode isolate from which the pur bacteria had been isolated, and the other five received the nematode isolate from which the yel bacteria had been isolated. There were thus six treatments in total, as shown in Table 4.14.

Statistics. Data for the three segments were combined to give a single reading per plate for numbers of nematodes in yel or pur. To see whether the particular bacterial isolates on the plate affected the nematodes' choice, the percentage of nematodes in the *P.t. cinerea* was analysed by GLM with bacterial combination and nematode isolate (nested within bacterial combination) as the factors.

To see whether the type of bacteria that was carried by the nematodes affected their choice on the agar plates, the percentage of nematodes in the *P.t. cinerea* was analysed by GLM with nematode isolate as a random factor nested within the subspecies of symbiont carried by the nematodes (either yel or pur).

4.2.4 Competition in liquid culture (co-culture)

Three combinations of *P. t. cinerea* and *P. t. temperata* isolates from north Bull Island were used (pur1 and yel3, pur2 and yel1, pur3 and yel2).

Each isolate was cultured for 24 hours at 27°C (Section 2.2.3) and the cell density was adjusted as necessary with sterile nutrient broth to $4X10^5$ cells/ml.

In the Co-culturing experiment 500 µl cell culture was added to 20 ml nutrient broth in a 100 ml flask. There were three treatments for each pair of isolates: For both control isolates Pur and Yel, 500 µl for each isolate were inoculated and cultured separately, however in the combination 50/50 Pur/Yel (250 µl, 250 µl) isolates were cultured both together in one flask. All flasks were incubated at 27°C with shaking at 100-200 rpm for 24 hours.

A serial dilution was made, and 50 µl aliquots were spread on nutrient agar plates. Three replicates of at least three different dilutions were spread. The plates were incubated at 27°C for 48hrs, then plates were observed for the colour and the number of bacteria colony type in each dish. The plates were counted in the range of 100-200 colonies.

Statistical analysis: For each trial, the number of CFU in the control (single isolate) cultures was compared using 2-sample t-tests. In trials where there was no difference ($P > 0.05$) between controls (pur and yel on their own), a one-sample t-test was used to test whether the proportion of CFU of one type in the co-culture differed from 0.5. If both bacterial subspecies

grow equally well together, then each is expected to make up 50% of the colonies from the co-culture and a departure from this would indicate that one subspecies is outcompeting the other.

4.2.5 Developing molecular method to distinguish between *P. temperata* subspecies *Photorhabdus* Genome sequencing (pur1 and yel3)

The aim of the work was to identify the unique nucleotide sequence and design primers for *P. t. temperata* and *P. t. cinerea*, that can be used for PCR analysis of *in vitro* and *in vivo* co-cultures of the two subspecies.

Cultured cells of *P. t. temperata* yel 1 and *P. t. cinerea* pur1 at a concentration of 1 X 10^9 bacteria cells per ml were collected by centrifuging 1-1.5 ml at 5000 xg (Sigma 1-15 Micro centrifuge) for 1 minute. The DNA was extracted using Fungal/ Bacterial Mini prep kit (Zymo Spin llc column) protocol. To confirm the presence of genome DNA a 2% agarose gel was run to check there was a single a band of DNA. The concentration was checked on NanoDrop (ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, USA)). Concentration was adjusted to 20 to 80 ng/ml and sent for sequencing by Microbes NG (University of Birmingham).

Genome analysis was carried out by Nsilico Life Science Ltd. Further analysis was carried out by the Institute of Molecular Biosciences, Goethe University Frankfurt. The pur1 and yel3 genomes were annotated using *Photorhabdus asymbiotica* subspecies *asymbiotica* ATCC 43949 (P.a.a.ATCC 43949) and *Photorhabdus temperata* subspecies *thracensis* DSM 15199 (P.t.t. DSM 15199).

Primer design and PCR Optimisation

A number of unique coding sequences were identified in each isolate and one unique sequence from each isolate was selected as a candidate for use in the PCR analysis. The

candidate coding sequences were suggested by Nick Tobias from Goethe University Frankfurt. The pur1 sequence (leucine responsive regulatory protein) was 465 base pair and yel3 (hypothetical protein) was 1293 base pairs. Primer design was carried out using the Primer-BLAST programme (National Centre for Biotechnology Information (NCBI) https://www.ncbi.nlm.nih.gov/tools/primer-blast/) to design appropriate primers for both Pur1 and Yel3 isolates.

The nucleotide sequence was searched against enterobacteriaceae and five other families because enterobacteriaceae are found in *G. mellonella* larvae (Yuen and Ausubel, 2014). For example bacteria found in *G. mellonella* gut flora include *Enterococcus faecium* (Lebreton *et al.*, 2011), *Streptococcus faecium* (Jarosz, 1989), *Staphylococcus epidermidis*, *Micrococcus epidermidis*, *Streptococcus faecalis* and *Arthrobacter* species (Gilliam and Lorenz, 1983).

Criteria for selecting primers

The criteria for choosing primer pairs were a) length of the product (120 to 220 bp), b) low probability of unintended template, and c) low probability of unintended template being present in the *G. mellonella*. Primers were designed for each of these sequences using Primer-BLAST from NCBI.

Selected primers shown in Table 4.1.

Table 4.1 Primer-BLAST result from NCBI (https://www.ncbi.nlm.nih.gov/tools/primer-blast/).

Primer	Sequence	Expected product length	supplier
Pur1-pp5 F primer	**GATCGGAGCGGGTCTTTCAT**	184 bp	Eurofins
Pur1-pp5 R primer	**GAAACGGCGGTAAGAGTGGA**	184 bp	Eurofins
yel3-pp1 F primer	**CAGGACATTGATGCCCAAGAGA**	258 bp	Eurofins
yel3-pp1 R primer	**GTTGATAGGAAGGGCAGGGG**	258 bp	Eurofins
yel3-pp 6 F primer	**ATCAAATTGGGCGTTATTGCCG**	142 bp	Eurofins
yel3-pp 6 R primer	**CTTGGGCATCAATGTCCTGTGAA**	142 bp	Eurofins

Testing novel primers for distinguishing *P. temperata* subspecies

Preliminary tests of the new primers were carried out using DNA extracted from cultures of each *P. temperata* subspecies, alone or in mixtures of the two subspecies. Then, primers were tested on tissues (IJs and infected *G. mellonella*). PCR mixtures and annealing temperature were adjusted during the investigation in an effort to reduce non-specific binding.

The PCR mixes used are shown in Tables 4.2 & 4.3. The reagents that were used were kept on ice. An appropriate master mix was prepared for several reactions made up in a 1.5 ml micro centrifuge tube. Individual reactions were carried out in 0.5 ml PCR tubes (Fisher Scientific, Ireland) using 22.5 µl of master mix and 2.5 µl of DNA extract (20 ng/µl). The contents of the tubes were mixed by vortexing and briefly centrifuged.

The PCR cycle conditions were carried out individually for each primer pair using the Eppendorf Mastercycler gradient machine, as described in (Fischer-Le Saux *et al.*, 1999a). Standard cycle conditions are shown in Table 4.4. Following gradient PCR, the annealing temperature for pur1 pp5 was increased to 69.1^{o}C.

The PCR product was visualised on a gel. For each reaction, 5 µl PCR product was mixed with 1 µl of 5x loading buffer (Bioline, UK) and loaded on a 1% gel [3g agarose (Life Technologies) dissolved in 150 ml 1x TEA buffer, SYBR safe DNA gel stain 20µl] and run at 120V for 1 hour. The running buffer was 10X TAE 50 ml with 450 ml dH_2O. Gels were visualised by the G: Box with GeneSnap software (Syngene).

Table 4.2 PCR reaction mixture 1 used for amplification of *P. temperata* DNA using subspecies-specific primers. Reactions were carried out in 25 µl volumes.

Reagents	Volume(µl)	Final concentration	Supplier
5x MyTaq Reaction Buffer	5	1x	Bioline
Forward primer(10 µM)	0.5	0.2 µM	Eurofins MWG Operon
Reverse primer (10 µM)	0.5	0.2 µM	Eurofins MWG Operon
Taq Polymerase (5 units/µl)	0.125	0.025 U/µl	Bioline
Dd H_2O	16.375	water	
DNA template (20ng/µl)	2.5	2 ng/µl	
Total	25		

Table 4.3 PCR mixture 2 used for amplification of *P. temperata* DNA using subspecies-specific primers. Reactions were carried out in 25 µl volumes.

Reagents	Volume (µl)	Final concentration	Supplier
buffer (10x)	2.5	1x	Sigma
$MgCl_2$ (25mM)	2	2 mM	Sigma-Aldrich
dNTP Mix (10 mM)	0.5	0.2 mM	Metabion
Forward primer (10 µM)	2.5	1 µM	Eurofins MWG Operon
Reverse primer (10 µM)	2.5	1 µM	Eurofins MWG Operon
Dd H_2O	12.375		
Taq Polymerase (5 units/µl)	0.125	0.025 U/µl	Sigma Aldrich
DNA template (20 ng/µl)	2.5	2 ng/µl	
Total	25µl		

Table 4.4 Standard PCR cycle conditions for amplification of *P. temperata* DNA using subspecies-specific primers. Lid temperature was set to 95°C.

Stage	Cycles	Temperature	Duration
Initial denaturation	1	95°C	5 min
Denaturation	30	95°C	1min
Annealing temp		60°C	1 min
Extension		72°C	2 min
Final extension	1	72°C	3 min
Hold	1	4°C	Indefinite

1. Testing the primers

Firstly, pur1-pp5 primers were tested on Pur DNA and yel3-pp1 primers were tested on yel DNA to confirm that they worked. PCR mix **1** (Table 4.2) and standard PCR conditions (Table 4. 4) were used for both primer pairs.

Next, the primers were tested using DNA from both *P. temperata* subspecies to test whether they would amplify one another (switching). Each primer pair (Pur1-pp5 and yel3-pp1) was tested with each of the six Bull Island isolates of *Photorhabdus temperata.* PCR mix 1 (Table 2) and standard PCR conditions (Table 4.4) were used for both primer pairs.

2. Gradient polymerase chain reaction (PCR) to optimise annealing temperature

Gradient PCR was used to optimise the appropriate temperature for the subspecies-specific primers Pur1-pp5 and yel3-pp 6, to eliminate non-specific binding of primers. PCR mixture 2 Table 4.3 was used for the amplification for both primer pairs.

The gradient PCR nominal annealing temperature, based on the primers' melting temperature (TM), was 59.4^{o}C (Pur1-pp5 primer set), and the temperature range was between 49.4 and 69.9 ^{o}C. The gradient PCR nominal temperature for yel3-pp6 primer pair was 60.6^{O}C, temperature range was between 50.6 to 71.1^{O}C. Gradient was +/- 10^{O} C of nominal temperature in each case. Other conditions were as in Table 4.4.

3. Testing primers on mixtures of DNA from both *P. temperata* subspecies

Both pur1-pp5 and yel3-pp6 primers were tested against a mixture of *P. temperata* DNA. Each primer was tested against pure (100%) DNA from *P. t. temperata* (pur1) and *P. t. cinerea* (yel3), and a 50:50 mix of DNA from both subspecies. For the 100% pur1 or 100% yel3 there was 2.5µl DNA, and with the mixture of DNA (50% pur1 & 50% yel1) there was 1.25 µl pur1 DNA + 1.25 µl ye13 DNA. Those reaction templates were made for both primers individually.

PCR mixture 2 (Table 4.3) was used for amplification of both primer sets. PCR cycle conditions for amplification using yel3-pp1 was standard (Table 4.4) while for pur1-pp5 primers the annealing temperature was set to 69.1°C (the optimum determined by gradient PCR).

4. Testing novel primers for distinguishing *P. temperata* subspecies in infected *G. mellonella*

The aim of this experiment was to see if the primers would detect and identify *P. temperata* subspecies from infected insects. *G. mellonella* larvae were infected with either *H. downesi* pur1, yel3 or a 50:50 mix of the two. Four days post-infection, DNA was extracted as described in (section 2.4) and amplified using pur1-pp1 and yel3-pp6 primer

PCR mixture 2 was used for amplification of symbiotic bacteria DNA (Table 4.3) using both primers Pur1-pp5 (expected amplicon 184bp) and Yel3-pp6 (expected amplicon 142bp). The cycle conditions for Yel3-pp6 were as before (Table 4.4) while conditions for Pur1-pp5 were changed with annealing temperature to 69.1°C**.**

16S primers were used as a positive control to confirm that bacterial DNA was present (Table 2.2, Section 2.4.1)

5. Testing novel primers for distinguishing *P. temperata* subspecies present in *Heterorhabditis downesi* infective juveniles

The aim of this experiment was to test the ability of the primers to detect symbiont DNA directly from tissue samples, including when a mixture of symbiont subspecies was present in the treatments. The IJs were surface sterilised with hyamine and rinsed three times with sterile PBS. IJs were either pure Pur1 or Yel3 or mixed in different ratios (Table 4.5).

Table 4.5 Number of *H. downesi* IJs, either single or mixed isolates, used for detection of symbiotic bacteria

H. downesi isolate	Number of IJs
Pur1	300 IJs
Yel3	300 IJs
Pur1/Yel3	150 IJs/150 IJs
Pur1/Yel3	290 IJs/Yel3 10IJs
Pur1/Yel3	10 IJs/Yel3 290 IJs

The DNA was extracted using the Qiagen Blood and Tissue kit protocol as described in chapter 2 except for the initial treatment of the IJs. IJs were surface sterilised with hyamine then centrifuged for 10 minutes at 5000 x g and the supernatant was removed. After that 180 µl buffer AL was added to the pelleted IJs, then the IJs were crushed with a micro-pestle and 20 µl proteinase K was added. The homogenate was mixed by vortexing, and then the homogenate was lysed for 3 hrs at 65 ^{o}C in a shaking water bath and vortexed every 15 minutes. The remainder of the DNA extraction was described as in Section 2.4.

PCR mixture 2 was used for amplification of symbiotic bacteria DNA (Table 4.3) using both primers Pur1-pp5 (expected amplicon 184bp) and Yel3-pp6 (expected amplicon 142bp). The cycle conditions for Yel3-pp6 were as before (Table 4.4) while conditions for Pur1-pp5 were changed with annealing temperature to 69.1^{o}C**.**

6. New set of primer design for PCR optimisation

According to the failure of the previous primers tested new sets of primers were designed, using the same candidate-coding sequences as before. Primer design for pur1 and yel3 was carried out with the help of Dr Conor Meade of Maynooth University, using the Primer-BLAST programme (National Centre for Biotechnology Information (NCBI) https://www.ncbi.nlm.nih.gov/tools/primer-blast/) in the Geneious basic graphical user

interface (Kearse *et al.*, 2012). Selected primers are shown in Table 4.6. One primer pair for Pur1 with an expected product length of 84 bp, and two primer pairs for Yel3, both with an expected product length of 83 bp.

Table 4.6 Primer-BLAST result using Geneious.

Primer	Sequence	Expected product length	supplier
Pur1-349 F primer	**CTCCACTCTTACCGCCGTTT**	84 bp	Eurofins
Pur1-432 R primer	**TTCCATCGGTACCTCGGTCT**	84 bp	Eurofins
yel3-297 F primer	**CAGCAAAGGCAATATCGGGC**	83 bp	Eurofins
yel3-379 R primer	**GAAGGGCAGGGGAGATTAGC**	83 bp	Eurofins
yel3-552 F primer	**TCAAGGGGAACAGGCAATCC**	83 bp	Eurofins
yel3-660 R primer	**TCTGGCAGTAGGTAGCAGGT**	83 bp	Eurofins

F denotes forward primer, and R reverse primer

The primers were tested using DNA extracted from *P. temperata cinerea* (pur1) and *P. temperata temperata* (yel3). PCR mixtures and annealing temperature were adjusted during the investigation in an effort to reduce non-specific binding. PCR mixture 3 (Table 4.7) was used for the amplification for all primer pairs. This differs to mixture 2 by having only half the amount of primer.

Gradient PCR was used to optimise the appropriate temperature for the subspecies-specific primers Pur1-349F/pur1-432R, yel3-297F/yel3-379R and yel3-552F/yel3-660R, to eliminate non-specific binding of primers. The gradient PCR nominal annealing temperature, based on the primers' melting temperature (TM), was 59.4ºC for the Pur1-349F/pur1-432R primer set, and the temperature range was between 50.0 and 70.5 ºC. The gradient PCR nominal temperature for yel3-297F/yel3-379R and yel3-552F/yel3-660R pairs was 61.4^{O}C, temperature range was between 50.0 to 70.5^{O}C. Gradient was +/- 10^{O} C of nominal temperature in each case. Other conditions were as in Table 4.8. Following gradient PCR the annealing temperature for Pur1-349F/pur1-432R was decreased to 60.0 ^{O}C

Table 4.7 PCR mixture 3 used for amplification of *P. temperata* DNA using subspecies-specific primers. Reactions were carried out in 25 µl volumes.

Reagents	Volume (µl)	Volume (µl)	Final concentration	Supplier
buffer (10x)	2.5	80	1	Sigma-Aldrich
MgCl2 (25mM)	2	64	2	Sigma-Aldrich
dNTP Mix (10 mM)	0.5	16	0.2	Metabion
Forward primer (10 µM)	1.25	40	0.5	Eurofins MWG Operon
Reverse primer (10 µM)	1.25	40	0.5	Eurofins MWG Operon
Molecular grade water	14.875	476		
Taq Polymerase (5 units/µl)	0.125	4	0.025	Sigma Aldrich
DNA template (20 ng/µl)	2.5	as required		
Total vol	25			

Table 4.8 Standard PCR cycle conditions for amplification of *P. temperata* DNA using subspecies-specific primers. Lid temperature was set to 95°C.

Stage	cycles	Temperature	Duration
Initial denaturation	1	95°C	5 min
Denaturation		95°C	1min
Annealing (Gradient)	30	nominal 60.0°C	1 min
Extension		72°C	2 min
Final extension	1	72°C	7 min
Hold	1	4°C	Indefinite

4.3 Results

4.3.1 Effect of environmental conditions on competition between subspecies of *Photorhabdus temperata* co-infecting the same insects

In round 1 all IJs from insects that had been kept in dry conditions carried *P. temperata* but in each of the three mixtures of isolates with moist condition several IJs had no *P. temperata* colony forming units (Table 4.9). In round 3 also several IJs had no *P. temperata* colony forming units in both moist and dry condition (Table 4.10).

In the first infection round, the majority of IJs emerging from cadavers infected with two of the combinations of strains, pur1/yel3 and pur3/yel2 carried *P. t. cinerea* (Fig 4.2). Only one

combination (pur2/yel1) produced a more balanced mix of bacteria. By round 3, *P. t. cinerea* predominated in all treatments, including pur2/yel3 (Fig 4.2).

In round 1, most of the treatments included a small proportion of IJs carrying both subspecies. For pur2/yel1 emerging from cadavers kept under dry conditions this represented 40% of the IJs that carried bacteria (Fig 4.2). In most of the IJs that were colonised by both subspecies, there were more CFU identified as *P.t. cinerea* than *P.t. temperata* (mean $\pm$ SE: *cinerea*: 63.0 $\pm$ 9.89; *temperata* 23.7 $\pm$ 4.18) and there was a significant difference between subspecies (Mann Whitney test, W = 625.5, $P < 0.001$). Fewer IJs carried both types of bacteria in round 3 (Fig 4.3).

Comparing numbers of IJs carrying Pur or Yel bacteria in dry and moist condition, there was a significant difference in the mixture of Pur2Yel1 isolates (Fisher Exact test, $P = 0.003$). The number of IJs carrying *P.t. temperata* (yel1) was higher in the moist condition than in the dry condition (Fig. 4.3), However, there was no significance difference in Pur1Yel3 (Fisher Exact test ($P = 0.73$) and a marginal difference in Pur3 Yel2 (Fisher Exact test, $P = 0.046$).

Table 4.9 The number of IJs that have no *P. temperata* in three mixed isolates in moist condition (round 1)

IJs with No *P. temperata*			
Condition	**pur1yel3**	**Pur2Yel1**	**Pur3Yel2**
Moist	1/50	6/50	4/50
Dry	0/50	0/50	0/50

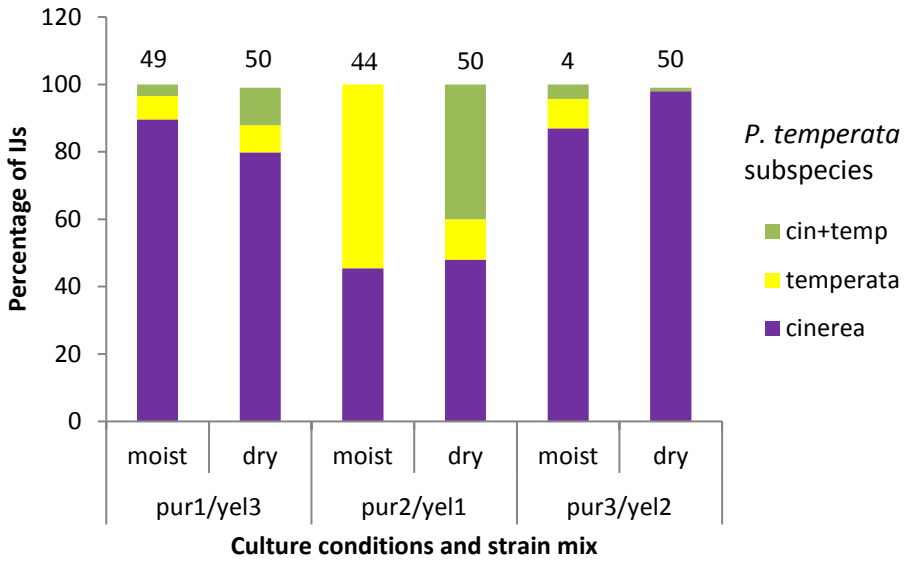


Fig. 4.2 Percentage of *H. downesi* IJs carrying *P. t. cinerea*, *P. t. temperata* or a mix of both subspecies following one round of culture in insects infected with 50:50 mixtures of IJs and then maintained in either moist or dry conditions.

Table 4.10 Number of IJs with no *P. temperata* colony forming units in three mixed isolates in moist and dry condition (round 3)

IJs with No *P. temperata*			
Condition	**pur1yel3**	**yel2pur1**	**Pur3Yel2**
Moist	2 / 50	4 / 50	5 / 50
Dry	5 / 50	1 / 50	1 / 50

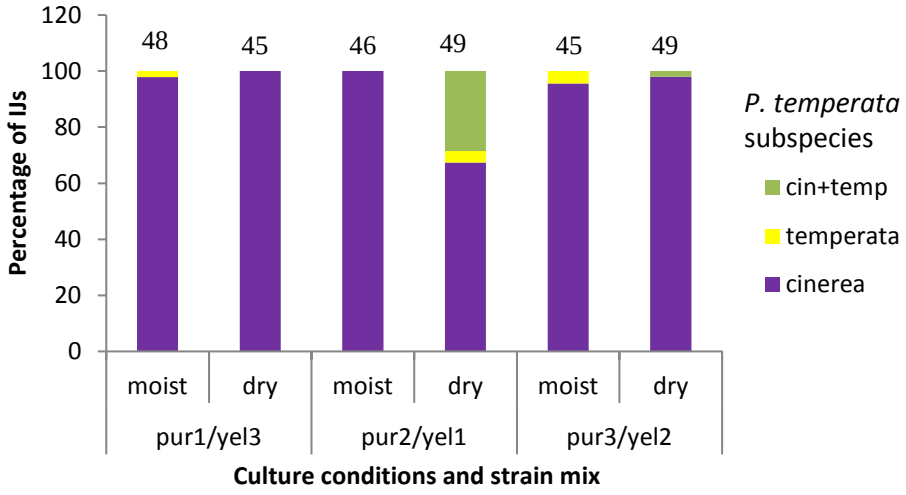


Fig. 4.3 Percentage of *H. downesi* IJs carrying *P. t. cinerea*, *P. t. temperata* or a mix of both subspecies following three rounds of culture in insects infected with 50:50 mixtures of IJs and then maintained in either moist or dry conditions.

4.3.2 Colonisation of IJs in cadavers with mixed bacterial infection (in vivo)

Most of the mother worms from cadavers that had been co-infected with pur1 and yel3 nematodes were colonised by only one subspecies of *P. temperata*, as assessed by crushing the offspring IJs that emerged from them. The data was examined firstly by cadaver (Table 4.11) and secondly by grouping hermaphrodites based on the identity of symbiont. Out of the 52 mothers tested, only 6 carried both subspecies (Table 4.11). There was a significant difference in the total number of bacteria carried by IJs from the six different cadavers (One Way ANOVA $F_{5.37} = 7.11$, $P < 0.001$), ranging from an average of just 66 to over 700 bacteria/IJ depending on the cadaver (Table 4.12).

Table 4.11 Number of mother worms colonised by *P. t. temperata* (Yel), *P. t. cinerea* (Pur) or both, and mean number of bacteria (both subspecies) carried by IJs from each cadaver. Means followed by the same letter are not significantly different (Tukey test, following One way ANOVA $F_{5.37} = 7.11$, $P < 0.001$).

	Number of mother worms				Total Number of *P. temperata* /IJ (yel) & (pur)			
Cadaver	**N**	**yel only**	**pur only**	**Yel & pur**	**N**	**Mean**	**SE**	
P3D2	**14**	9	2	3	**10**	706.2	65	a
P1B1	**6**	6			**5**	567.6	66.3	ab
P2B5	**6**	1	5		**6**	442.7	147.2	abc
P1A6	**14**	3	9	2	**10**	437.6	111.8	abc
P1D3	**6**		5	1	**6**	122.0	34.7	bc
P1C4	**6**	6			**6**	66.0	32.3	c
Total	**52**	25	21	6				

In terms of the number of bacteria per IJ in each mother worm (Table 4.12), there was no significant difference between mother-worms carrying *P. t. temperata* only, *P. t. cinerea* only, or both subspecies (One Way ANOVA $F_{2,40} = 0.07$, $P = 0.933$). All had approximately 400 bacteria/IJ (Table 4.12). The six mothers where both subspecies were found carried on average 386 bacteria/IJ. They had nearly twice as many *P.t. cinerea* than *P.t. temperata* (Table.4.13) and when these data were analysed using paired t test there was a significant difference at $P < 0.10$, but not at $P < 0.05$ ($T = -2.22$, $P = 0.078$).

Table 4.12 Number of *P. temperata* per IJ (pur only , yel only or both) in each mother worm, data analysed using One way ANOVA $F_{2,40} = 0.07$, $P = 0.933$

Subspecies	**N**	**Mean**	**SE**
***P. t. temperata* only**	20	412.3	72.94
***P. t. cinerea* only**	17	441.1	86.45
Both	6	386.0	123.77

Table 4.13 Number of *P. temperata* per IJs in Mothers carrying both types, data analysed using Paired t. test, T = -2.22, *P* = 0.078

Subspecies	N	Mean	SE
P. t. temperata	6	133.0	38.69
P. t. cinerea	6	253.0	87.35

4.3.3 Preference of *H. downesi* for *P. temperata* subspecies on agar plates

Seven days after the plates were inoculated with IJs, a higher proportion of the first generation worms (hermaphrodites) were in the segments of the plate inoculated with *P.t. cinerea* (pur isolates) than in the segments with *P. t. temperata* (yel) isolates. This was true for each of the three combinations of yel/pur bacteria, and for each of the six *H. downesi* isolates (Table 4. 14). There was no difference between the three bacterial combinations in the percentage of nematodes found in pur bacteria (ANOVA $F_{2, 24} = 0.37$, $P = 0.721$), But there was a significant difference amongst the six nematode isolates in the percentage of nematodes in pur (ANOVA $F_{3, 24} = 2.91$, P = 0.026), ranging from 60.2 % of *H. downesi* yel3 to 80.4% of *H. downesi* pur3 (Table 4.14). Overall, 1452 of the 2150 nematodes counted (67.5%) were found in the pur bacteria, a highly significant departure from the 50% expected if the nematodes did not have a preference (one sample test for proportions, $P < 0.001$).

The number of first generation nematodes on the plate was not influenced by the bacterial combination on the plate (ANOVA $F_{2, 24} = 4.47$, P = 0.232), but there was a significant difference amongst the six nematode isolates in the numbers of nematodes per plate (ANOVA $F_{3, 24} = 10.39$, P < 0.001), ranging from 29.6 *H. downesi* pur3 to 98.2 *H. downesi* pur1 (Table

4.14). This could be either due to an error in the number of IJs placed on the plates, or a difference in the proportion of the IJs recovering and developing to adult.

Table 4.14 Proportion of *H. downesi* first generation hermaphrodites in the segments of the plate inoculated with *P. t. cinerea* bacteria on plates with three *P.t. cinerea* (pur) segments and three *P.t. temperata* (yel) segments and average number of nematodes per plate. Mean of five plates per nematode isolate. Within column, numbers followed by the same letter are not significantly different (at $P < 0.05$, Tukey's test).

P. temperata isolates	*H. downesi* isolate	Number nemas per plate (mean±SE)	% nemas in pur segments (mean±SE)
Pur1/yel3	Pur1	98.2 (4.12) A	69.6 (4.47) AB
Pur1/yel3	Yel3	86.2 (4.14) ABC	60.2 (3.36) B
Pur2/yel1	Pur2	58.8 (4.68) C	63.1 (7.42) AB
Pur2/yel1	Yel1	92.4 (9.68) AB	72.9 (4.13) AB
Pur3/yel2	Pur3	29.6 (1.81) D	80.4 (2.58) A
Pur3/yel2	Yel2	64.8 (9.48) BC	64.2 (3.53) AB

4.3.4 Competition in liquid culture (co-culture)

Table 4.15 shows the results of co-culture experiments in which there was no significant difference in CFU between the controls (Pur alone and Yel alone), in other words the two subspecies were growing equally well when alone, and so any differences in co-culture are expected to be due to the interaction between the two subspecies. For two of the mixtures (Pur2/Yel1 and Pur3/Yel2) CFU of the two bacterial subspecies were found in approximately equal numbers (Table 4.15). For Pur1Yel3 isolates nearly all (99%, Trial 2) or all (100%, Trial 3) of the CFU were *P. t. cinerea* and this differed from the 50% expected if both subspecies were present in equal numbers, (one sample t test, $P < 0.001$, Trial 2). The data of trial 3 could not be tested for significance as all values were 100%.

Table 4.15 Proportion of *Photorhabdus* made up by *P.t. cinerea* in co-culture inoculated with equal cell numbers of *P.t. cinerea* and *P.t. temperata.*

Isolates *P.t. cinerea/ P.t. temperata*	Trial	Proportion CFU *P. t. cinerea* Mean (SE)	Probability that proportion = 0.5 T (2 DF)	*P*
Pur1/Yel3	2	0.99 (0.005)	78.69	<0.001
	3	1.00 (-)	Cannot test	
Pur2/yel1	2	0.60 (0.041)	2.03	0.179
	3	0.61 (0.69)	1.61	0.249
	4	0.49 (0.040)	0.37	0.750
	6	0.50 (0.059)	0.88	0.471
Pur3/yel2	1	0.35 (0.056)	-2.17	0.162
	4	0.598 (0.217)	0.45	0.729
	5	0.43 (0.031)	-252	0.128

4.3.5 Developing molecular method to distinguish between *P. temperata* subspecies *Photorhabdus* Genome sequencing (pur1 and yel3)

The genomes of *P. t. temperata* pur1 and *P. t. cinerea* yel3 were sequenced by MicrobesNG and assembled by NSilico. Summary statistics are given in Table. 4.16. The full reports are included as Appendix 1 and Appendix 2.

Table 4.16. Summary statistics for *P. temperata* genomes

	P.t. cinerea pur1	*P. t. temperata* yel3
Genome size (bp)	238,875	238,875
Predicted genes count	245	229
Coding GC (%)	55.0	45.7

A search against the Virulence Factor Database (VFDB) showed that for pur1 15 sequences resulted in a BLAST hit, and for yel3 17 sequences resulted in a BLAST hit. The virulence factors identified in the genomes of the two isolates are compared in Table. 4.17.

Most of the hits were to Type III secretion system proteins. The two isolates shared 11 of the BLASTp hits in common; another three hits were to the same protein but matching *P. asymbiotica* subsp *asymbiotica* in the case of pur1 and *P. luminescens* subsp. *laumondii* TT01 in the case of yel3. Pur1 had one unique hit (type III secretion component protein sctp) and Yel3 had three unique hits (yopb/d chaperone sycd, and two hypothetical proteins plu3758 and plu3776) (Table 4.17).

Table 4.17 Comparison of virulence factors identified in genomes of *P. t. cinerea* pur1 and *P. t. temperata* yel3. Matches are to *Photorhabdus luminescens* subsp. *laumondii* TT01 or to *Photorhabdus asymbiotica* subsp. *asymbiotica* ATCC 43949

Entry	Both	Pur1	Yel3
Type III secretion protein SctV [laumondii TTO1]	+		
Type III secretion control protein SctW [laumondii TTO1]	+		
(sctN) type III secretion system ATPase [asymbiotica]	+		
(sctQ) type III secretion system protein [asymbiotica]	+		
(sctR) type III secretion system protein [asymbiotica}	+		
(sctT) type III secretion component protein sctt [asymbiotica]	+		
(rhaS) transcriptional activator proteinExsA/virf [asymbiotica]	+		
(sctC) type III secretion outer membrane protein pscc [asymbiotica]	+		
(sctD) type III secretion component protein sctd [asymbiotica]	+		
(sctH) type III secretion component protein scth [asymbiotica]	+		
(sctL) type III secretion system protein [asymbiotica]	+		
(sctJ) type III secretion component protein sctj	+	asym	TT01
(sctK) type III secretion component protein sctk	+	asym	TT01
(sctU) type III secretion component protein sctu	+	asym	TT01
(sctP) type III secretion component protein sctp [asymbiotica]		+	
(sycD) yopb/d chaperone sycd [asymbiotica]			+
(plu3758) hypothetical protein [laumondii TTO1]			+
(plu3776) hypothetical protein [laumondii TTO1]			+

Primer design and PCR optimisation

The targeted regions were leucine-responsive regulatory protein (for *P. t. cinerea*) and a hypothetical protein (for *P. t. temperata*). The Primer-BLAST search returned a number of primer pairs for each nucleotide sequence, such as pur1 primer pair 5, product length 184 bp; primer pair 8, product length 220 bp; primer pair 9, product length 188 bp. Similarly, the Primer-BLAST search for yel3 returned primer pair1, product length 258 bp; and primer pair 6, product length 142 bp. Some of the primer pairs were excluded because they had unintended templates including enterobacteriaceae such as *Streptococcus* species and *Pseudomonas* species. The primer pairs that were selected are mentioned in the methods section (Table 4. 1).

1. **Testing novel primers for distinguishing *P. temperata* subspecies and optimising PCR conditions.**

PCR was used to amplify DNA from pur and yel isolates in a primer specific band. The result showed that for both primers the product size was as expected: pur1-pp5 is 184bp and yel3-pp1 is 258 bp (Fig 4.4**)**. However, testing Pur1-pp5 and yel3- pp1 on DNA from both subspecies (switching) showed non-specific binding of pur1-pp5 primer with yel isolates (for example lanes 4 and 6) and also nonspecific binding of yel3-pp1 primers with pur3 (for example lanes 1 and 3) (Fig 4.5**).**

In order to eliminate the non-specific binding, three steps were taken. Firstly, the reaction mix was changed to mix 2, with the $MgCl_2$, dNTP and buffer added separately, instead of the MyTaq Reaction Buffer (mix 1). Secondly, gradient PCR was used to optimise the annealing temperature for primer Pur1-pp5. Thirdly, yel3-pp1 was replaced by yel3-pp6. Yel3-pp6 primers gave satisfactory results when used with mix 2 (amplified target region of yel DNA but did not show non-specific binding; gel not shown).

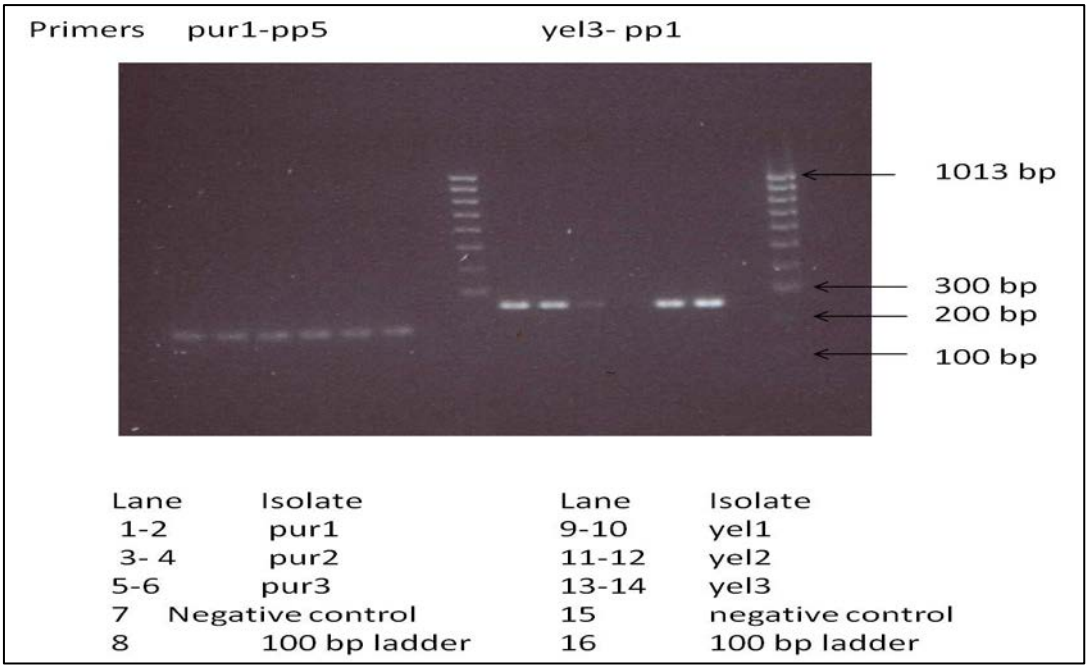


Fig 4.4 Gel analysis of *P. t. cinerea* (pur1, pur2, pur3) and *P. t. temperata* (yel1, yel2, yel3). Target sequences were Leucine-responsive regulatory protein (*P. t. cinerea*) and a hypothetical protein (*P. t. temperata*). Primers were designed using Primer-Blast **(**http://www.ncbi.nlm.nih.gov/tools/primer-blast/) and the target sequences were 184 bp (pur1-pp5) and 258 bp (yel3-pp1).

Testing primers pur1-pp5 and yel3-pp1 for *P. temperata* spp.

Lane	Isolate
1	pur1
2	pur2
3	pur3
4	yel1
5	yel2
6	Yel3
7	Negative control
8	100bp ladder

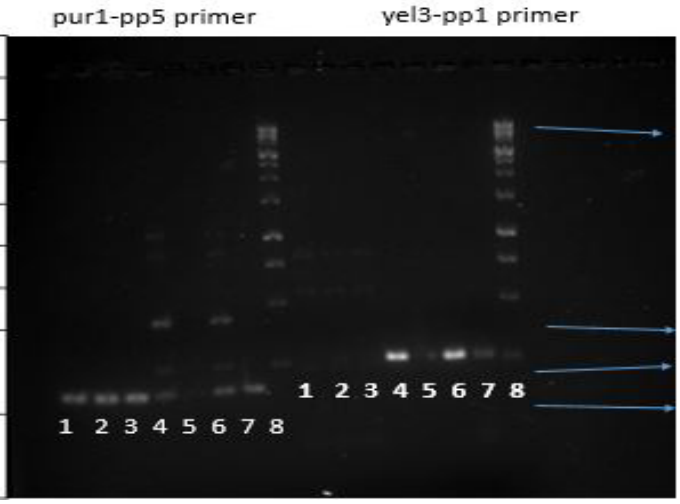


Fig 4.5 Gel analysis of *P. t. cinerea* (pur1, pur2, pur3) and *P. t. temperata* (yel1, yel2, yel3). Target sequences were Leucine-responsive regulatory protein (*P. t. cinerea*) and a hypothetical protein (*P. t. temperata*). Primers were designed using Primer-Blast **(**http://www.ncbi.nlm.nih.gov/tools/primer-blast/) and the target sequences were 184 bp (pur1-pp5) and 258 bp (yel3-pp1).

2. Gradient PCR: pur1-pp5 primers

The primer Pur1-pp5 was tested against the yel DNA *P. t. temperata* isolates to eliminate non-specific binding of pur1-pp5 primer in yel isolates. The optimal temperature found was ranged from 67.5^{o}C to 69.1^{o}C (Fig 4.6) (Table 4.18).

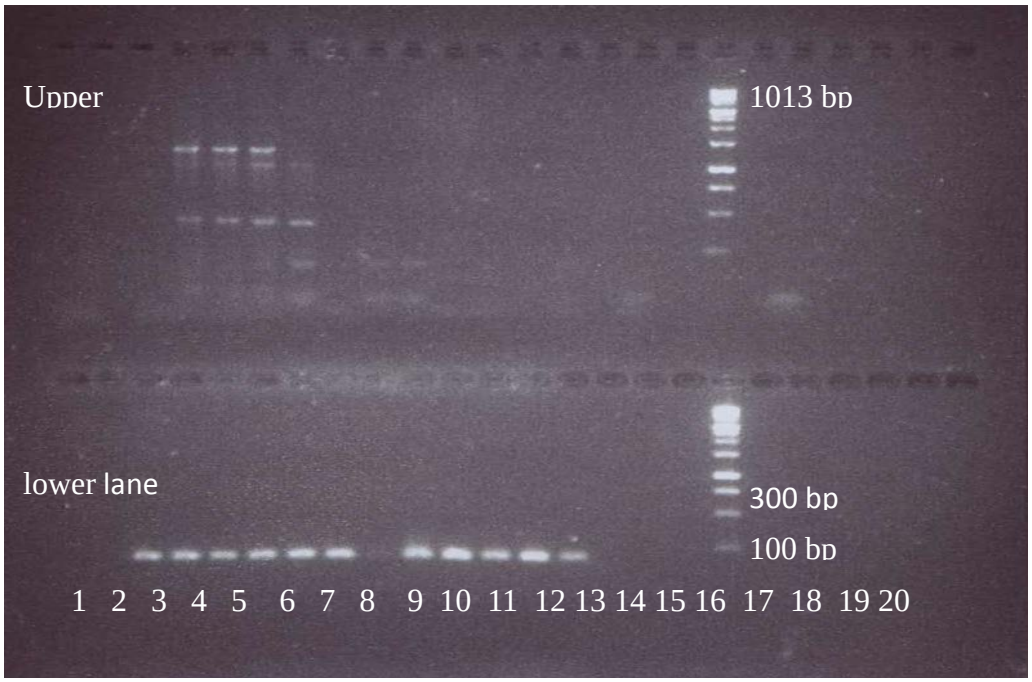


Fig 4.6 Gel analysis of *P. t. cinerea* (pur1) and *P. t. temperata* (yel1) DNA amplification in gradient PCR using (pur1-pp5 primer)**.** Target sequence was Leucine-responsive regulatory protein (*P. t. cinerea*) Primers were designed using Primer-Blast **(**http://www.ncbi.nlm.nih.gov/tools/primer-blast/) and the target sequences were 184 bp (pur1-pp5 primer) & (100 BP ladder). (Key to lanes in Table 4.18)

Table 4.18 Show that the lanes of the Gel loading using pur1-pp5 primer, the primer was used to amplify DNA extracted from infected *G. mellonella* with pur1 and yel1 100%.

Upper Lane	**Isolate**	**Temp ºC**	**Lower Lane**	**Isolate**	**Temp ºC**
1	ctrl	49.4	**1**	ctrl	60.2
2	empty		**2**	empty	
3	yel1 DNA	49.4	**3**	pur1 DNA	49.4
4	yel1 DNA	49.7	**4**	pur1 DNA	49.7
5	yel1 DNA	50.8	**5**	pur1 DNA	50.8
6	yel1 DNA	52.6	**6**	pur1 DNA	52.6
7	yel1 DNA	54.9	**7**	pur1 DNA	54.9
8	yel1 DNA	57.5	**8**	pur1 DNA	57.5
9	yel1 DNA	60.2	**9**	pur1 DNA	60.2
10	yel1 DNA	62.9	**10**	pur1 DNA	62.9
11	yel1 DNA	65.4	**11**	pur1 DNA	65.4
12	yel1 DNA	67.5	**12**	pur1 DNA	67.5
13	yel1 DNA	69.1	**13**	pur1 DNA	69.1
14	yel1 DNA	69.9	**14**	pur1 DNA	69.9
15	empty		**15**	empty	
16	Ctrl	45.9	**16**	Ctrl	62.9
17	empty		**17**	empty	
18	100bp Ladder		**18**	100bp Ladder	
19	empty		**19**	empty	
20	Ctrl	57.5	**20**	Ctrl	69.6

3. Testing primers on mixtures of DNA from both *P. temperata* subspecies

The mixture of DNA from both pur and yel isolates was amplified using both primers pur1-pp5 and yel3-pp6 (Fig 4.7**).** Pur1-pp5 primers amplified samples of 100% pur1 DNA (Lanes 1 and 2) but not yel1 DNA (lanes 7 and 8); yel3-pp 6 primers amplified samples of 100% yel1 DNA (lanes 3 and 4) but not pur1 DNA (lanes5 and 6). Samples containing a mix of 50% pur1 and 50% yel1 DNA were amplified by pur1-pp5 primer (lanes 9 and 10) and by yel3-pp6 primer (lanes 11 and 12). The difference between the primers was the product length by base pair; Pur1-pp5 has a larger expected product than yel3-pp6, which also shows in the gel picture.

Lane	Isolate	primer
1	pur1	Pur1-pp5
2	pur1	Pur1-pp5
3	yel1	Yel3-pp6
4	yel1	Yel3-pp6
5	pur1	Yel3-pp6
6	pur1	Yel3-pp6
7	yel1	Pur1-pp5
8	yel1	Pur1-pp5
9	yel1&pur1	Pur1-pp5
10	yel1&pur1	Pur1-pp5
11	yel1&pur1	Yel3-pp6
12	yel1&pur1	Yel3-pp6
13	(ctrl)	Pur1-pp5
14	(ctrl)	Yel3-pp6
15	100 bp ladder	

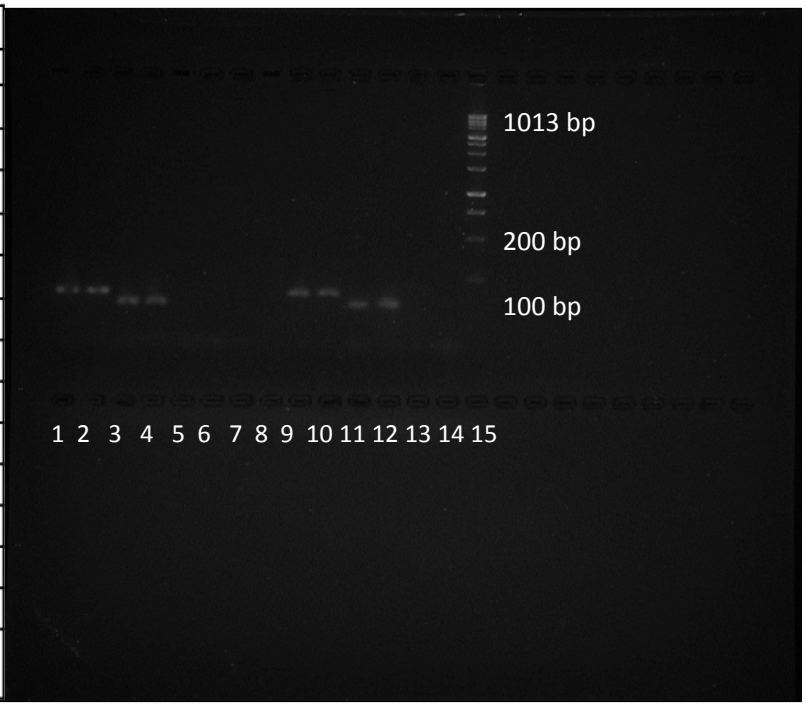


Fig 4.7 Gel analysis of *P. t. cinerea* (pur1) and *P. t. temperata* (yel1) Bacterial DNA**.** Target sequences were Leucine-responsive regulatory protein (*P. t. cinerea*) and a hypothetical protein (*P. t. temperata*). Primers were designed using Primer-Blast (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) and the target sequences were 184 bp (pur1-pp5 primer) & 142 bp (yel3-pp6 primer), the ladder was (1000 bp ladder).

4. Testing novel primers (pur1-pp5 and yel3-pp6) for distinguishing *P. temperata* subspecies in infected insects (*G. mellonella*)

Pur1-pp5 and yel3-pp6 primers were tested against DNA extracted from *G. mellonella* infected with *P. t. cinerea* (pur1) and *P. t. temperata* (yel3) to test subspecies-specific amplification (Fig 4.8) (Table 4.19). The primers successfully amplified pure DNA in a subspecies-specific manner (Upper lanes 5 and 6 for pur1-pp1 and lower gel lanes 5 and 6 for yel3-pp6). 16S primer confirmed there was bacterial DNA present in the extracts from at least some of the infected insects, including insects with pur1 only (upper lanes 17, 18) and a mix of pur1 and yel3 (lower gel, lanes 17-20) but did not detect bacterial DNA in either of the insects infected by yel3 alone (upper lanes 19, 20). The novel primers amplified *P. temperata* DNA extracted

from insects infected with a mix of pur1 and yel3. Pur1-pp1was successful in amplifying DNA from several insects (upper lanes 8-14) and yel3-pp6 was successful in samples 15 and 16 (lower lanes). This may mean that *P. t. cinerea* was dominant in some of the infected insects and *P. t. temperata* was dominant in others.

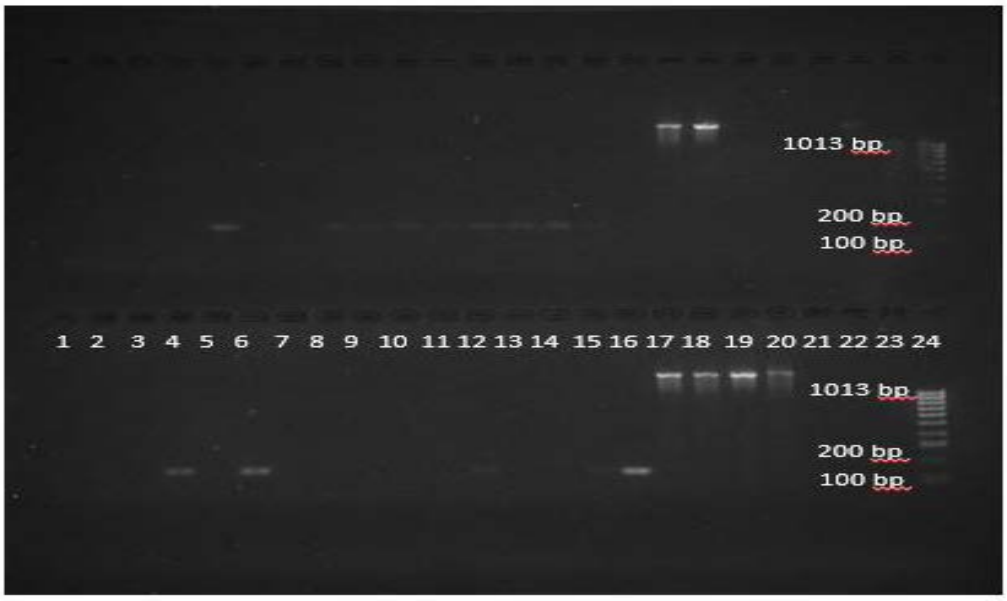


Fig 4.8 Gel analysis of *P. t. cinerea* (pur1) and *P. t. temperata* (yel3) DNA extracted from infected *G. mellonella*. Target sequences were Leucine-responsive regulatory protein (*P. t. cinerea*) and a hypothetical protein (*P. t. temperata*). Primers were designed using Primer-Blast **(**http://www.ncbi.nlm.nih.gov/tools/primer-blast/) and the target sequences were 184 bp (pur1-pp5 primer), 142 bp (yel3-pp6 primer) and (16S primer), the ladder was (100 bp ladder & 1000 bp). Key to lanes in Table 4. 19.

Table 4.19 Gel loading lanes using pur1-pp5 primer, yel3-pp 6 primer and (16S primer as a positive controls) primers were used to amplify DNA extracted from infected *G. mellonella* with pur1 and yel3 100% or 50:50 mix of both isolates. Colour specific binding.

Upper Lane	**Isolate**	**primer**	**Lower Lane**	**Isolate**	**primer**
1	purple insect	Pur1-pp5	1	purple insect	yel3-pp6
2	purple insect	Pur1-pp5	2	purple insect	yel3-pp6
3	yel1ow insect	Pur1-pp5	3	yel1ow insect	yel3-pp6
4	Yellow insect	Pur1-pp5	4	Yellow insect	yel3-pp6
5	pur1 DNA	Pur1-pp5	5	pur1 DNA	yel3-pp6
6	Yel3 DNA	Pur1-pp5	6	Yel3 DNA	yel3-pp6
7	Purple/yellow insect	Pur1-pp5	7	Purple/yellow insect	yel3-pp6
8	Purple/yellow insect	Pur1-pp5	8	Purple/yellow insect	yel3-pp6
9	Purple/yellow insect	Pur1-pp5	9	Purple/yellow insect	yel3-pp6
10	Purple/yellow insect	Pur1-pp5	10	Purple/yellow insect	yel3-pp6
11	Purple/yellow insect	Pur1-pp5	11	Purple/yellow insect	yel3-pp6
12	Purple/yellow insect	Pur1-pp5	12	Purple/yellow insect	yel3-pp6
13	Purple/yellow insect	Pur1-pp5	13	Purple/yellow insect	yel3-pp6
14	Purple/yellow insect	Pur1-pp5	14	Purple/yellow insect	yel3-pp6
15	Purple/yellow insect	Pur1-pp5	15	Purple/yellow insect	yel3-pp6
16	Purple/yellow insect	Pur1-pp5	16	Purple/yellow insect	yel3-pp6
17	purple insect	16S	17	Purple/yellow insect	16S
18	purple insect	16S	18	Purple/yellow insect	16S
19	Yellow insect	16S	19	Purple/yellow insect	16S
20	Yellow insect	16S	20	Purple/yellow insect	16S
21	Pur1 DNA	16S	21	Purple/yellow insect	16S
22	Yel3 DNA	16S	22	Negative control	yel3-pp6
23	Negative control	Pur1-pp5	23	Negative control	16S
24	ladder	1000 bp ladder	24	ladder	1000 bp ladder

5. Testing novel primers (pur1-pp5 and yel5-pp6) to distinguish *P. temperata* subspecies present in *Heterorhabditis downesi* infective juveniles

The primers were tested against DNA extracted from 300 IJs as described in chapter 2. The DNA template was from pur1 IJs, DNA from Yel3 IJs and DNA from mixes of the two in various ratios. The primers were pur1-pp5 and yel5-pp6 and each primer was tested against all DNA template (Table 4.20). DNA extracted directly from bacteria was also included

Fig. 4.9 shows the gel analysis of PCR product. The yel3-pp6 primers successfully amplified samples including p1/y3 (150/150 IJs) and p1/y3 (10/290 IJs) (lanes 13,15) and bacterial DNA *P. t. temperata* (yel3) (lane 17) and p1/y3 bacterial DNA (lane 18) but the pur1-pp5 primers did not.

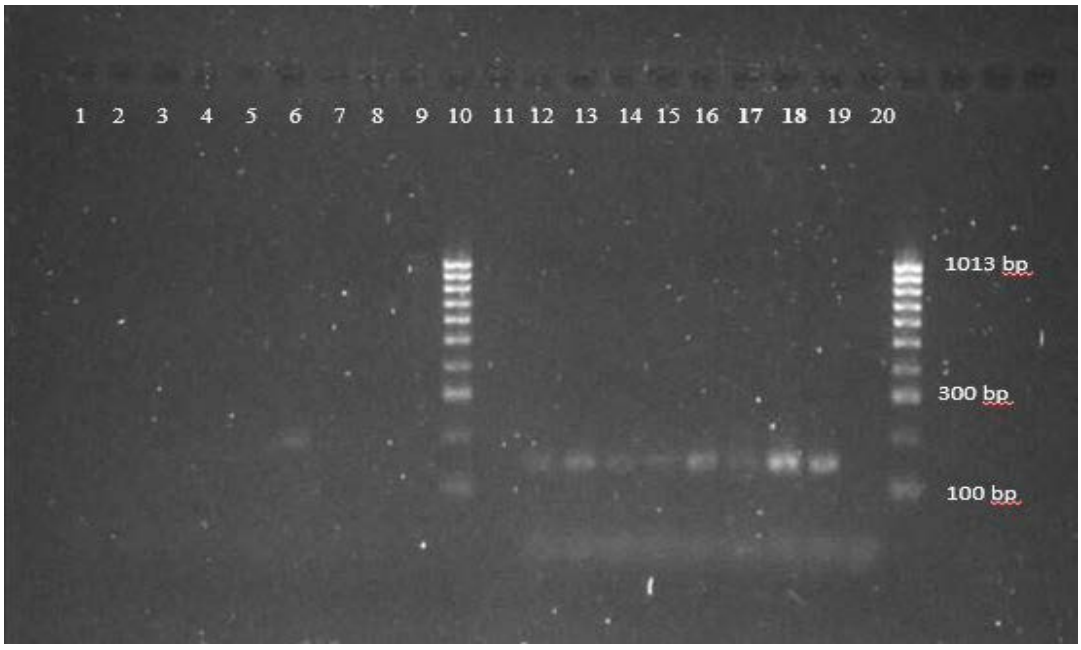


Fig 4.9 Gel analysis of mix of *P. t. cinerea* IJs (pur1, pur2, pur3) and *P. t. temperata* (yel1, yel2, yel3) IJs with different percentages. Target sequences were Leucine-responsive regulatory protein (*P. t. cinerea*) and a hypothetical protein (*P. t. temperata*). Primers were designed using Primer-Blast **(**http://www.ncbi.nlm.nih.gov/tools/primer-blast/) and the target sequences were 184 bp (pur1-pp5 primer) and 258 bp (yel3-pp6 primer). Key to lane in Table 4.20.

Table 4.20 Gel loading lanes using pur1-pp5 primer, yel3-pp 6 primer and primers were used to amplify bacterial DNA present in Heterorhabditis downesi infective juveniles with pur1 and yel3 100% or 50:50 mix of both isolates. Colour specific binding.

Lane	Isolate	Primer	Lane	Isolate	Primer
1	pur1, 300 IJs	Pur1-pp5	**11**	pur1, 300 IJs	Yel3-pp6
2	yel3, 300 IJs	Pur1-pp5	**12**	yel3, 300 IJs	Yel3-pp6
3	P1/y3, 150/150 IJs	Pur1-pp5	**13**	P1/y3, 150/150 IJs	Yel3-pp6
4	P1/y3, 290/10 IJs	Pur1-pp5	**14**	P1/y3, 290/10 IJs	Yel3-pp6
5	P1/y3, 10/290 IJs	Pur1-pp5	**15**	P1/y3, 10/290 IJs	Yel3-pp6
6	Bacterial Pur1 DNA	Pur1-pp5	**16**	Bacterial Pur1 DNA	Yel3-pp6
7	Bacterial yel3 DNA	Pur1-pp5	**17**	Bacterial yel3 DNA	Yel3-pp6
8	P1/y3 Bacterial DNA	Pur1-pp5	**18**	P1/y3 Bacterial DNA	Yel3-pp6
9	Negative Ctrl	Pur1-pp5	**19**	Negative Ctrl	Yel3-pp6
10	100 bp Ladder		**20**	100 bp Ladder	

6. New set of primer design for PCR Optimisation

In further work, results with these primer pairs were unreliable. Therefore, new primers were designed (Pur1-349F/pur1-432R, yel3-297F/yel3-379R and yel3-552F/yel3-660R) (Table 4.6). Gradient PCR was used to optimise the annealing temperature for both pur1 and yel3 isolates. However, this did not resolve the problems. Non-specific binding was found and results were unreliable for all three new primer pairs. Results for primer pair yel3-552F/yel3-660R are shown in Fig. 4.10, (Table 4.21).

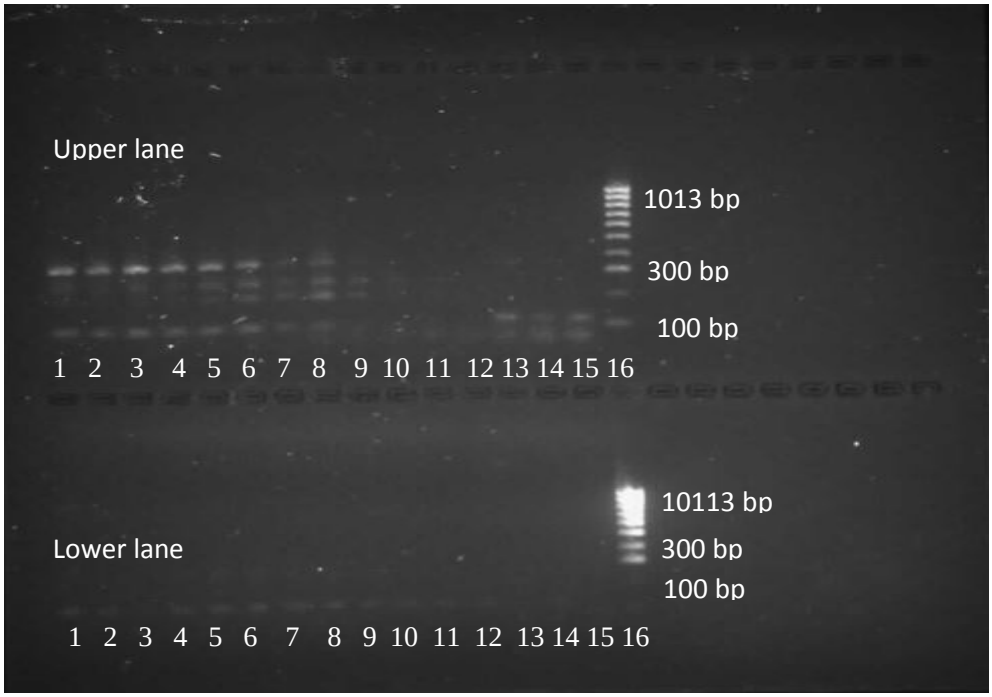


Fig 4.10 Gel analysis of *P. t. cinerea* DNA (pur1) and *P. t. temperata* (yel3) with primer pair **yel3-552F/yel3-660R**. Target sequences was a hypothetical protein (*P. t. temperata*). Primers were designed using **Geneious 9.0.' (Kearse *et al.*, 2012)** and the target sequence was 83 bp. Key to lanes is in Table 4. 21.

Table 4.21 Show that the lanes of the Gel loading using yel3-552F/yel3-660R, the primers was used to amplify the DNA.

Upper Lane	**Isolate**	**Temp °C**	**Lower Lane**	**Isolate**	**Temp °C**
1	Pur1	**50.0**	1	Yel3	**50.0**
2	Pur1	**50.3**	2	Yel3	**50.3**
3	Pur1	**51.4**	3	Yel3	**51.4**
4	Pur1	**53.2**	4	Yel3	**53.2**
5	Pur1	**55.5**	5	Yel3	**55.5**
6	Pur1	**58.1**	6	Yel3	**58.1**
7	Pur1	**60.1**	7	Yel3	**60.1**
8	Pur1	**63.5**	8	Yel3	**63.5**
9	Pur1	**66.0**	9	Yel3	**66.0**
10	Pur1	**68.1**	10	Yel3	**68.1**
11	Pur1	**69.7**	11	Yel3	**69.7**
12	Pur1	**70.5**	12	Yel3	**70.5**
13	Ctrl	**50.0**	13	Ctrl	**60.1**
14	Ctrl	**55.5**	14	Ctrl	**63.5**
15	Ctrl	**58.1**	15	Ctrl	**70.5**
16	100bp ladder		16	100bp ladder	

4.4 Discussion

Bacteria may compete in different ways: one species or strain may grow faster or produce antibiotics that inhibit the growth of competitors.

The results of the experiment in which cadavers were co-infected with pur and yel nematodes and then subjected to either moist or dry conditions did not confirm the hypothesis that *P.t cinerea* would predominate in emerging IJs, but only under dry conditions. In fact, *P. t. cinerea* predominated in emerging IJs under both moist and dry conditions. This was already seen after one round of infection in two of the three combinations of isolates tested, pur1/yel3 and pur3/yel2. By the third round of infection, *P. t. cinerea* predominated in emerging IJs in all three pairs of isolates, and in both wet and dry conditions. This is different to the findings of Maher (2014), who found that overall more than twice as many emerging IJs carried *P. t. temperata* than carried *P. t. cinerea*, when hosts were kept moist. The main difference between the two experiments is that Maher (2014) injected a 50:50 mixture of bacterial cells (Pur1 and yel3) and then allowed IJs to invade. In this experiment, the insects were infected using a 50:50 mix of pur and yel IJs. It is possible that by chance, in each of the three pairs of isolates used in the experiment, either more pur IJs invaded or they carried more bacteria than the yel IJs, and so the starting inoculum was unequal and pur bacteria dominated. Although as shown in Chapter 3, there is no consistent difference between pur and yel isolates in the number of bacteria carried by the IJs.

An interesting finding in the desiccation experiment was that several individual IJs carried both subspecies of symbiont. Ciche et al. (2008) described how *P. luminescens* colonises *H. bacteriophora*. First it colonises the rectal gland cells of the mother worm. Then the bacteria from these cells colonise the pre-IJs developing within the mother. Just one or two cells colonise each IJ and then multiply (Ciche *et al.*, 2008). If the same happens in *H. downesi*, then

it means that one cell of *P. t. temperata* and one cell of *P. t. cinerea* both successfully colonised some of the IJs in this experiment and so both subspecies must also have colonised the mother-worm. This was explored in a further experiment by again co-infecting insects with a mix of IJs (pur1 and yel3) but this time dissecting out some of the mother-worms at the "bagged" stage (full of developing IJs) and identifying which bacteria were carried by the IJs inside. Most mother-worms carried only one subspecies of bacteria, either yel or pur, but a small number (6 out of 52) carried both. In those that carried both, there was some evidence that *P.t. cinerea* was more successful in these co-infected mother worms, as there were nearly twice as many *P. t. cinerea* CFU per IJ than *P. t. temperata* CFU. However, the sample size was small and a significant difference was not demonstrated. In this experiment IJs were not crushed individually, therefore the result was not confirmed that both of them carried symbiont subspecies. However, IJ colonised by both symbiont subspecies in the first experiment must also have developed within a mother-worm colonised by both subspecies.

All six isolates of *H. downesi* preferred *P. t. cinerea* when offered a choice of the two *P. temperata* subspecies on the same agar plate. There was no difference between the three combinations of yel-pur isolates, suggesting that the attractiveness of pur for *H. downesi* is a feature of Bull Island *P. t. cinerea*. There was very little movement of nematodes from one segment of bacteria to another (data not shown), suggesting that it is the IJs that were placed on the plate that make the choice. At the time of counting 7 days after IJs were put on the plates they had developed into mature hermaphrodites, and shortly afterwards were observed to be "bagged" or full of IJs. Therefore, it is assumed that IJs developing in hermaphrodites on pur segments will be colonised by *P. t. cinerea*. Evolutionary theory suggests that when it is possible, hosts should select the best performing symbionts from a population of possible partners and there is evidence of this for several symbioses (Sachs *et al.*, 2011). The mechanisms underlying partner choice are unknown, but Hillman and Goodrich-Blair (2016)

suggest that a host could recognise and select a beneficial partner if the symbiont displays honest signals of its defensive or other capabilities, such as toxins or antibiotics. On the other hand, they may be choosing based on nutritive quality.

Mostly, when cultured alone in liquid medium, pur and yel bacteria grew equally well as judged by number of CFU when plated after 24 hours. Only those sets of trials where the controls (pur or yel alone) grew equally well were used to evaluate growth in co-culture. In co-culture, for two of the pairs of isolates, pur2/yel1 and pur3/yel2, both symbiont subspecies grew equally well as judged by the proportion of CFU of each subspecies when the co-culture was plated after 24 hours. However, in the third pair, pur1/yel3, pur1 (*P. t. cinerea*) predominated, representing 99-100% of the CFU. This advantage of pur1 over yel3 must be a feature of these particular isolates and is not a feature of the sub-species in general. Maher (2014) tested inhibition between members of the same three pairs of isolates, and found that pur2/yel1 each inhibited each other very slightly, but in the other two pairs there was no evidence of inhibition. Therefore, it is more likely that the advantage of pur1 over yel3 is due to a faster growth rate of pur1 relative to yel3 (competing for resources), rather than to the production of specific inhibitory factors.

Attempts to develop a method to distinguish between the two symbionts using molecular methods were unsuccessful. PCR is performed on a DNA template, which needs to be specific and different for the two subspecies. Using the genome sequences provided by MicrobesNG Nick Tobias identified unique coding sequences for pur1 (leucine responsive regulatory protein) and yel3 (a hypothetical protein). Six different primer pairs were then designed and tested – two for pur1 and four for yel2. These were designed based on three criteria: a low probability of amplifying DNA from other bacteria likely to be found in *G. mellonella*, a low probability of unintended template, and length of product (the amplicon) less than 200 base pairs. Conditions of the PCR reaction mix were varied during the course of this work. The

reaction mix contains the forward and reverse primers, dNTPs (which are the four nucleotide triphosphates), a heat stable polymerase (Taq polymerase) and magnesium ions ($MgCl_2$) in the buffer. At first MyTaq reaction buffer was used which contains the dNTP mix and $MgCl_2$ (Reaction Mix 1) but each of these ingredients were added later separately and also increased the concentration of the primers (reaction mix 2). Too much $MgCl_2$ can result in non-specific binding (C Meade, pers communication). Finally, an intermediate concentration of primer (Reaction mix 3) was used, also the annealing temperature for each primer pair needed to be optimised. The PCR reaction is performed by temperature cycling. First, high temperature is applied to separate (melt) the double-stranded DNA template. Then the temperature is lowered to let the primers anneal to the template, and finally the temperature is set to 72^{o}C which is the optimum for the polymerase that extends the primers by incorporating the dNTPs. The annealing temperature depends on the primers. Theoretically it should be a few degrees below the melting point of the two primers so that they form a stable complex with the target sequence but not with any other sequence (Kubista *et al.*, 2006). Initial PCR using the annealing temperature based on the melting point of the primers (provided by Eurofins Scientific) resulted in non-specific binding of the pur1 and yel3 templates when a mixture of DNA from both isolates was used as the template (Fig 4.4) Non-specific binding occurs if the annealing temperature is too low. The optimum annealing temperature can be higher than the calculated temperature (Prezioso, 2000). To try to eliminate the non-specific binding a gradient PCR was run with each primer pair, using a gradient of +/- 10^{o}C of the nominal temperature in each case to try to determine the optimum annealing temperature (Weitkamp and Crowe 2001). The optimum annealing temperature for each target sequence, (pur1 or yel 3), was chosen as the temperature at which the clearest band was produced on the gel, with no amplification of the other isolate. In spite of all of the modifications to primers, reaction mix and annealing temperature, it was not possible to reliably identify the subspecies present.

The size of the genomes provided by Microbes NG (238,875 bp) was small relative to genomes of other *Photorhabdus* that have been sequenced. The first genome sequenced was that of *P. luminescens* subsp. *laumondi* (isolate TT01) (Duchaud *et al.*, 2003). Other genomes sequenced include those of *P. asymbiotica* subsp. *asymbiotica* ATCC43949 (Wilkinson *et al.*, 2009) and *P. temperata temperata* strain M1021 (Park *et al.*, 2013). These genomes are typically more than 5 million base pairs – 20 times larger than the genome data for our strains from Microbes NG. Nevertheless, the analysis provided by NSilico provides some information about virulence factors, obtained by searching the Virulence Factor Database. This identified a number of elements of the type III secretion system. The type III secretion system (T3SS or "injectisome") is described as a complex nanomachine that allows bacteria to deliver protein effectors across cell membranes of eukaryotes (Cornelis, 2006, Kosarewicz *et al.*, 2012). Type III secretion systems occur in many species of Gram negative bacteria that interact with other organisms as pathogens or symbionts. The T3SS apparatus and secretion mechanisms are remarkably conserved (Deng *et al.*, 2017). Tobias (2016) compared the genomes of seven *Photorhabdus* subspecies, from three species. They reported that most strains maintained the entire T3SS system, but *P. temperata temperata* M1021 had lost three genes, *sctC, sctV* and *sctP*. The protein products SctC and SctV are described as core proteins in this T3SS, leading Tobias et al. (2016) to suggest that *P. t. temperata* M1021 contains a non-functional T3SS. Our *P. t. temperata* strain yel3 contained these core genes sctC and sctV (though not sctP) lending support to the alternative hypothesis of Tobias et al (2017) that the absence of these two genes in M1021 is due to an "assembly artefact".

More toxin genes were predicted in the *P. luminescens* genome than in any other bacterial genome sequence (Duchaud *et al.*, 2003). However, in addition to the T3SS complex, only a few other virulence genes were detected in our pur1 and yel3

genomes. Rhas, coding for a transcriptional activator protein was present in both genomes. In addition, sycd (a chaperone) and two genes for hypothetical proteins were present in the yel3 genome. None of the other virulence factors identified in *Photorhabdus* such as the Tc (toxin complex) or Mcf (makes caterpillars floppy) genes were identified. This is presumably due to the small size of the genome sequence provided by Microbes NG.

The virulence factor genes are described as being more similar either to *P. luminescens* subsp. *laumondi* TT01 or *P. asymbiotica* subsp. *asymbiotica* ATCC42949. For three of the T3SS genes, sctJ, sctK and sctU, the *P.t.cinerea* pur1 version was closer to *P.a. asymbiotica* and the *P. t. temperata* yel3 version was closer to *P. a. asmbiotica* (Table 4.16). This is in accordance with the more recent view of the relationships within *Photorhabdus*. Although *P.t. cinerea* was originally placed in the same phylogenetic clade as subspecies of *P. temperata* using the gyrB gene (Toth and Lakatos, 2008), by using a multigene approach it was shown to be more closely related to *P. asymbiotica asymbiotica* (Ferreira *et al.*, 2014).

Chapter 5

Cadaver Defence

5.1 Introduction

The bacterial symbionts of entomopathogenic nematodes kill the insect, convert it into food for the nematodes, and also protect the cadaver against microbes and scavenging animals. Cadavers infected by entomopathogenic nematodes do not putrefy, and this has been shown to be due to the production of antibiotics by the symbionts. *Xenorhabdus* spp produce several classes of molecule with antibiotic activity, such as indoles, xenorhabdins and xenocoumacins (Isaacson and Webster, 2002). The main antibiotics produced by *Photorhabdus* spp. are stilbenes, which are active against Gram-positive bacteria and fungi (Paul *et al.*, 1981, Richardson *et al.*, 1988, Li *et al.*, 1995). Cadavers infected by *Photorhabdus* are also protected against ants and other scavengers by "scavenger deterrent factor" (Zhou *et al.*, 2002, Gulcu *et al.*, 2012). Most *Photorhabdus* turn the cadaver a red, yellow or orange colour, due to the production of anthraquinone pigments (Richardson *et al.*, 1988). The red colour was shown to protect cadavers from scavenging by birds (Fenton *et al.*, 2011). A further possible defence against scavengers is bioluminescence. *Photorhabdus* spp. are the only terrestrial bioluminescent bacteria (Peat and Adams, 2008). The function of bioluminescence in *Photorhabdus* is unknown (Peat and Adams, 2008), but one possibility is that it deters scavengers or competitors. There is evidence that bioluminescence deters nematodes that might otherwise enter the cadaver and compete with the residents (Chuche et al., unpublished).

In this chapter, the role of *Photorhabdus temperata* subsp. *temperata* and *Photorhabdus temperata* subsp. *cinerea* in cadaver defence were explored. Maher (2014) showed that the Bull

Island isolates of both subspecies suppressed a range of bacteria and fungi, but the zone of suppression was larger with *P.t. cinerea* than with *P.t. temperata* isolates. Following that, an undergraduate student (Flynn, 2016) compared the antibiotic activity of two more pairs of isolates, one of each subspecies isolated from a site in County Cork, Ireland and one of each subspecies isolated from a site in Pembrokeshire, Wales. In the case of the pair from County Cork, the *P.t. cinerea* isolate had a larger zone of inhibition than the *P. t. temperata* isolate, as had been found for the Bull Island isolates. However, the *P.t. cinerea* isolate from Wales did not inhibit any of the three fungi and two bacterial species tested (Flynn, 2016). Therefore, in order to see whether this was a feature of *P. t. cinerea* from Wales in general, or just the isolate W26/2 tested by Flynn, the antibiotic activity of a number of other Welsh isolates of *P. t. cinerea* was tested.

An attempt was made to identify secondary metabolites with antimicrobial activity produced by the Bull Island isolates using reverse phase high performance liquid chromatography (RP-HPLC). HPLC is a powerful analytical technique used to separate, identify and quantify each component in a mixture. A solvent is forced under high pressure through a column packed with tiny particles of silica. In reverse phase, non-polar compounds in the mixture are slowed by the column, and therefore take longer to travel, while polar molecules travel quickly through the column. The molecules travel through the column at different rates depending on the identity of the molecules and the conditions used. The time taken by a compound to reach the detector is the retention time, which can help identify the compound. The size of the peak indicates the relative amount of each compound. The HPLC can be coupled to a mass spectrometer (MS). When the HPLC detector shows a peak, some of the material is diverted to the MS. There it will give a fragmentation pattern that can be compared against a database of known compounds.

The overall aim of this chapter is to explore the cadaver defences provided by *P.t. cinerea* and *P. t. temperata*. Specific objectives are to

(1) Test whether Welsh isolates of *P.t. cinerea* inhibit bacteria and fungi, based on the finding by Flynn (2016) that the Welsh isolate W26/2 did not. In order to do this, firstly several isolates of *H. downesi* that had been cryo-preserved in liquid nitrogen were thawed out. Then the symbionts were isolated and identity confirmed by sequencing. then antibiotic potential of six Welsh isolates of *P. t. cinerea* were compared using Bull Island isolate pur2 as a standard, testing against one bacterial species and two fungal species.

(2) Identify potential antibiotic compounds produced by Bull Island isolates of *P.t. cinerea* and *P. t. temperata.*

(3) Compare bioluminescence of *P. t. cinerea* and *P. t. temperata*.

5.2 Methods

5.2.1 Antibiosis and Antimycosis by *P. temperata*

Antibiosis test methods

P. temperata (50 µl of a 24hr culture) was spot-inoculated onto the centre of nutrient agar in a glass petri dish and incubated for 48 hrs at 27°C. In the fume hood *Photorhabdus* spot colonies were killed by flooding the lid of petri dish with chloroform and leaving the plate inverted over the chloroform for 30 minutes. After that the plate was left open for another 30 minutes to allow the chloroform to evaporate.

Soft nutrient agar was made (7 g agar / L dH_2O) and allowed to cool to 45°C. Then the cooled soft agar was inoculated with 1 ml *E. coli* (1 ml / 50 ml agar), and a layer of agar was

poured onto the plates with the *Photorhabdus*. The *E. coli* was obtained from the Preparation Laboratory, Maynooth University Biology Department. Then the plates were left half-covered by the lids in the laminar flow hood to set for 30 min and incubated for 24 h at 37°C. After the appropriate incubation time the measurement of the zone of inhibition was taken (Fig 5.1). The experiment was run twice with 3 replicate plates per isolate.

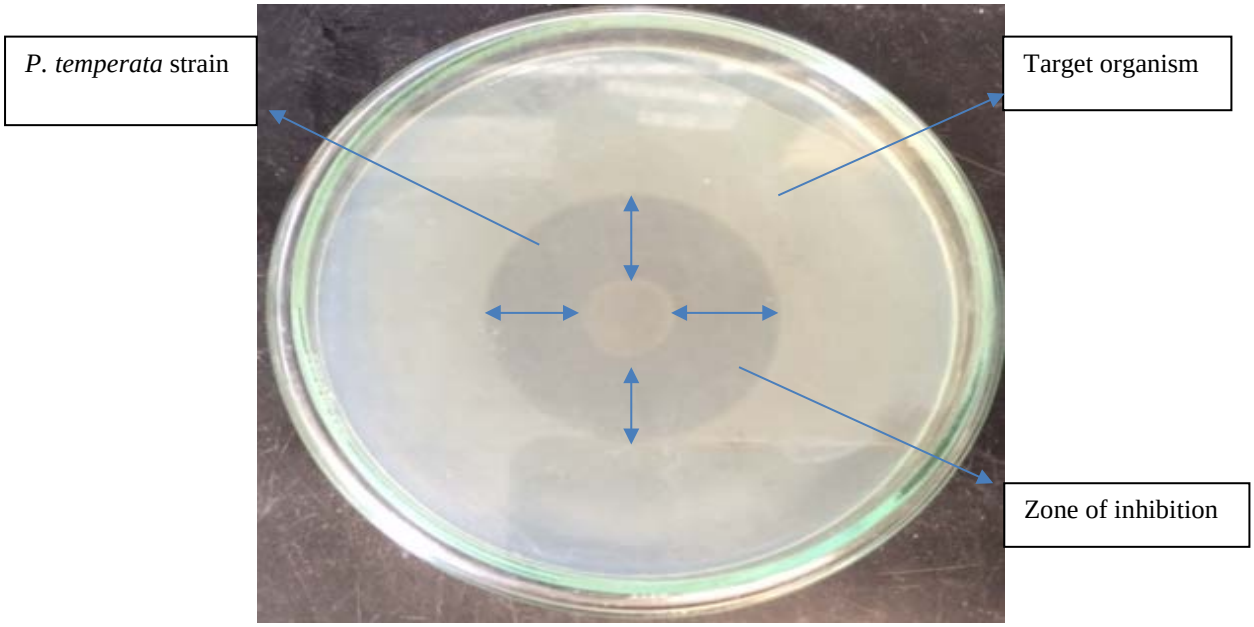


Fig. 5.1 Diagram explaining the zone of inhibition and main measuring point (blue arrows) against *E. coli*

Antimycosis assay

For the antimycosis experiment the method of Ansari *et al*. (2005) was used. A loopful of *P. temperata* was streaked onto nutrient agar plates in a line 5 cm long and 3 cm from one edge of a 9 cm nutrient agar plate and incubated for 48 hrs at 27°C in the dark.

After two days of incubation the plates were inoculated with a mycelial plug 0.5 cm in diameter using a 5 ml pipette tip to take the plug the fungi used were the entomopathogenic fungi

Beauveria bassiana and *Metarhizium anisopliae* Met 52. The mycelial plug was placed in line with midpoint of the bacteria streak and centered 3 cm from the wall of the Petri dish and the bacterial streak (Fig 5.2). Negative control plates were inoculated with only a plug of mycelium at each treatment. Plates were incubated at 25°C for 3 weeks. As soon as the fungus growth on the control plates reached the far side of the dish the test was terminated. After the appropriate incubation time, which was more than three weeks, the zone of inhibition was measured. The experiment was run twice with three replicate plates per treatment in each experiment.

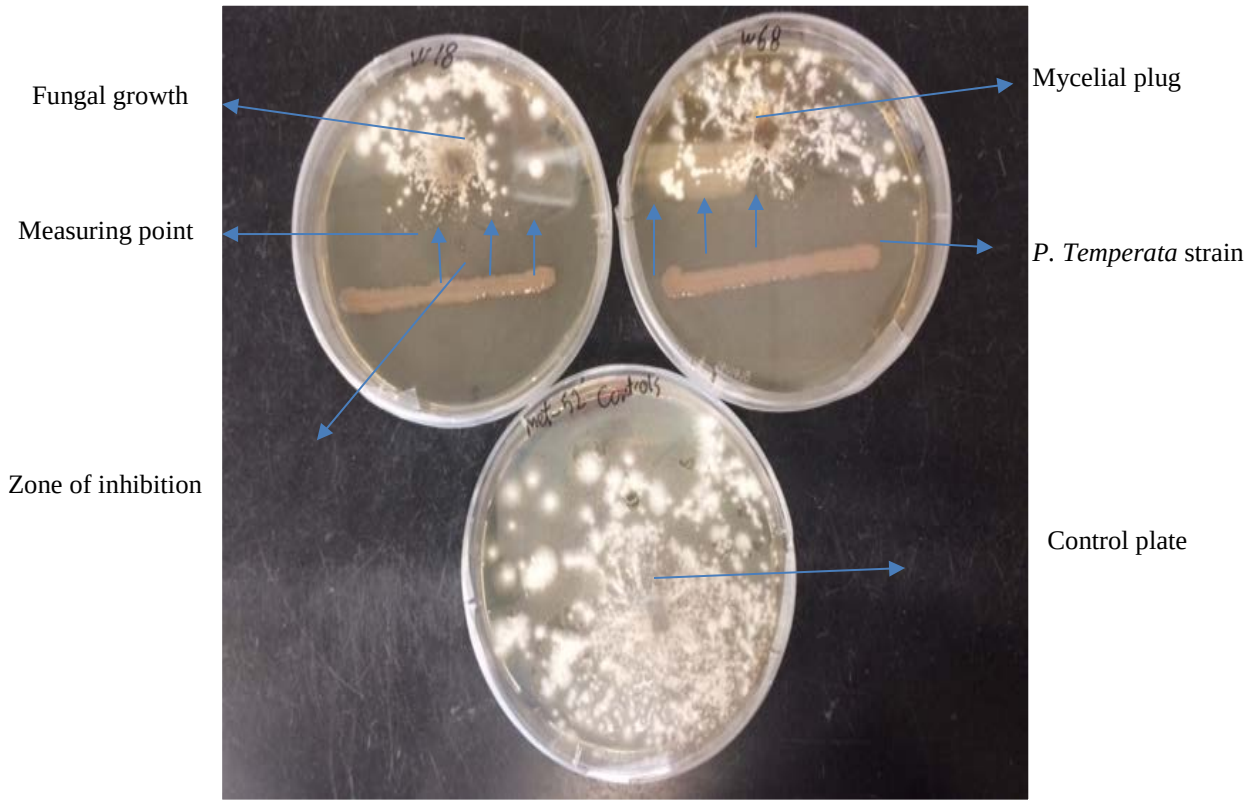


Fig. 5.2 Diagram showing experimental setup with *P. temperata* streak, mycelial plug, fungal growth from the inoculating mycelial plug, zone of inhibition and three measuring points (blue arrows) in Antimycosis test.

5.2.2 RP-HPLC of culture supernatant from *Photorhabdus* isolates

Preparation of samples for HPLC analysis

One colony of bacteria was put in 50 ml LB (Luria Broth) in a 250 ml flask in a shaking bath at 27°C to let them grow for 48 hours. The liquid culture was transferred to a 50 ml centrifuge tube and centrifuged at 3000 xg (Beckman GS-6 Centrifuge) for 10 minutes to pellet the bacteria. The supernatant was transferred to a clean 50 ml centrifuge tube and filtered twice through a 0.45µm Minisart Syringe filter then through a 0.2 µm Minisart Syringe filter then stored at 4°C. Prior to HPLC analysis 100µl of the culture filtrate was transferred to a 1.5 ml Eppendorf tube and 100µl of filtered (0.2µm) Milli Q water was added, mixed and the diluted filtrate was centrifuged at 14000 xg (Sigma 1-15 Micro centrifuge) for two minutes to remove contamination, then 100 µl was transferred to HPLC for analysis.

HPLC Solvent preparation

HPLC buffers were prepared as follows:

30 ml per sample Buffer A (1 L HPLC grade water, (use Milli-Q fitered water), 1 ml Trifluroacetic acid, 302031, Sigma);

15 ml per sample Buffer B (1 L HPLC grade Acetonitrile, 34998, Sigma; 1 ml Trifluroacetic acid, 302031, Sigma).

RP-HPLC Analysis

Samples were analysed by reverse phase high performance liquid chromatography (RP-HPLC) with UV detection (Shimadzu), using a C_{18} RP-HPLC column (Luna Omega-Polar C18; 5 mm particle size; 4.6 x 15 mm) at a flow rate of 1 ml/min. A mobile phase of water and acetonitrile, with 0.1 % (v/v) trifluoroacetic acid (TFA), was used under gradient conditions outlined in (Table 5.1). Injection volume was set to 20 µl and all wavelengths between 190-

800 nm were monitored. Samples from *Photorhabdus* isolates were analysed along with an aliquot of the culture broth as a negative control.

Table 5.1 RP-HPLC gradient

Time (min)	0	5	35	40	41	50
% Acetonitrile	5	5	100	100	5	5

5.2.3 Measuring bioluminescence

Insects (*G. mellonella*) were infected with 50 *Heterorhabditis* IJs each, in wells of multiwell plates. They were incubated at 20^{o}C. Two measures of bioluminescence were made: the average light intensity per cadaver, and the emission spectrum.

Bioluminescence intensity measurements were made using G:Box. Five minute exposure photographs of cadavers were taken using a Syngene G:Box Chemi HR16 BioImaging System in the dark and using the software GeneSnap 7.12 (SynGene, Canbridge, UK). The pictures were then exported as uncompressed TIFF files. The area and the mean gray value, corresponding to the light intensity, of each insect were then measured using the Image J 1.51f software. The mean gray is the sum of the gray values of all the pixels in the selection divided by the number of pixels. This measures the intensity of light at each pixel of the insect and gives the average intensity for each insect.

The emission spectrum was recorded using a Clariostar plate reader. This measures light intensity at each wavelength from 400 to 740 nm. Measurements are made at a single point of the cadaver. Both types of measurements were made on days 3, 4 and 5 post infection. Two Bull Island isolates were used: *H. downesi* pur1 (*P.t. cinerea*) and yel3 (*P. t. temperata*). There were 24 insects per isolate.

5.3 Results

5.3.1 Antibiosis and Antimycosis

All of the six Welsh isolates recovered from cryopreservation were identified as *P.t. cinerea* using 16S rRNA gene sequencing. They were compared with *P.t. cinerea* pur2 in antibiosis and antimycosis tests.

Antibiosis tests

General linear model (GLM) analysis showed that there was significant interaction between isolate and trial ($F_{6,28.}$ = 28.68 , P < 0.001). Therefore, each trial was analysed separately. There were significant differences among the isolates in both trials (Trial 1: one-way ANOVA $F_{6,14}$ =17.73, P < 0.001; Trial 2: One- way ANOVA, $F_{5,12}$ = 4.83, P = 0.012). Especially for the isolate W48 the zone of inhibition was lower against *E. coli* compared with most of the other isolates in Trial 2, and in Trial 1 there was no zone of inhibition found (Fig 5.3).

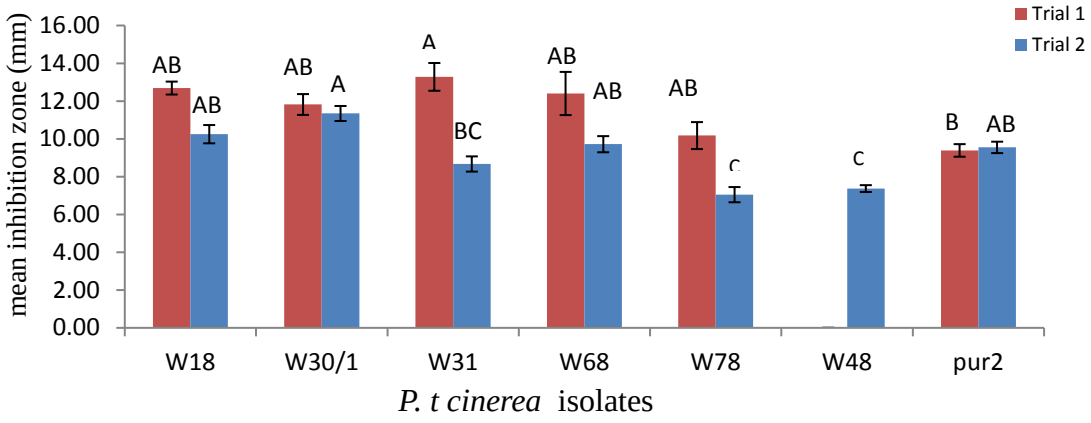


Fig. 5.3 Mean (±SE) size of zone inhibition for *P. temperata* against *E. coli* (Trial 1: one-way ANOVA $F_{6,14}$ =17.73, P < 0.001; Trial 2: One- way ANOVA, $F_{5,12}$ = 4.83, P = 0.012) Within trial, bars with the same letters are not significantly different (Tukey's test)

Antimycosis Assay

There was no interaction between isolate and trial for *Beauveria bassiana* (GLM, $F_{6,28}$ = 1.8, *P* = 0.134). Therefore the data for both trials were combined for analysis using One-way ANOVA. There were significant differences in the zone of inhibition among the isolates for *Beauveria bassiana* ($F_{6,35}$ = 4.99, *P* = 0.001). For example, the zone of inhibition was high for W48 and low for W78, but no strain was significantly different to Pur2 (Fig 5.4)

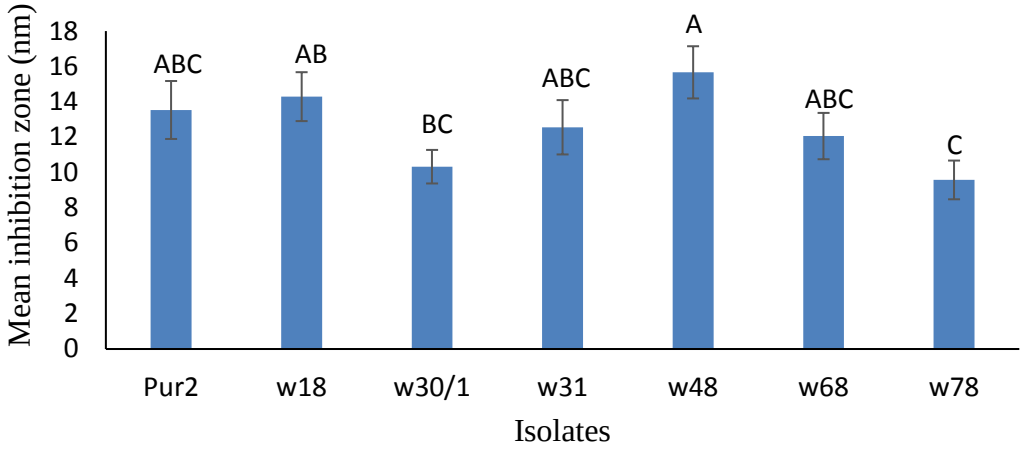


Fig. 5.4 Mean (±SE) zone inhibition of the *P. temperata* isolates against *Beauveria bassiana* (two trials) (Oneway ANOVA, $F_{6,35}$ = 4.99, *P* = 0.001). Bars with the same letters are not significantly different (Tukey's test)

Also for *Metarhizium anisopliae*, General linear model analysis (GLM) showed there was no interaction between isolate and trial ($F_{6, 28}$ = 0.92, *P* = 0.497). Therefore the data for both trials were combined for analysis using one-way ANOVA. There were significant difference in the inhibition zone among the isolates for *Metarhizium* 52 ($F_{6,35}$ = 8.59, *P* < 0.001), especially for the isolate W78 the zone of inhibition was low against *Metarhrizium* 52 compared with other isolates. None of the isolates differed from Pur2 (Fig 5.5).

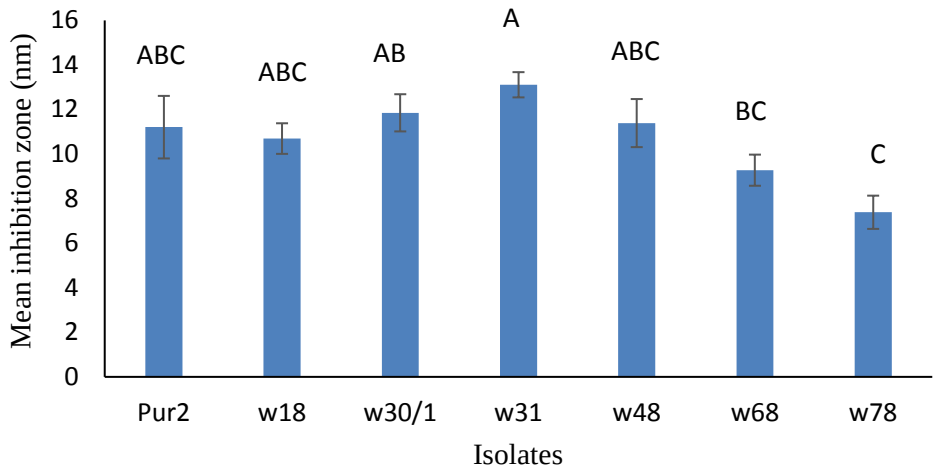


Fig. 5.5 Mean (±SE) zone of inhibition of the *P. temperata* isolates against *Metarhizium* 52 fungus (two trials), there was a significant different a between isolates. One-way ANOVA ($F_{6,35} = 8.59$, $P < 0.001$). Bars with the same letters are not significantly different (Tukey's test)

5.3.2 RP-HPLC of culture supernatants from *Photorhabdus* isolates

RP-HPLC analysis was used to compare the profile of secreted metabolites among the six Bull Island isolates *P. t. cinerea* Purple and *P. t. temperata* Yellow (Fig 5.6). All wavelengths were reviewed but no clear grouping of the different strains was apparent from the HPLC analysis. Many of the detected molecular species appeared to originate from the media, as observed in the negative control (green signal; Fig 5.6). Using RP-HPLC, no differentiation can be made between the two analysed subspecies, indicating that any differentially produced metabolites may be intracellular or at levels below the sensitivity of the methodology and equipment used at Maynooth University.

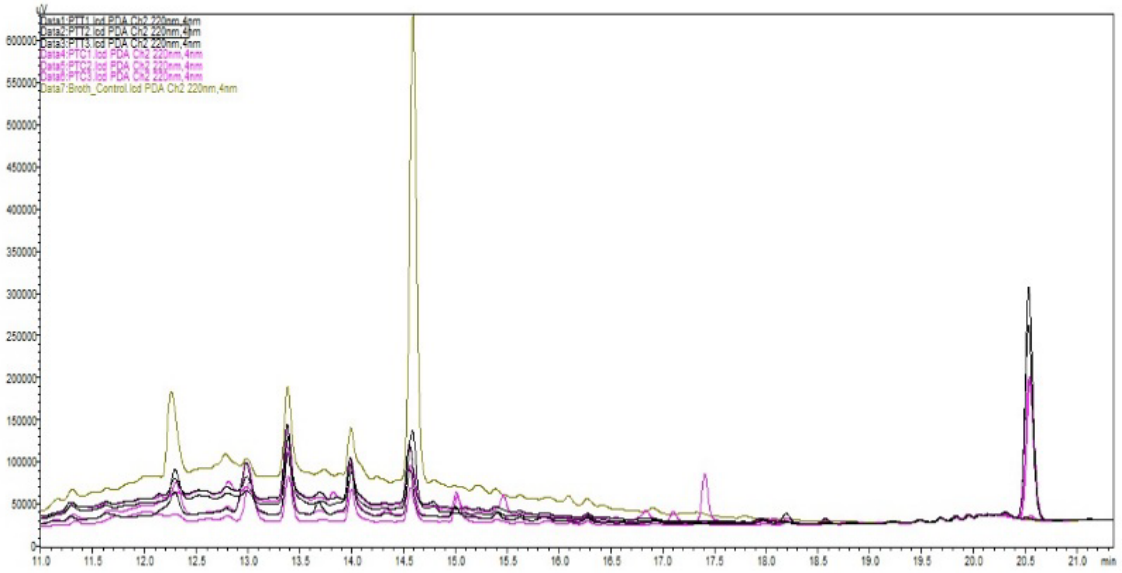
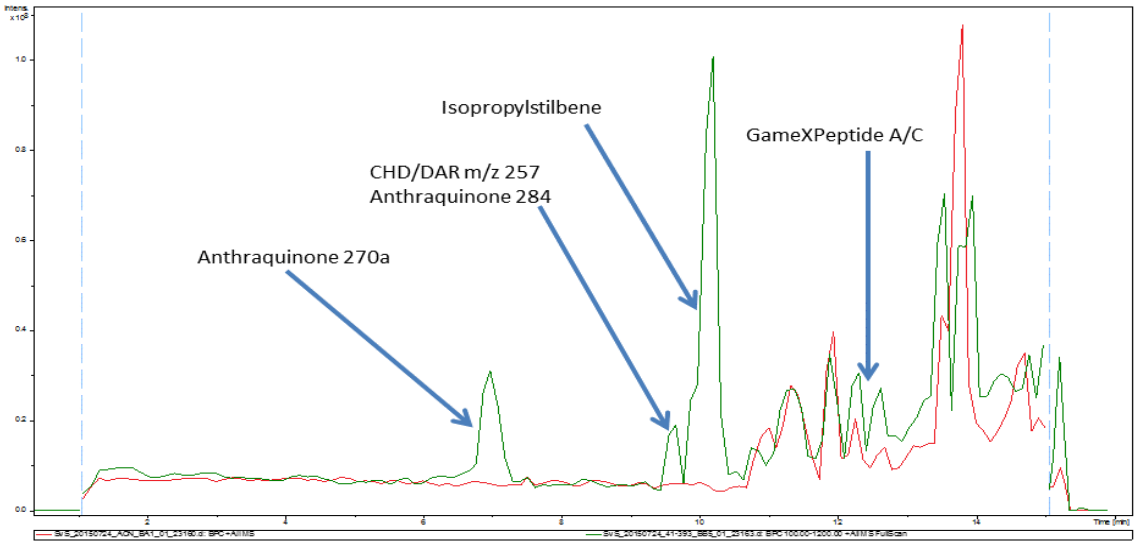


Fig. 5.6 RP-HPLC analysis showing culture supernatants from 6 *Photorhabdus* isolates overlaid. *P. t. cinerea* Purple isolates are indicated by purple trace and *P. t. temperata* Yellow isolates are in black. The green trace represents the culture media negative control. Wavelength shown is 220 nm.

Following the failure to detect metabolites in culture supernatant at Maynooth University, samples of each of the six Bull Island isolates were sent to Prof Helge Bode of Frankfurt University for analysis. Analysis at Frankfurt University was more successful. Representative chromatographs are shown in Fig 5.7. Nine metabolites were identified (Table 5.2). Isopropylstilbene was produced by all six isolates. Mevalagmapeptide A was produced by some but not all isolates, of both subspecies. Four metabolites (GameXPeptide A, GameXPeptide C, anthraquinone 284 and anthraquinone 270a) were produced by all *P. t. temperata isolates*, but not by any *P. t. cinerea* isolates, and one metabolite (dihydro-isopropylstilbene) was produced by all *P. t. cinerea* isolates, but not by any *P. t. temperata* isolates. The structures of the metabolites are shown in Fig 5.8.

Representative overview: *P. t. temperata-yel* sample 393 (green), blank (red)
Base peak chromatogram Rt=1-15min m/z=100-1200



Representative overview: *P. t. cinerea-pur3 (2)* (blue), blank (red)
Base peak chromatogram Rt=1-15min m/z=100-1200

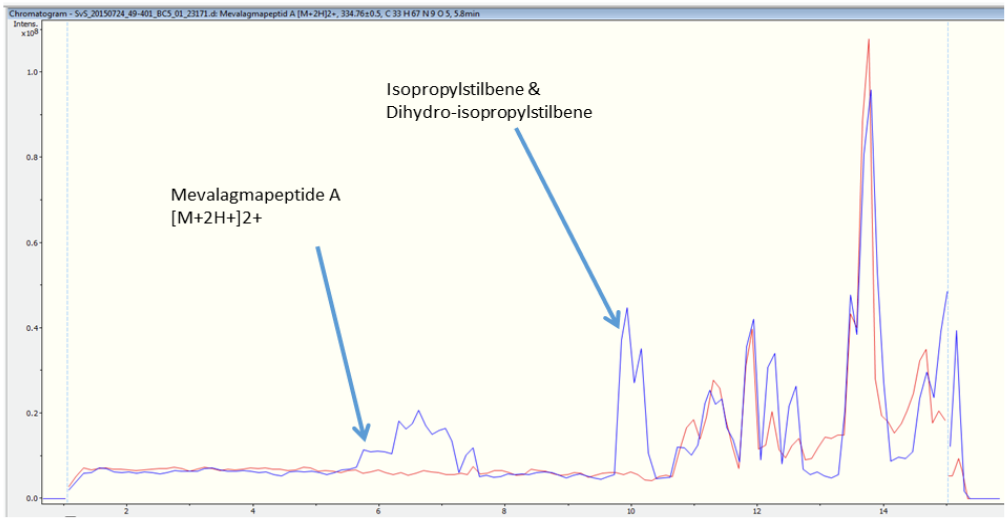


Fig. 5.7 Representative chromatographs of *P. t. temperata* (yel1) and *P.t. cinerea* (pur3)

Table. 5.2 Metabolites identified in culture supernatant of *P. t. temperata and P. t. cinerea* isolates (two replicate cultures of each isolate). m/z is the mass/charge ratio.

Metabolite	*P. t. temperata*						*P. t. cinerea*					
	Yel1		Yel2		Yel3		Pur1		Pur2		Pur3	
	1	2	1	2	1	2	1	2	1	2	1	2
GameXPeptide A	x	x	x	x	x	x						
GameXPeptide C	x	x	x	x	x	x						
isopropylstilbene (m/z 253 [M-H]-; m/z 255 [M+H]+)	x	x	x	x	x	x	x	x	x	x		x
dihydro-isopropylstilbene (m/z 255 [M-H]-; 27 7[M+H]+)							x	x	x	x	x	x
cyclohexandione/dialkylresorcinole m/z 257 [M+H]+	x	x	x	x	x	x						
Mevalagmapeptide A [M+H]+	x					x				x		x
Mevalagmapeptide A [M+2H]2+	x					x	x		x	x		x
Anthraquinone 284 (m/z 285 [M+H]+)	x	x	x	x	x	x						
Anthraquinone 270a (m/z 269 [M-H]-)	x	x	x	x	x	x						

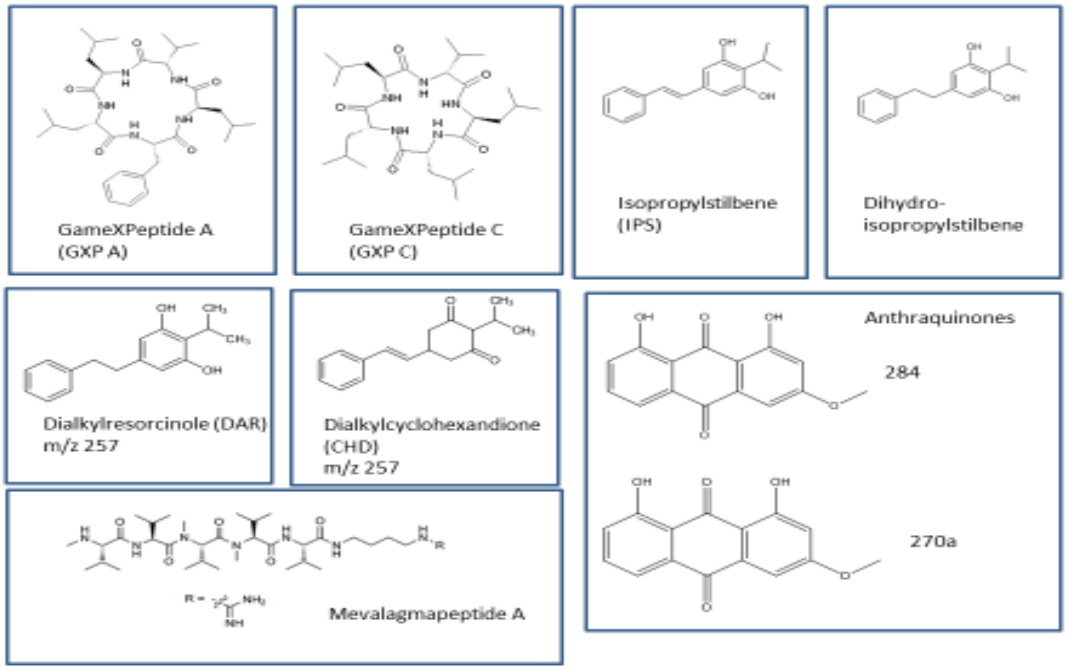


Fig. 5.8 Structures of the metabolites identified in culture supernatant of *P. t. temperata and P. t. cinerea* isolates.

5.3.3 Bioluminescence

When the average light intensity of cadavers infected by *Photorhabdus* was measured 3, 4 or 5 days post-infection, there was a highly significant difference between isolates on each day (Mann Whitney U test, $P < 0.001$ in each case). *P. t. temperata* yel3 was always brighter than *P.t. cinerea* pur1 (Fig.5.9). There was a decline in light intensity with time, especially for Pur1.

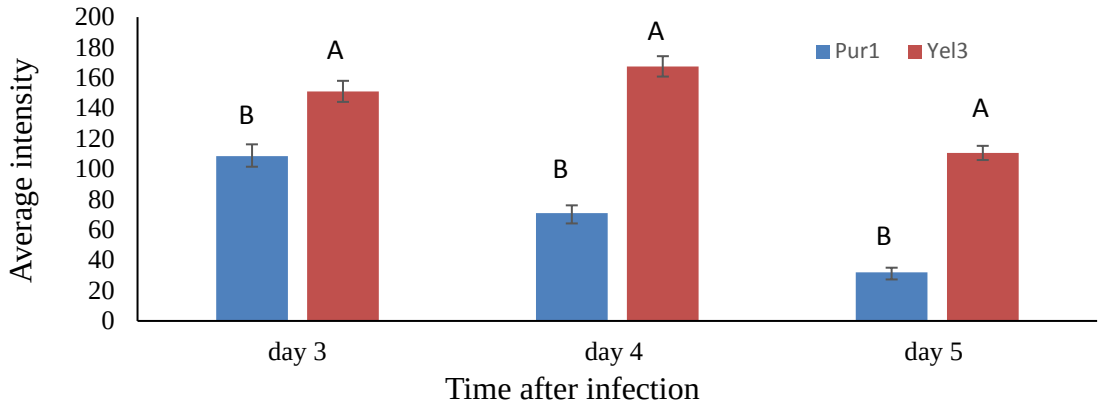


Fig. 5.9 Mean (+/- SE) light intensity of cadavers infected by *P. t cinerea* Pur1 and *P. t. temperata* Yel3 measured 3, 4 and 5 days post infection with Heterorhabditis downesi IJs. Within each day, bars accompanied by the same letter are not significantly different (Mann Whitney U test)

The emission spectra for Pur1 and yel3 differed slightly. Pur1 appeared to have two peaks, one at about 480 nm and one at about 510 nm, while yel1 tended to have a single peak at about 510 nm. (Fig 5.10**)**. The wavelength at which light emission peaked for each cadaver on days 3, 4 and 5 after infection is shown in Fig 5.11. This shows that cadavers infected with Yel3 always had maximum light emission at 505-510 nm. At day3, cadavers infected with Pur1 had a peak either at 475-480 or 495-510 but by day 3 all Pur1 cadavers peaked at 475-485. Cadaver appearance on 3, 4 and 5 days after infection is shown in Fig 5.12. Cadavers infected with *P.t. cinerea* Pur1 were colourless at the time of death and then became deep purple by Day 5 after infection. Cadavers infected with *P.t. temperata* Yel3 were orange on day 3, and bacame yellow by day 5 (Fig.5.12).

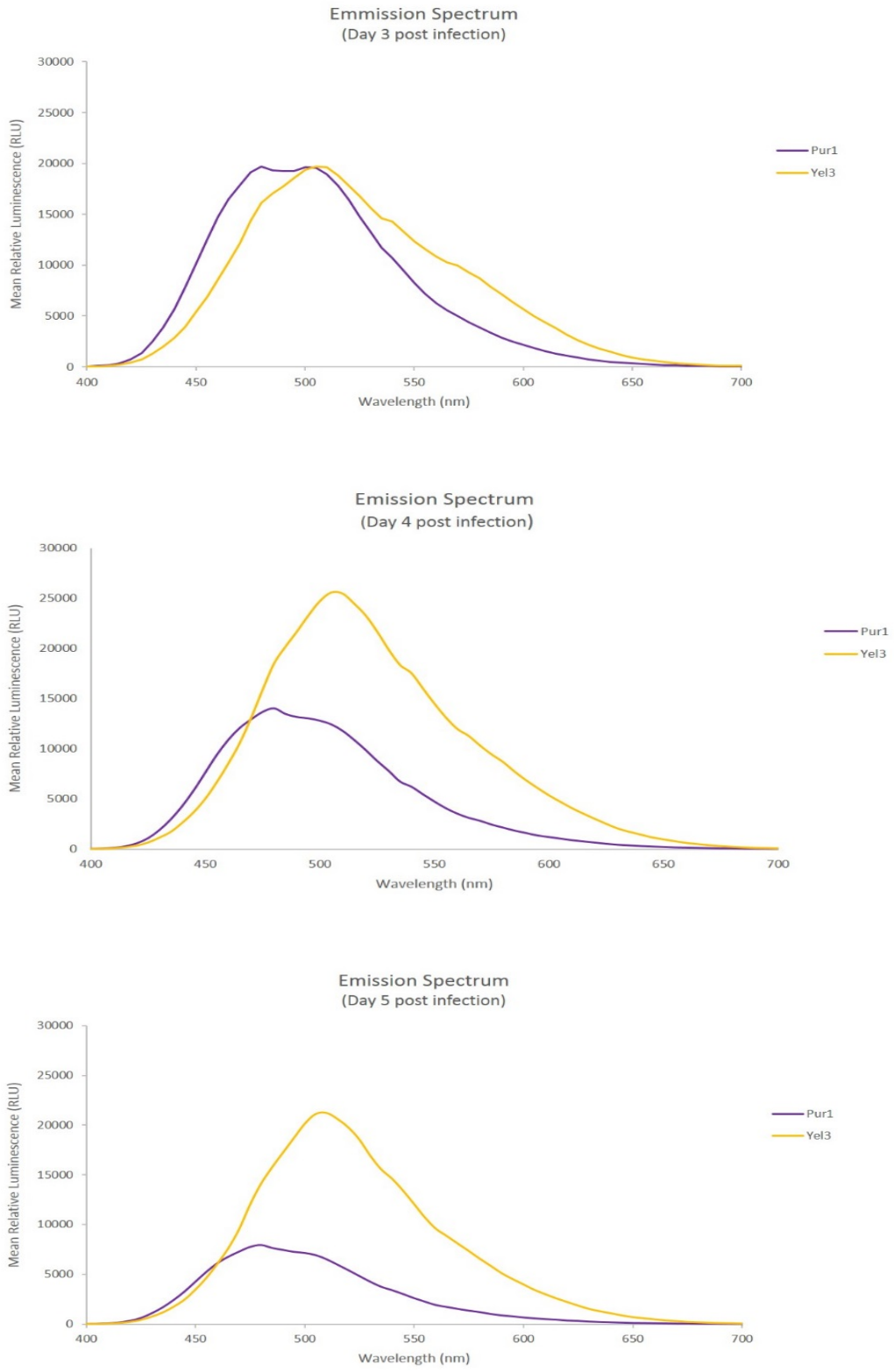


Fig. 5.10 Emission spectra for bioluminescence from cadavers infected with either *P.t. cinerea* (pur1)or *P.t. temperata* (yel3) 3,4 or 5 days post infection. Each line is mean of 24 cadavers.

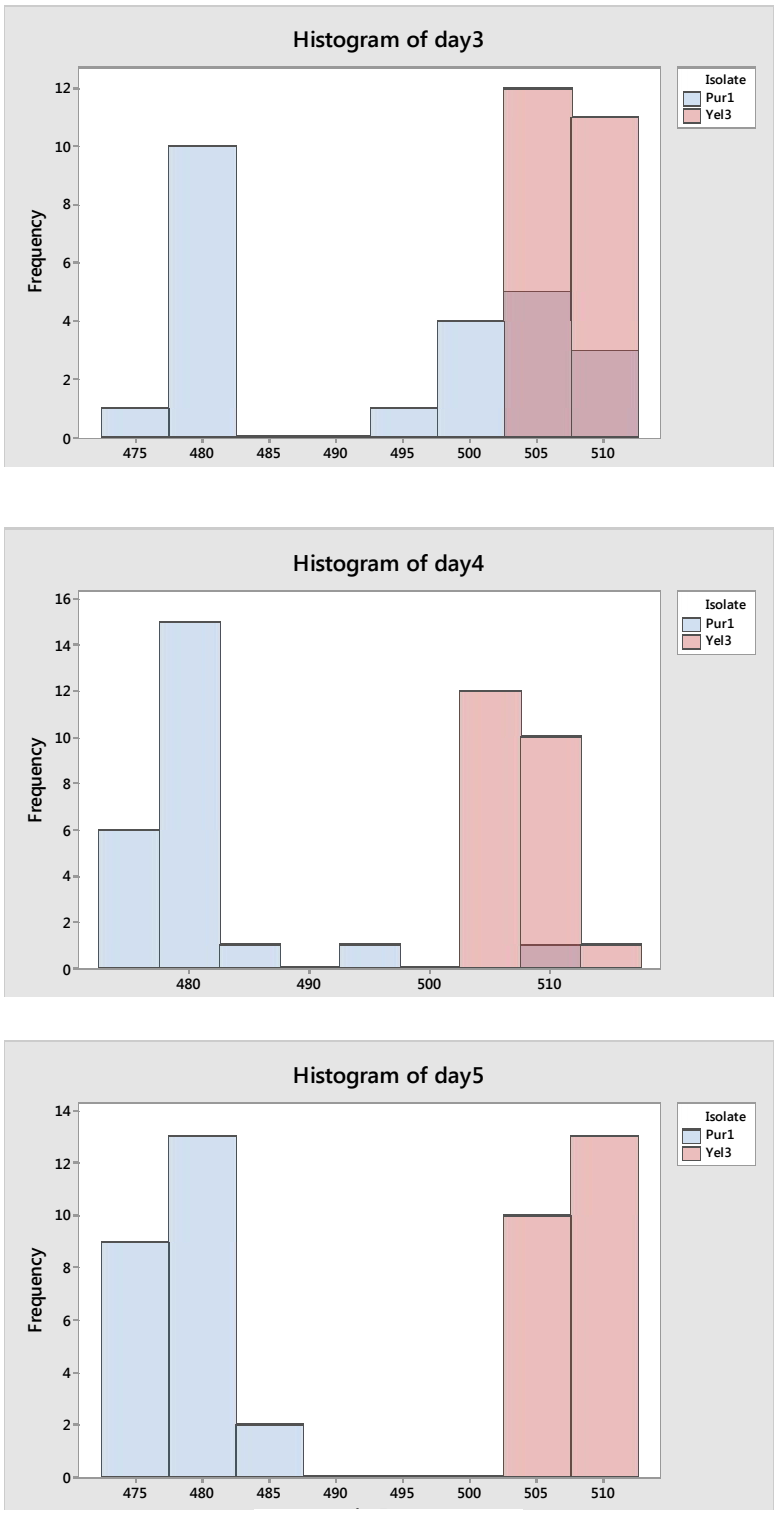


Fig. 5.11 Histograms showing the wavelength at which the highest light intensity was recorded for each cadaver, measured 3, 4 and 5 days after infection. Cadavers were infected with either *P.t. cinerea* Pur1 or *P.t. temperata* Yel3.

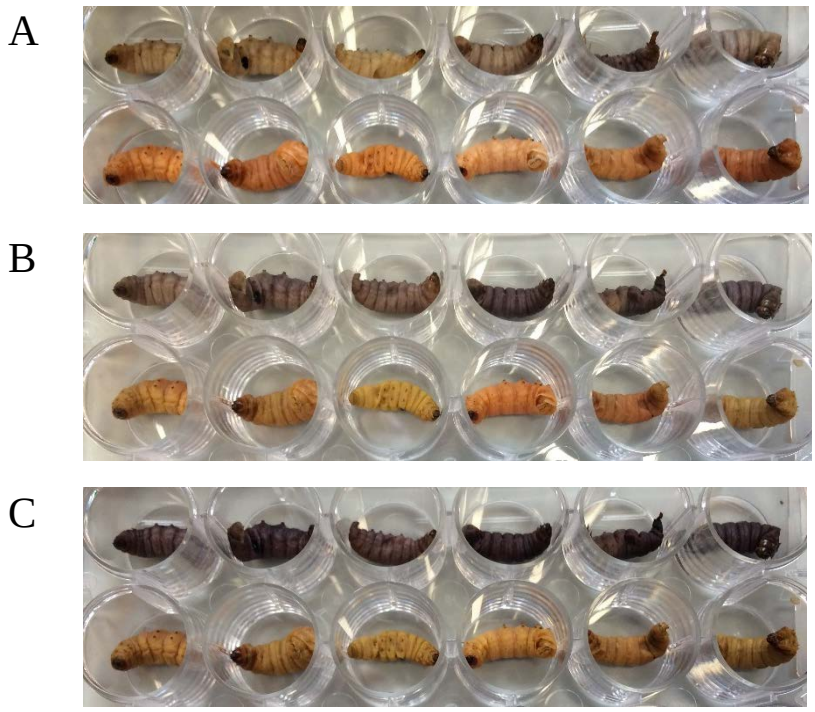


Fig. 5.12 Cadaver appearance on 3 (A), 4 (B) and 5 (C) days after infection. Upper row cadavers were infected with *P.t. cinerea* Pur1, lower row *P.t. temperata* Yel3 in each case.

5.4 Discussion

All six Welsh isolates of *P. t. cinerea* showed antibacterial and antifungal activity, unlike the W26/2 isolate tested by Flynn (2016). Therefore, the finding by Flynn was due to the particular isolate W26/2 that she used, and was not a feature of Welsh isolates of *P.t. cinerea* in general. There was some variation in performance of the isolates. For example, W48 was relatively weak against *E. coli* and W78 tended to be poor against the fungi. However, in general, the Welsh isolates were as good as Bull Island isolate pur2, and sometimes better than it. This indicates that the relatively good antibiotic performance of *P.t. cinerea* demonstrated by Maher (2014) is probably a feature of the subspecies and not just the Bull Island isolates that she tested.

Nine secondary metabolites were identified in culture filtrate of the Bull Island *Photorhabdus* isolates. Only one of these, isopropylstilbene, was produced by all six isolates. One (dihydro-isopropylstilebene) was produced by all three pur isolates but not by the yel isolates. Five metabolites were produced by all three yel isolates but not by pur. These were two GameXPeptides, two anthraquinones and cyclohexandione/dialkylresorcinole. Mevalagmapeptides were produced in small amounts by some isolates of each subspecies.

Stilbenes are the major antibiotic molecules produced by *Photorhabdus* spp in laboratory culture (Li *et al.*, 1995, Hu *et al.*, 1997, Richardson *et al.*, 1988, Hu and Webster, 2000, Hu *et al.*, 2006). Stilbenes are simple molecules with complex ecological functions (Bode, 2009). They have antibiotic activity against Gram-positive bacteria and fungi and also inhibit phenol oxidase, which is part of the insect immune system (Eleftherianos *et al.*, 2007). Isopropylstilbene was produced by both yel and pur isolates. This is a major secondary metabolite produced by all strains of *Photorhabdus* investigated by Joyce et al. (2008). It also acts as a signal molecule for recovery of *Heterorhabditis* IJs to normal development (Joyce *et*

al., 2008). In addition, pur isolates produced a second stilbene, dihydro-isopropylstilbene. This compound was shown to have strong antifungal activity against several fungal species (Li *et al.*, 1995) and also to be nematicidal (Hu *et al.*, 1999). It is not possible to conclude whether this stilbene is responsible for the greater antibiotic activity of pur isolates relative to yel isolates demonstrated by Maher (2014) and Flynn (2016). Both subspecies were active against all bacterial and fungal species tested. It may also be that pur isolates produce greater quantities of stilbenes, and that accounts for their higher activity.

Another major group of secondary metabolites produced by *Photorhabdus* spp are the anthraquinone pigments (Hu *et al.*, 1998, Li *et al.*, 1995, Richardson *et al.*, 1988). These are responsible for the yellow-red colour of most isolates and also have some antimicrobial activity (Li *et al.*, 1995). It is hypothesized that the anthraquinones are responsible for deterring birds, ants and other scavengers (Bode, 2009, Vizcaino *et al.*, 2014), based on a similar role for these substances in insects (Pankewitz and Hilker, 2008). Anthraquinones were not detected in the culture supernatant of pur isolates and the absence of yellow or red pigmentation from cadavers infected with these isolates or other isolates of *P.t. cinerea* suggests that anthraquinones are not produced in vivo, either. It is worth investigating how well insects infected with *P. t. cinerea* deter scavengers.

Yel isolates produced cyclohexandione/dialkylresorcinol. These molecules were shown to have a role in cell-to-cell communication (quorum sensing) in *P. asymbiotica* (Brameyer *et al.*, 2015). Brameyer et al. (2015) analysed over 90 different strains of *Photorhabdus* by HPLC/MS, and these molecules were only produced by *P. asymbiotica* (Brameyer *et al.*, 2015). Therefore, it is unexpected to find them produced by *P.t. temperata*, but not by *P.t. cinerea* which is more closely related to *P. asymbiotica* (Ferreira *et al.*, 2014).

Yel isolates also produced GameXPeptide A and GameXPeptide C. These are cyclic peptides (Table 5.2.) first described by Bode et al. (2012). Bode et al (2012) described four GameXPeptides A, B, C and D produced by *P. luminescens* TT01 in liquid culture. Later, four more GameXPeptides were identified when TT01 was grown in *G. mellonella* (Nollmann *et al.*, 2015). Also, it was reported that most strains of both *Photorhabdus* and *Xenorhabdus* can produce GameXPeptides (Nollmann *et al.*, 2015). GameXPeptides showed no antibacterial activity, through GameXPeptides A, B and D showed anti-*Plasmodium* activity (Challinor and Bode, 2015).

Mevalagmapeptide A was detected in some yel and pur isolates, but not always in both replicates. Mevalagmapeptides are linear peptides (Fig.5.8) recently identified in *P. luminescens* (Challinor and Bode, 2015).

Cadavers infected with *P.t. temperata* yel3 were brighter (emitted light more intensely) than cadavers infected with *P.t. cinerea* pur1. The difference may be partly due to differences in the purity of the *Photorhabdus* culture within the insect, but since the pur isolates have greater antibiotic potential than the yel isolates (Maher, 2014), it is expected that pur cadavers would be more purely *Photorhabdus* than yel cadavers, and so should be brighter on that basis. The difference in intensity between subspecies is in agreement with findings by Hyrsl et al (2004). They measured bioluminescence intensity in various species of *Photorhabdu*s, and showed that *P. temperata* exhibited higher intensity than the *P. asymbiotica* clade. Bull Island subspecies belong to different clades: *P. t.. temperata* belongs to the *P. temperata* clade while *P. t. cinerea* belongs to the *P. asymbiotica* clade (Ferreira *et al.*, 2014).

Luminous bacteria including *Photorhabdus* all have the same emission spectrum, centred around a wavelength of 490 nm (Hyrsl *et al.*, 2004, Peat and Adams, 2008), but in

some cases, the emission is modified by the presence of other molecules (Haddock *et al.*, 2010). In this experiment, almost no cadaver infected with *Photorhabdus* had a maximum at 490 nm. Cadavers infected with yel3 always had a higher maximum (505-510 nm). Some cadavers infected with pur1 had similar high maximum on day 3 but by five days after infection, maximum emission of all pur1 cadavers was 485 nm or lower. This must be due to the presence of different molecules including pigments within the cadavers, and to changes in their expression over time, as illustrated in Fig 5.12.

The function of bioluminescence in *Photorhabdus* is not known. Peat and Adams (2008) review the possible functions which include mopping up oxygen which allows the bacteria to outcompete obligate aerobic bacteria, or stimulation of DNA repair. Ecological functions that have been proposed include scavenger deterrence (Baur *et al.*, 1998), and there is some evidence of this for *P. t. temperata* strain K122 (Chuche et al. unpublished). The higher light intensity of yel3 may make it more efficient at scavenger deterrence than pur1. It is unclear whether the difference in emission spectra would also have an effect on scavenger deterrence. It would be important to carry out similar tests with the other Bull Island isolates to confirm that the differences detected between pur1 and yel3 are characteristic of the subspecies and not just these two isolates.

Chapter 6

General Discussion

The overall aim of this project was to explore the relationship between *H. downesi* and the two symbionts that it carries on Bull Island, and the relationship (co-existence and competition) between the two symbionts. It was previously thought that the relationship between EPN and their symbionts was very specific – each nematode can carry only one bacterial species (Adams *et al.*, 2006). This still seems to be mainly true for *Steinernema-Xenorhabdus* (Murfin *et al.*, 2015), but it is becoming clear that the relationship is not so specific for *Heterhabditis-Photorhabdus*, as shown in Table 1.2 Rolston et al. (2005) found that on Bull Island, *H. downesi* carried two phenotypically different types of *Photorhabdus* ("yellow" and "purple"). Maher (2014) identified the yellow as *P.t. temperata* and the purple as *P.t. cinerea*. In both studies *P.t. cinerea* dominated in the dry area at the front of the dunes (Maher, 2014, Rolston *et al.*, 2005). These same two *Photorhabdus* subspecies are also associated with *H. downesi* in Poland, where they are also found to be co-existing at the same sites (Toth and Lakatos, 2008, Kazimierczak *et al.*, 2017). Although they are both classified as subspecies of *P. temperata*, a multi-gene sequencing approach shows that *P. t. cinerea* is more closely related to *P. heterorhabditis* than to *P. temperata* (Ferreira *et al.*, 2014) and so the two symbionts associated with *H. downesi* are more likely to be two different species. There are two main questions, which are linked to each other: what are the advantages for *H. downesi* of associating with one or the other symbiont, and how do the symbionts compete with each other?

In chapter 3, it was shown that under normal (moist) conditions, *H. downesi* isolates carrying either yel or pur bacteria produced equal number of IJs, indicating that both symbionts

are equally good at turning the insect into food for the nematodes. *H. downesi* yel and pur isolates also carried equal numbers of their symbiont, and so they should deliver the same dose of bacteria to an insect when they invade. Maher (2014) previously showed that *H. downesi* yel and pur isolates were equally effective at killing four different species of insect. There were sometimes differences in the speed of kill of *G. mellonella* larvae infected with yel and pur isolates, but these differences were small and were not consistent. So in terms of the main functions of killing an insect and turning it into food, both symbiont subspecies seem to be equally good as a partner for *H. downesi*. However, under dry conditions, cadavers infected with pur isolates produced more IJs than those infected with yel isolates, indicating that associating with *P.t. cinerea* may be an advantage to *H. downesi* in places with dry soil, such as the poorly vegetated front part of the dunes on Bull Island. In terms of the water loss it was shown that the *P.t. cinerea* slows the rate at which water is lost from the cadaver. This could give more nematodes the chance to develop to the IJ stage, which is the resistant stage (Shapiro-Ilan *et al.*, 2014), before the cadaver becomes too dry. Or it could give the IJs more time to prepare physiologically for drying (Grewal *et al.*, 2006). It would be interesting to investigate how *P.t. cinerea* slows the rate of water loss from cadavers: perhaps through production of different exopolysaccharide molecules (Greene *et al.*, 2016, Rossi *et al.*, 2012, Kondakova *et al.*, 2015), as suggested in Chapter 3, or by changing the properties of the cuticle to make it less permeable to water.

By its partnership with *P. t. cinerea*, *H. downesi* can extend its niche into drier parts of the dune system on Bull Island. Bacterial symbionts are increasingly recognised as mediators of ecologically important traits of animals, with significant involvement in the host's adaptation to its niche (Douglas, 2009, Feldhaar, 2011, Duperron *et al.*, 2013, Thrall *et al.*, 2007). Where horizontal transfer is possible, as in *Heterorhabditis*, uptake of a new symbiont may result in the acquisition of new traits (Oliver *et al.*, 2010, Henry *et al.*, 2013). In aphids, horizontal

transfer of facultative symbionts is associated with aphid lineages colonizing new ecological niches, including novel host plant species and climatic regions (Henry *et al.*, 2013). The possibility of symbiont acquisition facilitating niche expansion has received little attention in entomopathogenic nematodes previously.

P.t. cinerea protected IJs from desiccation in cadavers better than *P.t. temperata*. Therefore, the hypothesise was if wax-moths were co-infected with both symbiont subspecies and then dried, more IJs would emerge from cadavers in which *P.t. cinerea* predominated than from those in which *P.t. temperata* predominated, and therefore the proportion of IJs carrying *P.t. cinerea* in the population should increase over several selection rounds. This might be what happens under natural conditions at Bull Island to create the observed distribution with *P.t. cinerea* in the drier, front part of the dunes. The hypothesis was tested by co-infecting wax-moths with IJs carrying yel and pur IJs and reared them under desiccating or normal conditions for three generations. The phenotype of the bacteria carried by IJs from the first and third selection round (Chapter 4) was assessed. The results of the experiment did not support the hypothesis. *P.t. cinerea* dominated in two of the three combinations of strains in the first selection round. In the third selection round, *P.t. cinerea* predominated in all three strain combinations. The prediction was that *P.t. cinerea* would predominate only in cadavers that were kept in dry conditions, but *P.t. cinerea* predominated under both wet and dry conditions. This may suggest that *P.t. cinerea* is outcompeting *P.t. temperata* in the insect host, either through its growth rate or by being better at colonising the developing nematodes. This is different to the results reported by Maher (2014) who found that more of the IJs emerging from co-infected hosts carried *P.t. temperata*. Maher used only the pur1/yel3 pair of strains. In this experiment one of the pairs were pur predominated even at the first selection round. Differences in methodology between the two experiments may have contributed to the difference in results

between the two studies. Differences in the bacteria themselves may also have contributed. Bacteria may deteriorate or change in other ways over time in laboratory culture (Hopper *et al.*, 1993, Bilgrami *et al.*, 2006, Blackburn *et al.*, 2016). It may also be that even a small difference in the proportions of the yel/pur present in the cadaver at the start of the infection may give an advantage to the strain that is present in higher numbers. I had hoped to be able to quantify the proportions of each symbiont subspecies present in the cadavers through developing PCR tools, but unfortunately this was not successful (Chapter 4). The co-infection experiment could be repeated if reliable quantitative PCR methods are developed for this pair of symbionts.

A second co-infection experiment with one pair of isolates was carried out, and looked at colonisation of mother-worms and their IJs. So equal numbers of mother-worms were found colonised by *P. t cinerea* and *P.t. temperata* (as judged by crushing and plating the IJs that developed inside them) (Chapter 4). Interestingly, in this experiment nearly all of the mother worms had only one or the other symbiont. Only 12% of the mother worms carried both symbiont subspecies. It is not clear why so few of them carried both symbionts. Colonisation of *Heterorhabditis* by *Photorhabdus* begins with colonisation of the mother and then the pre-IJs developing inside her (Ciche *et al.*, 2008). It may be that the two symbiont subspecies compete to colonise the mother worm's rectal gland cells, and one subspecies inhibits the other. Interestingly, when a choice was given to the symbiont on agar plates, first generation *H. downesi* preferred *P.t. cinerea* and fed in the segments of the plate where this subspecies was present (Chapter 4). If a mother-worm feeds entirely on one symbiont then she can only be colonised by that symbiont, and all the IJs that develop inside her will also be colonised by that symbiont. The feeding choice experiment was artificial, with each symbiont in a separate region of a plate. It is possible that IJs carrying different symbionts might invade a host at different ends of the insect. In that case each symbiont could colonise a different region of the

insect, and the mother nematodes could choose which bacteria to feed on. But it is more likely that the two symbionts are more evenly mixed within the insect cadaver.

The fact that *H. downesi* prefer to feed on *P.t. cinerea* raises some interesting questions. Firstly, is it the IJ or the developing adult that chooses? IJs of EPN respond to chemical cues associated with their hosts, and use these cues to find hosts and to decide whether to enter or not (Lewis *et al.*, 2006, Ramos-Rodríguez *et al.*, 2007, Dillman *et al.*, 2012). There is little known about responses of EPN adults to chemical cues, apart from the fact that male *Steinernema* are attracted to female sex pheromones (Lewis and Shapiro-Ilan, 2002). In the preference experiment, very little movement of nematodes was observed either within or between sections of the plates once the nematodes were large enough to be observed, and there were not many nematode trails between segments. Therefore, it seems likely that the choice was made by the IJs, and the adults stayed where they were. The second question is, why do the IJs prefer *P.t. cinerea*? Both symbiont subspecies seemed to be equally good at providing food for reproduction of *H. downesi* as shown in Chapter 3. Hillman and Goodrich-Blair (2016) propose that when microbes benefit their host by killing competitors, predators or parasites, natural selection should favour the transmission of microbes with the most beneficial traits, and the host's ability to pre-assess a symbiont's beneficial traits would be a selective advantage. Normally the recognition would be at the level of the bacterial surface interacting with host cells (Easom *et al.*, 2010, Hillman and Goodrich-Blair, 2016), for example, recognition between *Photorhabdus* and the rectal gland cells in *Heterorhabditis* (Ciche *et al.*, 2008), but in this experiment there seems to be recognition at the behavioural level also.

There was little evidence that one symbiont subspecies or the other suppressed the other when grown together in liquid culture (Chapter 4). This indicates that they are not producing bacteriocins such as lumicins that are active against related species or subspecies (Sharma *et*

al., 2002, Ciezki *et al.*, 2017). In one of the three yel-pur pairs tested, pur (*P.t. cinerea*) dominated, but not in the other two pairs, indicating that dominance over *P.t. temperata* is not a feature of the subspecies in general. Maher (2014) showed that *P.t. cinerea* from Bull Island produced a wider zone of inhibition against bacteria and fungi than *P.t. temperata*. This was also shown for another pair of *P.t. cinerea* and *P.t. temperata* isolates from Cork in Ireland but was not shown for a pair of isolates from Wales where the *P.t. cinerea* showed no inhibition (Flynn, 2016). However, it was confirmed that the Welsh isolates of *P.t. cinerea* have good antibiotic properties (Chapter 5). The main antibiotics produced by *Photorhabdus* are stilbenes which are active against Gram-positive bacteria and fungi (Paul *et al.*, 1981, Richardson *et al.*, 1988, Li *et al.*, 1995) and also inhibit the insect immune system (Eleftherianos *et al.*, 2007). All six Bull Island *Photorhabdus* isolates produced isopropylstilbene. The pur isolates also produced dihydro-isopropylstilbene (Chapter 5), and this may partly account for the greater antibiotic activity of *P. t. cinerea*. The yel isolates produced anthraquinones, which are pigments with some antimicrobial activity (Li *et al.*, 1995) and may also be the "scavenger deterrent" molecule responsible for deterring birds, ants and other scavengers (Pankewitz and Hilker, 2008) (Chapter 5). One *P. t. temperata* isolate, yel3, was found to bioluminescence more strongly than *P.t. cinerea* pur1, and the emission spectra of bioluminescence measured from infected cadavers differed between the two isolates (Chapter 5). Observations made throughout the project confirm that all three yel isolates glow more brightly than pur isolates when observed in the dark. The significance of bioluminescence in *Photorhabdus* is not known, though it is suggested to deter scavengers (Baur *et al.*, 1998).

In conclusion, two subspecies of *P. temperata* (which are probably more truly two separate species of *Photorhabdus*) occur together with *H. downesi*. They do not compete strongly against each other when grown together in liquid culture or in insects, but may

compete to be “packaged” by the nematodes. It seems that the main differences between the two subspecies relate to defence of the cadaver and of the nematodes inside it. *P.t. cinerea* gives better protection against desiccation and microbes. The results from Chapter 5 suggest that *P.t. temperata* might give better protection against scavengers (insects, birds or mammals) by anthraquinones and brighter bioluminescence. This should be tested. If this is found to be the case, then it could be that *P.t. cinerea* is favoured in conditions where desiccation and/ or microbes are the major threats, and *P.t. temperata* is favoured where scavengers are more of a problem.

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Appendix

Analysis of 2783 yel3 with Reference Genome Photorhabdus temperata subsp. temperata Meg1 (enterobacteria)

Pipeline completed.

Pipeline ID: `30531`

Date Completed: Wed, 17 Feb 2016 09:36

{pauljwalsh@gmail.com}

Disclaimer

The results presented in this report were produced by the tools listed below. The tools and report generation were managed by *Simplicity*™ [1].

Reads

Filename	2783-yel3_1_trimmed.fastq.gz	2783-yel3_2_trimmed.fastq.gz
Total Sequences	460,186	460,186
Sequence length	36-251	36-251
%GC	44	44
Encoding	Sanger / Illumina 1.9	Sanger / Illumina 1.9
Reads Library type		Paired ends

Table 1: Summary.

Reads Quality

FastQC [2] aims to provide a simple way to do some quality control checks on raw sequence data coming from high throughput sequencing pipelines. It provides a modular set of analyses, which you can use to give a quick impression of whether your data has any problems of which you should be aware before doing any further analysis.

Filename	2783-yel3_1_trimmed.fastq.gz	2783-yel3_2_trimmed.fastq.gz
Basic Statistics	PASS	PASS
Per base sequence quality scores	PASS	PASS
Per tile sequence quality	PASS	PASS
Per sequence quality	PASS	PASS

scores		
Per base sequence content	FAIL	FAIL
Per sequence GC content	PASS	PASS
Per base N content	PASS	PASS
Sequence Length Distribution	WARN	WARN
Sequence Duplication Levels	PASS	PASS

Table 2: Information on FASTQ files.

FastQC aims to provide a QC report, which can spot problems which originate either in the sequencer or in the starting library material. It is important to stress that although the FastQC analysis results appear to give a pass/fail result, these evaluations must be taken in the context of what you expect from your library. A *normal* sample as far as FastQC is concerned is random and diverse. Some experiments may be expected to produce libraries, which are biased in particular ways. You should treat the summary evaluations therefore as pointers to where you should concentrate your attention and understand why your library may not look random and diverse.

Per base sequence Quality: WARN = A warning will be issued if the lower quartile for any base is less than 10, or if the median for any base is less than 25. FAIL = This module will raise a failure if the lower quartile for any base is less than 5 or if the median for any base is less than 20.

Per tile sequence quality: WARN = A warning will be issued if any tile shows a mean Phred score more than 2 less than the mean for that base across all tiles. FAIL = This module will issue a warning if any tile shows a mean Phred score more than 5 less than the mean for that base across all tiles.

Per sequence quality scores: WARN = A warning is raised if the most frequently observed mean quality is below 27 - this equates to a 0.2% error rate. FAIL = An error is raised if the most frequently observed mean quality is below 20 - this equates to a 1% error rate.

Per base sequence content: WARN = This module issues a warning if the difference between A and T, or G and C is greater than 10% in any position. FAIL = This module will fail if the difference between A and T, or G and C is greater than 20% in any position.

Per base GC content: WARN = This module issues a warning it the GC content of any base strays more than 5% from the mean GC content. FAIL = This module will fail if the GC content of any base strays more than 10% from the mean GC content.

Per sequence GC content: WARN = A warning is raised if the sum of the deviations from the normal distribution represents more than 15% of the reads. FAIL = This module will indicate a failure if the sum of the deviations from the normal distribution represents more than 30% of the reads.

Per base N content: This module raises a warning if any position shows an N content of >5%. FAIL = This module will raise an error if any position shows an N content of >20%.

Sequence Length Distribution: WARN = This module will raise a warning if all sequences are not the same length. FAIL = This module will raise an error if any of the sequences have zero length.

Sequence Duplication Levels: WARN = This module will issue a warning if non-unique sequences make up more than 20% of the total. FAIL = This module will issue a error if non-unique sequences make up more than 50% of the total.

Sequence quality for 2783-yel3_1_trimmed

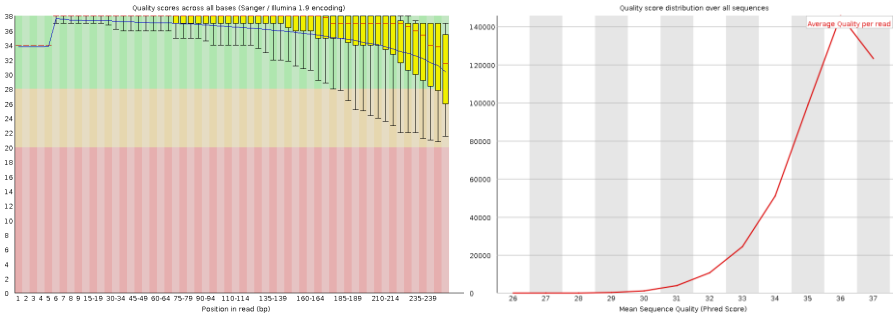

Figure 1: Per base (left) and sequence (right) quality for 2783-yel3_1_trimmed.

For each position a Box Whisker type plot is drawn for the per base quality (left). The elements of the plot are as follows:

- The central red line is the median value.
- The yellow box represents the inter-quartile range (25-75%).
- The upper and lower whiskers represent the 10% and 90% points.

The per sequence quality score report allows you to see if a subset of your sequences have low quality values. It is often the case that a subset of sequences will have poor quality, often because they are poorly imaged (on the edge of the field of view etc), however these should represent only a small percentage of the total sequences.

Sequence quality for 2783-yel3_2_trimmed

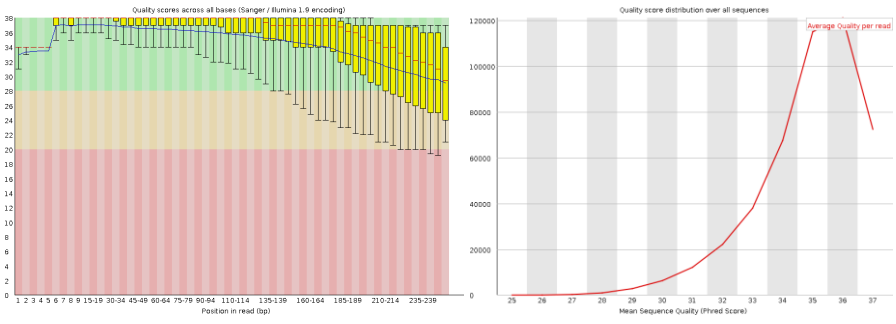

Figure 2: Per base (left) and sequence (right) quality for 2783-yel3_2_trimmed.

Read cleaning

The *PrinSeq* [3] ensures that the data used for downstream analysis is not compromised by low-quality sequences, PCR duplicates or sequence artifacts that might lead to erroneous conclusions. *PrinSeq* filtered out reads with a mean quality value lower than

10 and with any base quality value lower than **5**. It **removed** bases with ambiguity (Ns), **removed any PCR duplicates** and any reads with an **entropy value under 0.30**.

Adapter trimming

The adapter trimming stage was used to automatically detect and efficiently remove tag sequences (*e.g.* Adaptor or WTA tags) from genomic and metagenomic datasets. *TagCleaner* [4] was used to predict the potential adaptors in each file. **No adaptor or tag was detected**.

Trimmomatic [5] removed adapter sequences from DNA high-throughput sequencing data and also removes low quality regions of sequences while keeping paired end files synchronised. This is necessary when the reads are longer than the molecule that is sequenced and when sequence tags are present. Not removing adapter and sequence tags can hinder assembly, mapping of the reads and influence SNP calling and other downstream analyses. *Trimmomatic* [5] trimmed adapter sequences with a maximum allowed error rate of **10%** and a minimum length of trimmed reads of **0**.

Genome mapping

Genome mapping was performed by *Bowtie2* [6]. The *Bowtie* package enables ultrafast and memory-efficient alignment of large sets of sequencing reads to a reference sequence. The package contains tools for building indexes of reference genomes and for aligning short reads using the index as a guide. The **no** mismatches in seed alignment of **20**nt was allowed. Intervals between seed reads were *1* and *0.50*. For reads with repetitive seeds, try **3** sets of seeds. Give up extending after **20** failed extends in a row. The alignment was optimise for **local** alignment (ends might be soft clipped).

```
460088 reads; of these:
  454466 (98.78%) were paired; of these:
    445034 (97.92%) aligned concordantly 0 times
    9051 (1.99%) aligned concordantly exactly 1 time
    381 (0.08%) aligned concordantly >1 times
    ----
    445034 pairs aligned concordantly 0 times; of these:
      9872 (2.22%) aligned discordantly 1 time
    ----
    435162 pairs aligned 0 times concordantly or discordantly; of these:
      870324 mates make up the pairs; of these:
        867404 (99.66%) aligned 0 times
        1702 (0.20%) aligned exactly 1 time
        1218 (0.14%) aligned >1 times
  5622 (1.22%) were unpaired; of these:
    5359 (95.32%) aligned 0 times
    245 (4.36%) aligned exactly 1 time
    18 (0.32%) aligned >1 times
4.57% overall alignment rate
```

The average coverage across every base is 31.045 bases (Stdev 25.603).

The number of SNPs (single nucleotide polymorphisms) found was ** 1055** and the number of InDels (insertions and deletions) found was 8. Only gaps of less than 10 bases are considered for an insert or deletion.

Evaluate genome assemblies

The *QUAST* [7] program is used to evaluate assemblies. The scaffolds file generated by the assembly tool was evaluated.

Assembly	map_genome	map_genome broken
# contigs (>= 0 bp)	1	1
# contigs (>= 1000 bp)	1	1
Total length (>= 0 bp)	238875	238875
Total length (>= 1000 bp)	238875	238875
# contigs	1	1
Largest contig	238875	238875
Total length	238875	238875
GC (%)	42.82	42.82
N50	238875	238875
N75	238875	238875
L50	1	1
L75	1	1
# N's per 100 kbp	5124.02	5124.02

Table 3: Scaffolds were generated by the assembly tool and the information for this table was generated by QUAST.

GC (%) is the total number of G and C nucleotides in the assembly, divided by the total length of the assembly. N50 is the length for which the collection of all contigs of that length or longer covers at least half an assembly. N75 is defined similarly with 75% instead of 50%. L50 (L75) is the number of contigs as long as N50 (N75, NG50, NG75) In other words, L50, for example, is the minimal number of contigs that cover half the assembly. The scaffolds produced by assembly tool were combined to create a draft genome.

Genome finishing

CONTIGuator [8] performs a mapping step against the reference genome using the BLAST algorithm. The results are analysed taking into account the presence of more than one replicon, thus ensuring that no contigs are mapped to more than one replicon.

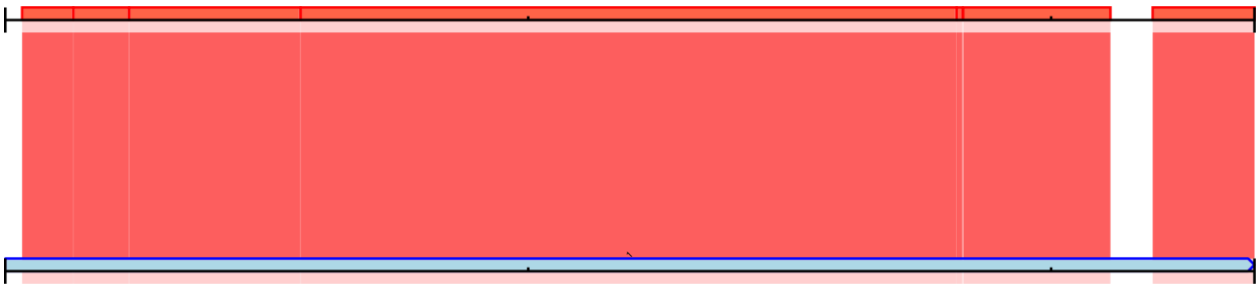

Figure 3: A image showing the reference genome on top with the mapped contigs underneath.

Gene prediction

GLIMMER [9] is a system for finding genes in microbial DNA, especially the genomes of Bacteria and Archaea. GLIMMER (Gene Locator and Interpolated Markov ModelER) uses interpolated Markov models to identify coding regions. The topology used was circular and the genetic code used was **11** (The Bacterial, Archaeal and Plant Plastid code).

Field name	Value
Genome size	238,875
Predicted genes count	229
Coding GC	45.7%

Table 4: Coding gene summary

Gene composition

EMBOSS CUSP [10] calculates a codon usage table from draft genome sequence.

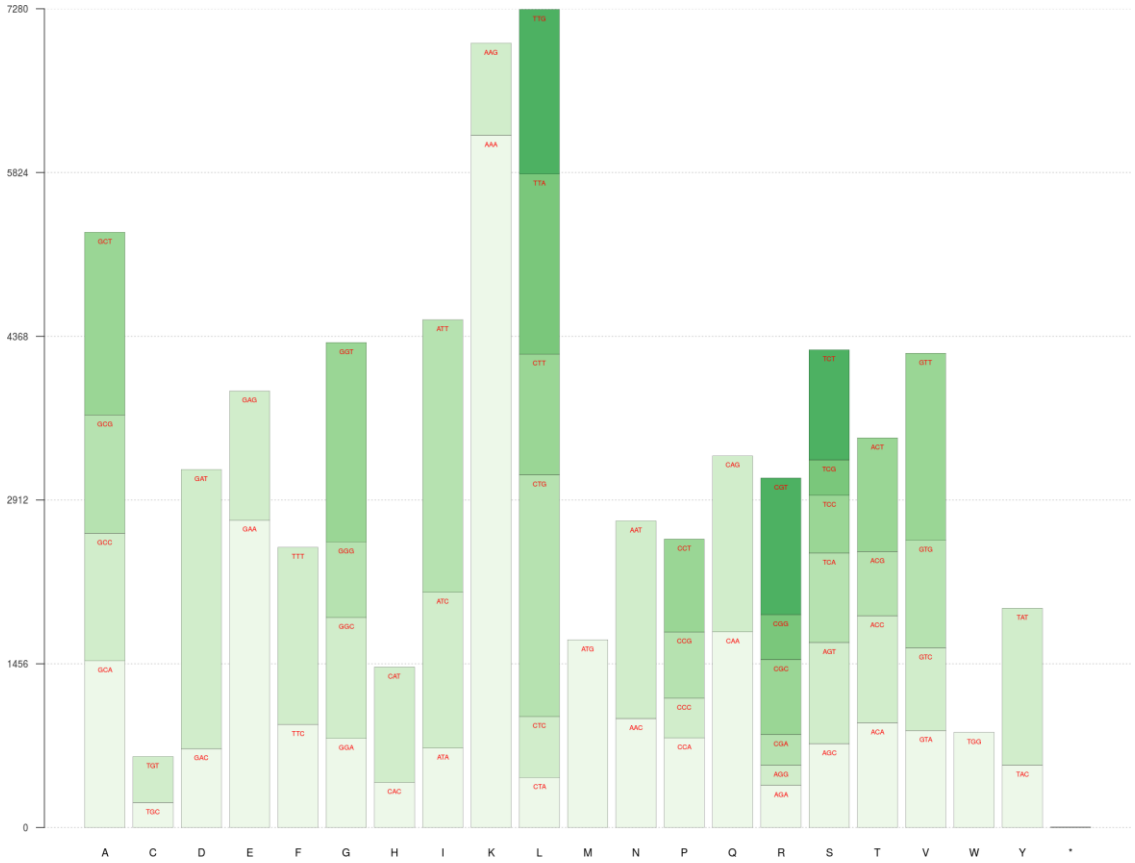

Figure 4: The image shows a summary of codon usage frequency.

Genome structure

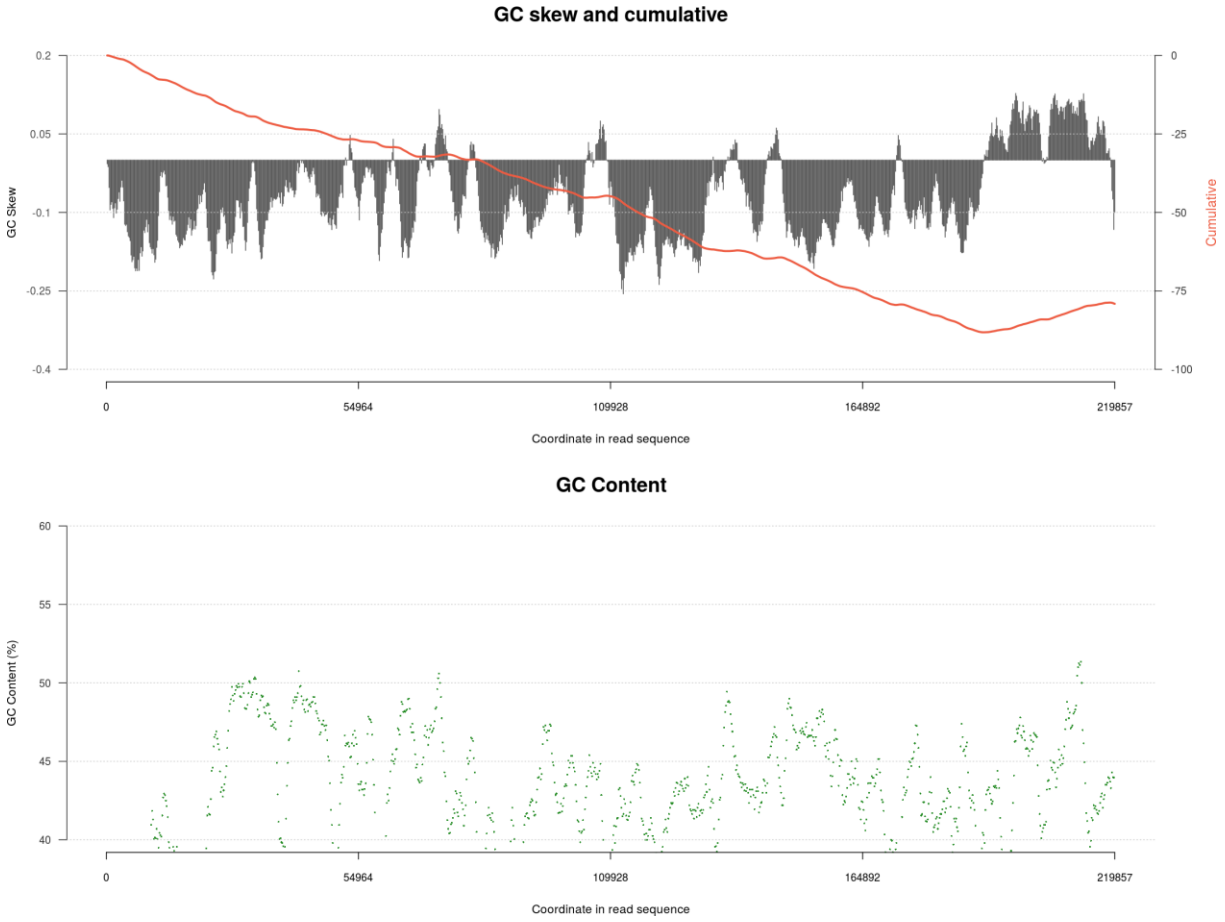

Figure 5: Summary of CG skew along the genome.

GView [11] is useful for producing high-quality genome maps for microbial genomes. The following image is a genome map produced by GView using the predicted ORFs.

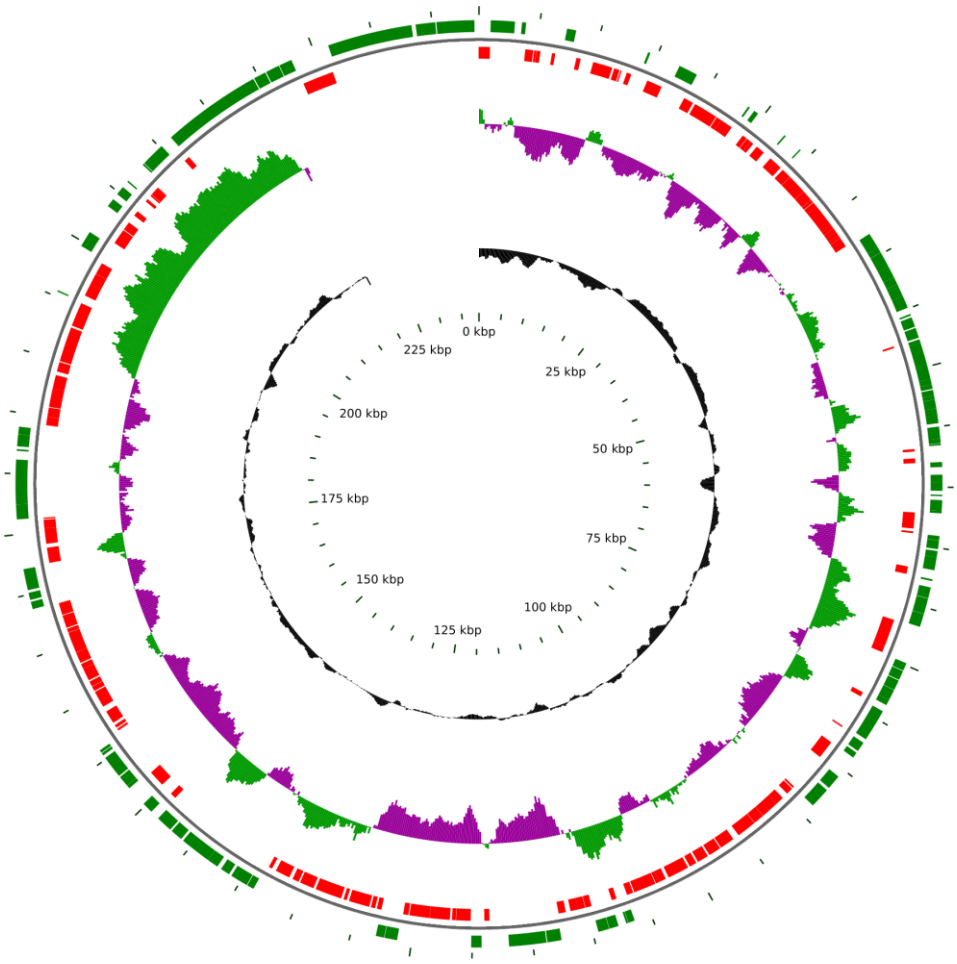

Figure 6: Genomic atlas. From the outer circle inward, coding regions are marked on the first two rings: outside the dividing line if encoded on the positive strand and inside the dividing line if encoded on the negative strand. The third ring shows the CG skew, with sharp changes in skew occurring at the origin and terminus of replication. The innermost graph shows local CG content measured in a sliding window as a black plot.

Genome annotation

Whole genome annotation is the process of identifying features of interest in a genomic sequence, and labelling them with useful information. *Annotator* annotates bacterial, archaeal and viral genomes and produce detailed output files. *GLIMMER* [9] prediction were used to identify the CDS location.

Feature	Count
CDS	228
misc RNA	3
gene	233
tRNA	2

Table 5: Details of each feature.

Sequence similarity search with Gene Ontology

A search was performed using the BLASTp [12] program against the **uniprot_sprot_bacteria** database. The **BLOSUM62** scoring matrix was used with genetic code **11**, a gap opening of **11** and extension of **2**, an expect-value cut off of **1e-1**. The minimum percentage of identify threshold was **80** and the minimum alignment length threshold was **150**. The output was limited to **5** alignments. Number of sequences that resulted in BLAST hits: **34**.

The top hit for each report was recorded and the organism name and protein name for each report noted. The accession number in the top hit for each BLAST report was submitted to the Gene Ontology (GO) database [13] and each term identified was recorded. Presented below are summary charts and tables for all the top hits with matching terms in the GO database.

Most likely species: *Photorhabdus luminescens*

Closest sub-species/strain: laumondii strain TT01

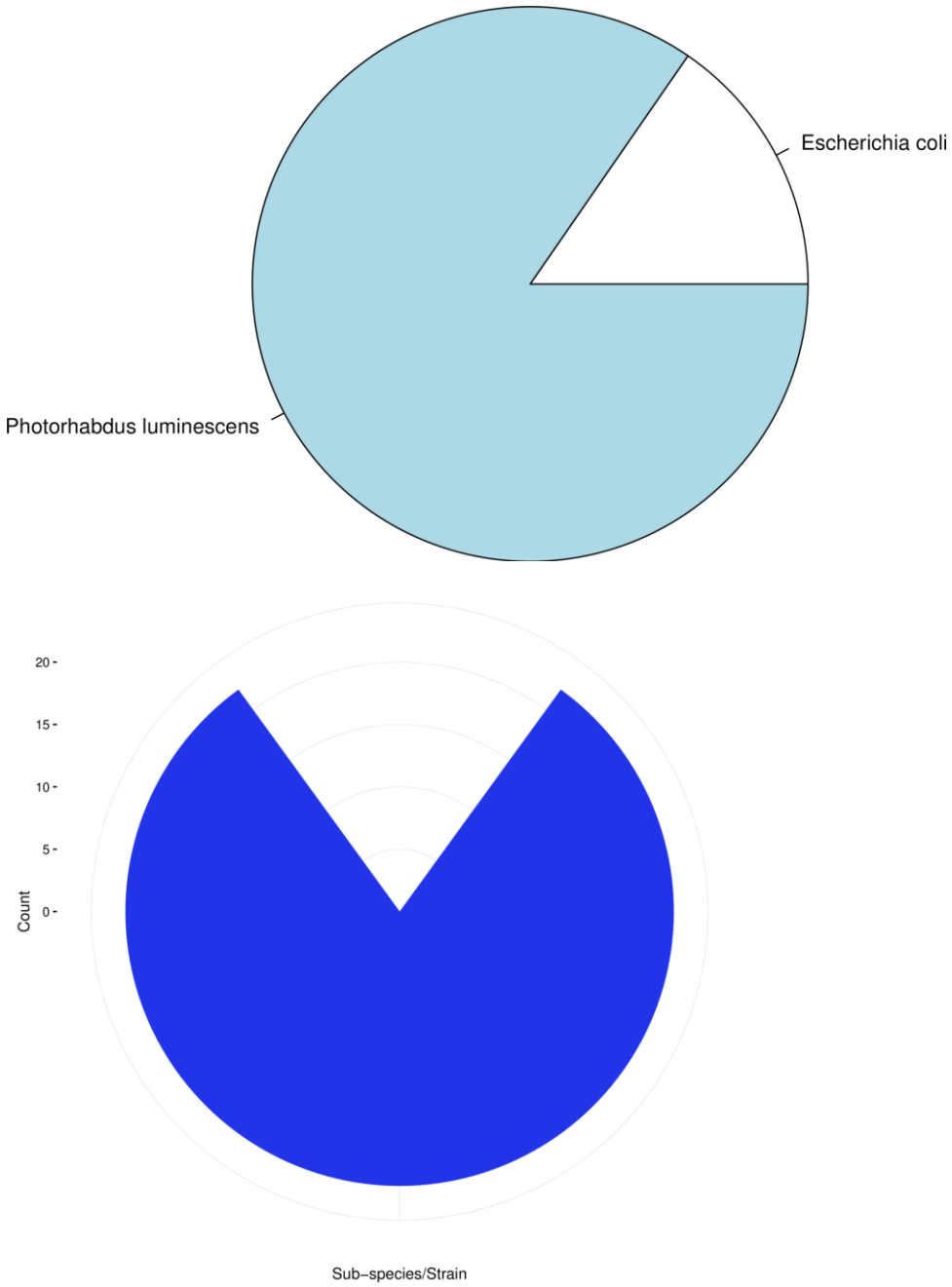

Figure 7: (left) Pie chart summary of top organism hits and (right) Bar chart summary of top sub-species/strain hits.

Gene Ontology Function	Count
cytoplasm	16
integral component of membrane	5
membrane	5
plasma membrane	5
cytosol	2
integral component of plasma membrane	2
cytochrome o ubiquinol oxidase complex	1

intracellular membrane-bounded organelle	1
respiratory chain	1
riboflavin synthase complex	1

Figure 8: Gene Ontology terms for C associated with the top hits.

Gene Ontology Function	Count
metal ion binding	13
ATP binding	11
nucleotide binding	11
transferase activity	11
hydrolase activity	9
catalytic activity	4
zinc ion binding	4
isomerase activity	3
kinase activity	3
oxidoreductase activity	3

Figure 9: Gene Ontology terms for F associated with the top hits.

Gene Ontology Function	Count
metabolic process	5
protein transport	4
transport	4
ion transport	3
oxidation-reduction process	3
pathogenesis	3
phosphorylation	3
protein folding	3
queuosine biosynthetic process	3
AMP salvage	2

Figure 10: Gene Ontology terms for P associated with the top hits.

Virulence factors database

VFDB [14] is an integrated and comprehensive database of virulence factors for bacterial pathogens (also including Chlamydia and Mycoplasma). A search was performed using the BLASTp [12] program against the **VFs**. database. The **BLOSUM62** scoring matrix was used with genetic code **11**, a gap opening of **11** and extension of **2**, an expect-value cut off of **1e-1**. The minimum percentage of identify threshold was **80** and the minimum alignment length threshold was **150**. The output was limited to **5** alignments.

Number of sequences that resulted in BLAST hits: **17**.

ORF	% Identity	Query Len	Hit Len	Entry
00044 [31854 - 31345]	90	169	169	VFG041649(gi:253988542) (sycD) yopb/d chaperone sycd [T3SS-1 (SS033)] [Photorhabdus asymbiotica subsp. asymbiotica ATCC 43949]
00045 [32802 - 31864]	80	312	304	VFG041825(gi:37527620) (plu3758) hypothetical protein [T3SS (SS035)] [Photorhabdus luminescens subsp. laumondii TTO1]
00048 [35680 - 33563]	95	705	705	VFG041828(gi:37527623) (sctV) Type III secretion protein SctV [T3SS (SS035)] [Photorhabdus luminescens subsp. laumondii TTO1]
00053 [37879 - 37007]	94	290	290	VFG041820(gi:37527628) (sctW) Type III secretion control protein SctW [T3SS (SS035)] [Photorhabdus luminescens subsp. laumondii TTO1]
00054 [38067 - 39392]	98	441	441	VFG041657(gi:253988533) (sctN) type III secretion system ATPase [T3SS-1 (SS033)] [Photorhabdus asymbiotica subsp. asymbiotica ATCC 43949]
00057 [41214 - 42143]	85	309	309	VFG041661(gi:253988530) (sctQ) type III secretion system protein [T3SS-1 (SS033)] [Photorhabdus asymbiotica subsp. asymbiotica ATCC 43949]
00058 [42140 - 42793]	96	217	217	VFG041660(gi:253988529) (sctR) type III secretion system protein [T3SS-1 (SS033)] [Photorhabdus asymbiotica subsp. asymbiotica ATCC 43949]
00060 [43059 - 43847]	92	262	262	VFG041668(gi:253988527) (sctT) type III secretion component protein sctt [T3SS-1 (SS033)] [Photorhabdus asymbiotica subsp. asymbiotica ATCC 43949]
00061 [43844 - 44899]	93	351	351	VFG041817(gi:37527636) (sctU) Type III secretion component protein SctU [T3SS (SS035)] [Photorhabdus luminescens subsp. laumondii TTO1]
00063 [45750 - 46568]	94	272	272	VFG041670(gi:253988521) (rhaS) transcriptional activator proteinExsA/virf [T3SS-1 (SS033)] [Photorhabdus asymbiotica subsp. asymbiotica ATCC 43949]
00066 [46683 - 47519]	83	278	277	VFG041810(gi:37527638) (plu3776) hypothetical protein [T3SS (SS035)] [Photorhabdus luminescens subsp. laumondii TTO1]
00069	88	609	618	VFG041673(gi:253988518) (sctC) type III

[47953 - 49782]				secretion outer membrane protein pscc [T3SS-1 (SS033)] [Photorhabdus asymbiotica subsp. asymbiotica ATCC 43949]
00070 [49779 - 51050]	87	423	424	VFG041675(gi:253988517) (sctD) type III secretion component protein sctd [T3SS-1 (SS033)] [Photorhabdus asymbiotica subsp. asymbiotica ATCC 43949]
00074 [51960 - 52421]	83	153	188	VFG041681(gi:253988514) (sctH) type III secretion component protein scth [T3SS-1 (SS033)] [Photorhabdus asymbiotica subsp. asymbiotica ATCC 43949]
00076 [52766 - 53503]	95	245	245	VFG041803(gi:37527647) (sctJ) Type III secretion component protein SctJ [T3SS (SS035)] [Photorhabdus luminescens subsp. laumondii TTO1]
00078 [53503 - 54129]	87	208	208	VFG041804(gi:37527648) (sctK) Type III secretion component protein SctK [T3SS (SS035)] [Photorhabdus luminescens subsp. laumondii TTO1]
00079 [54108 - 54725]	93	205	205	VFG041677(gi:253988510) (sctL) type III secretion system protein [T3SS-1 (SS033)] [Photorhabdus asymbiotica subsp. asymbiotica ATCC 43949]

Table 6: Sequences that had BLASTphits.

16S Phylogeny

The sequence of 16S rRNA gene was retrieved using Metaxa [15] and the **SILVA SSU** database [16].

No full size 16S sequence was identified.

Versions

Module	Application	Version
cusp		2.1
	cusp	
glimmer		2.1
	tigr-glimmer	
	transeq	
quast		2.1
	quast	3.0
metaxa		2.2
	metaxa	1.1.2
	raxml	
	clustalo	

	plot	1.4
readqc		2.1
	fastqc	0.11.3
annotator		1.1
full_gview		2.1
	gview	1.7
genome_mapper		2.2
	bowtie	
	bowtie2	
	bowtie-build	
	bowtie2-build	
	samtools	1.2
	bcftools	1.2
	vcfutils.pl	1.2
	seqtk	
blast_GO		2.2
	blastp	
filtering		2.4
	prinseq-lite.pl	0.20.4
	prinseq-graphs.pl	0.20.4
contiguator		2.1
	contiguator	2.7
clipping		2.5
	tagcleaner.pl	0.16
	fastq-mcf	r823
blast		2.2
	blastn	
	blastp	
	transeq	

Table 7: List of the scripts and tools used to generate this results.

Database	Release
uniprot_trembl	2016-02-10
VFs	2016-02-09

Table 8: List of the databases used to generate this results.

References

1. NSilico LifeScience Ltd: ***Simplicity*™, Version 1.5**. Cork, Ireland; 2015.

2. Andrews S: **FastQC: A Quality Control Tool for High Throughput Sequence Data**. Cambridge, UK; 2010.

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12. Altschul SF, Madden TL, Schäffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ: **Gapped BLAST and PSI-BLAST: a New Generation of Protein Database Search Programs**. *Nucleic Acids Res* 1997, **25**:3389–402.

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16. Quast C, Pruesse E, Yilmaz P, Gerken J, Schweer T, Yarza P, Peplies J, Glöckner FO: **The SILVA Ribosomal RNA Gene Database Project: Improved Data Processing and Web-Based Tools**. *Nucleic Acids Res* 2013, **41**(Database issue):D590–6.

Analysis of 2782-pur1 with Reference Genome Photorhabdus temperata subsp. temperata Meg1 (enterobacteria)

Pipeline completed.

Pipeline ID: `30530`

Date Completed: Wed, 17 Feb 2016 09:37

{pauljwalsh@gmail.com}

Disclaimer

The results presented in this report were produced by the tools listed below. The tools and report generation were managed by *Simplicity*[TM] [1].

Reads

Filename	2782-pur1_1_trimmed.fastq.gz	2782-pur1_2_trimmed.fastq.gz
Total Sequences	465,721	465,721
Sequence length	36-251	36-251
%GC	43	43
Encoding	Sanger / Illumina 1.9	Sanger / Illumina 1.9
Reads Library type		Paired ends

Table 1: Summary.

Reads Quality

FastQC [2] aims to provide a simple way to do some quality control checks on raw sequence data coming from high throughput sequencing pipelines. It provides a modular set of analyses, which you can use to give a quick impression of whether your data has any problems of which you should be aware before doing any further analysis.

Filename	2782-pur1_1_trimmed.fastq.gz	2782-pur1_2_trimmed.fastq.gz
Basic Statistics	PASS	PASS
Per base sequence quality	PASS	PASS

scores		
Per tile sequence quality	PASS	PASS
Per sequence quality scores	PASS	PASS
Per base sequence content	FAIL	FAIL
Per sequence GC content	PASS	PASS
Per base N content	PASS	PASS
Sequence Length Distribution	WARN	WARN
Sequence Duplication Levels	PASS	PASS

Table 2: Information on FASTQ files.

FastQC aims to provide a QC report, which can spot problems which originate either in the sequencer or in the starting library material. It is important to stress that although the FastQC analysis results appear to give a pass/fail result, these evaluations must be taken in the context of what you expect from your library. A *normal* sample as far as FastQC is concerned is random and diverse. Some experiments may be expected to produce libraries, which are biased in particular ways. You should treat the summary evaluations therefore as pointers to where you should concentrate your attention and understand why your library may not look random and diverse.

Per base sequence Quality: WARN = A warning will be issued if the lower quartile for any base is less than 10, or if the median for any base is less than 25. FAIL = This module will raise a failure if the lower quartile for any base is less than 5 or if the median for any base is less than 20.

Per tile sequence quality: WARN = A warning will be issued if any tile shows a mean Phred score more than 2 less than the mean for that base across all tiles. FAIL = This module will issue a warning if any tile shows a mean Phred score more than 5 less than the mean for that base across all tiles.

Per sequence quality scores: WARN = A warning is raised if the most frequently observed mean quality is below 27 - this equates to a 0.2% error rate. FAIL = An error is raised if the most frequently observed mean quality is below 20 - this equates to a 1% error rate.

Per base sequence content: WARN = This module issues a warning if the difference between A and T, or G and C is greater than 10% in any position. FAIL = This module will fail if the difference between A and T, or G and C is greater than 20% in any position.

Per base GC content: WARN = This module issues a warning it the GC content of any base strays more than 5% from the mean GC content. FAIL = This module will fail if the GC content of any base strays more than 10% from the mean GC content.

Per sequence GC content: WARN = A warning is raised if the sum of the deviations from the normal distribution represents more than 15% of the reads. FAIL = This

module will indicate a failure if the sum of the deviations from the normal distribution represents more than 30% of the reads.

Per base N content: This module raises a warning if any position shows an N content of >5%. FAIL = This module will raise an error if any position shows an N content of >20%.

Sequence Length Distribution: WARN = This module will raise a warning if all sequences are not the same length. FAIL = This module will raise an error if any of the sequences have zero length.

Sequence Duplication Levels: WARN = This module will issue a warning if non-unique sequences make up more than 20% of the total. FAIL = This module will issue a error if non-unique sequences make up more than 50% of the total.

Sequence quality for 2782-pur1_1_trimmed

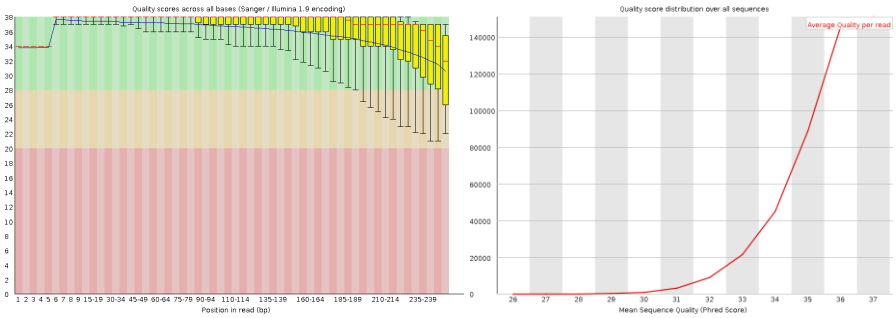

Figure 1: Per base (left) and sequence (right) quality for 2782-pur1_1_trimmed.

For each position a Box Whisker type plot is drawn for the per base quality (left). The elements of the plot are as follows:

- The central red line is the median value.
- The yellow box represents the inter-quartile range (25-75%).
- The upper and lower whiskers represent the 10% and 90% points.

The per sequence quality score report allows you to see if a subset of your sequences have low quality values. It is often the case that a subset of sequences will have poor quality, often because they are poorly imaged (on the edge of the field of view etc), however these should represent only a small percentage of the total sequences.

Sequence quality for 2782-pur1_2_trimmed

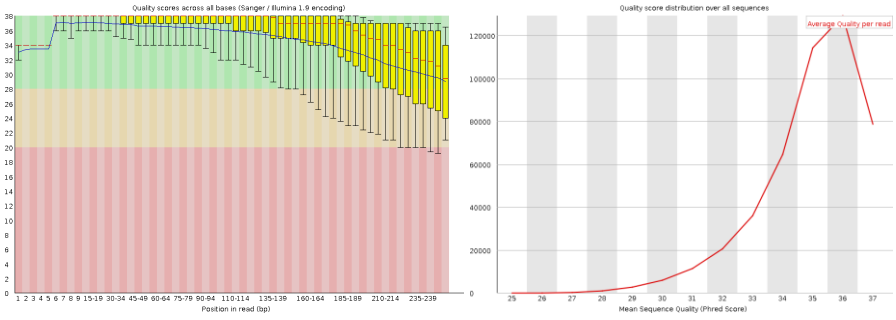

Figure 2: Per base (left) and sequence (right) quality for 2782-pur1_2_trimmed.

Read cleaning

The *PrinSeq* [3] ensures that the data used for downstream analysis is not compromised by low-quality sequences, PCR duplicates or sequence artifacts that might lead to erroneous conclusions. *PrinSeq* filtered out reads with a mean quality value lower than **10** and with any base quality value lower than **5**. It **removed** bases with ambiguity (Ns), **removed any PCR duplicates** and any reads with an **entropy value under 0.30**.

Adapter trimming

The adapter trimming stage was used to automatically detect and efficiently remove tag sequences (*e.g.* Adaptor or WTA tags) from genomic and metagenomic datasets. *TagCleaner* [4] was used to predict the potential adaptors in each file. **No adaptor or tag was detected**.

Trimmomatic [5] removed adapter sequences from DNA high-throughput sequencing data and also removes low quality regions of sequences while keeping paired end files synchronised. This is necessary when the reads are longer than the molecule that is sequenced and when sequence tags are present. Not removing adapter and sequence tags can hinder assembly, mapping of the reads and influence SNP calling and other downstream analyses. *Trimmomatic* [5] trimmed adapter sequences with a maximum allowed error rate of **10%** and a minimum length of trimmed reads of **0**.

Genome mapping

Genome mapping was performed by *Bowtie2* [6]. The *Bowtie* package enables ultrafast and memory-efficient alignment of large sets of sequencing reads to a reference sequence. The package contains tools for building indexes of reference genomes and for aligning short reads using the index as a guide. The **no** mismatches in seed alignment of **20**nt was allowed. Intervals between seed reads were *1* and *0.50*. For reads with repetitive seeds, try **3** sets of seeds. Give up extending after **20** failed extends in a row. The alignment was optimise for **local** alignment (ends might be soft clipped).

```
465626 reads; of these:
  459208 (98.62%) were paired; of these:
    451370 (98.29%) aligned concordantly 0 times
    7456 (1.62%) aligned concordantly exactly 1 time
    382 (0.08%) aligned concordantly >1 times
    ----
    451370 pairs aligned concordantly 0 times; of these:
      4921 (1.09%) aligned discordantly 1 time
    ----
    446449 pairs aligned 0 times concordantly or discordantly; of these:
      892898 mates make up the pairs; of these:
        887921 (99.44%) aligned 0 times
        4026 (0.45%) aligned exactly 1 time
        951 (0.11%) aligned >1 times
  6418 (1.38%) were unpaired; of these:
    6231 (97.09%) aligned 0 times
    173 (2.70%) aligned exactly 1 time
    14 (0.22%) aligned >1 times
3.32% overall alignment rate
```

The average coverage across every base is 28.2082 bases (Stdev 28.6077).

The number of SNPs (single nucleotide polymorphisms) found was ** 19860** and the number of InDels (insertions and deletions) found was 24. Only gaps of less than 10 bases are considered for an insert or deletion.

Evaluate genome assemblies

The *QUAST* [7] program is used to evaluate assemblies. The scaffolds file generated by the assembly tool was evaluated.

Assembly	map_genome	map_genome broken
# contigs (>= 0 bp)	1	1
# contigs (>= 1000 bp)	1	1
Total length (>= 0 bp)	238875	238875
Total length (>= 1000 bp)	238875	238875
# contigs	1	1
Largest contig	238875	238875
Total length	238875	238875
GC (%)	42.54	42.54
N50	238875	238875
N75	238875	238875
L50	1	1
L75	1	1
# N's per 100 kbp	21777.50	21777.50

Table 3: Scaffolds were generated by the assembly tool and the information for this table was generated by QUAST.

GC (%) is the total number of G and C nucleotides in the assembly, divided by the total length of the assembly. N50 is the length for which the collection of all contigs of that length or longer covers at least half an assembly. N75 is defined similarly with 75% instead of 50%. L50 (L75) is the number of contigs as long as N50 (N75, NG50, NG75) In other words, L50, for example, is the minimal number of contigs that cover half the assembly. The scaffolds produced by assembly tool were combined to create a draft genome.

Genome finishing

CONTIGuator [8] performs a mapping step against the reference genome using the BLAST algorithm. The results are analysed taking into account the presence of more than one replicon, thus ensuring that no contigs are mapped to more than one replicon.

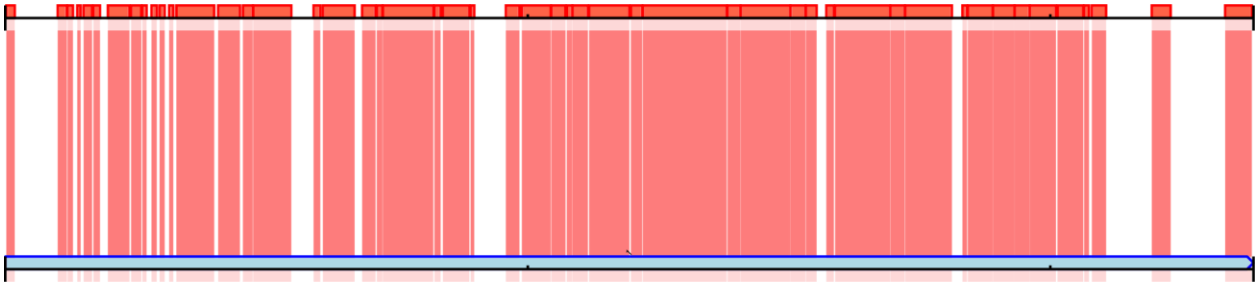

Figure 3: A image showing the reference genome on top with the mapped contigs underneath.

Gene prediction

GLIMMER [9] is a system for finding genes in microbial DNA, especially the genomes of Bacteria and Archaea. GLIMMER (Gene Locator and Interpolated Markov ModelER) uses interpolated Markov models to identify coding regions. The topology used was circular and the genetic code used was **11** (The Bacterial, Archaeal and Plant Plastid code).

Field name	Value
Genome size	238,875
Predicted genes count	245
Coding GC	55.0%

Table 4: Coding gene summary

Gene composition

EMBOSS CUSP [10] calculates a codon usage table from draft genome sequence.

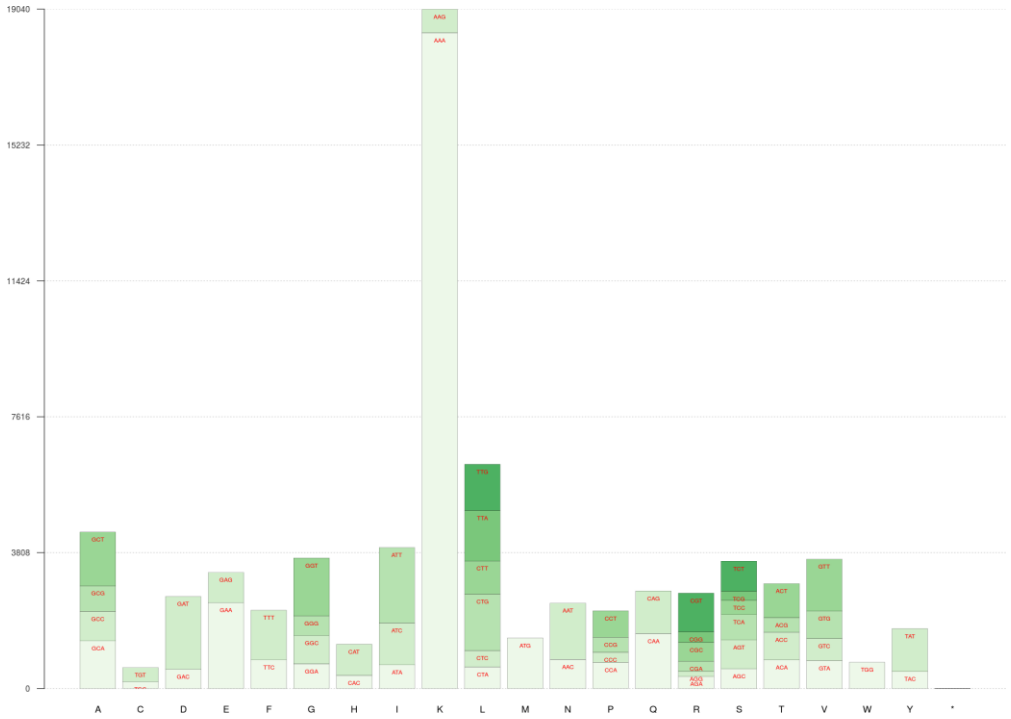

Figure 4: The image shows a summary of codon usage frequency.

Genome structure

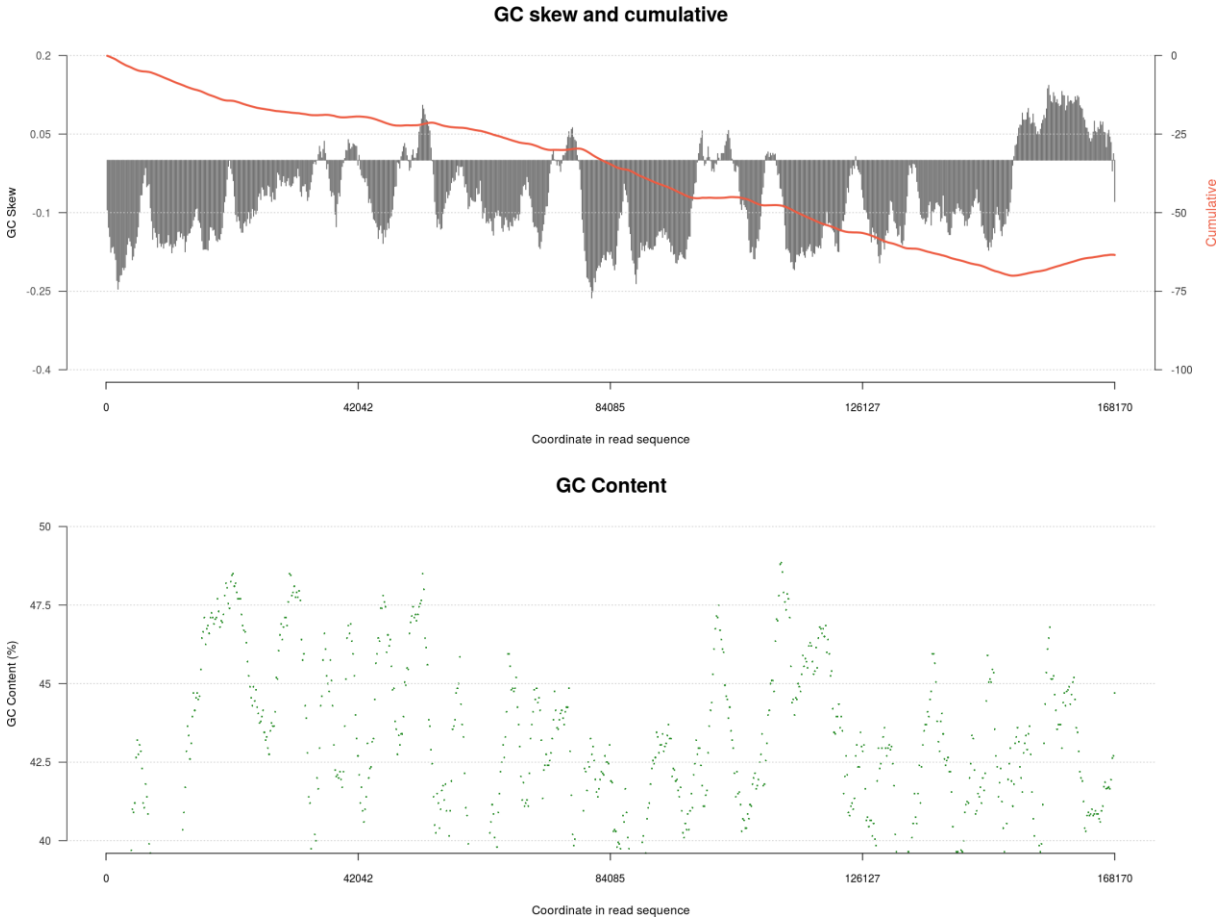

Figure 5: Summary of CG skew along the genome.

GView [11] is useful for producing high-quality genome maps for microbial genomes. The following image is a genome map produced by GView using the predicted ORFs.

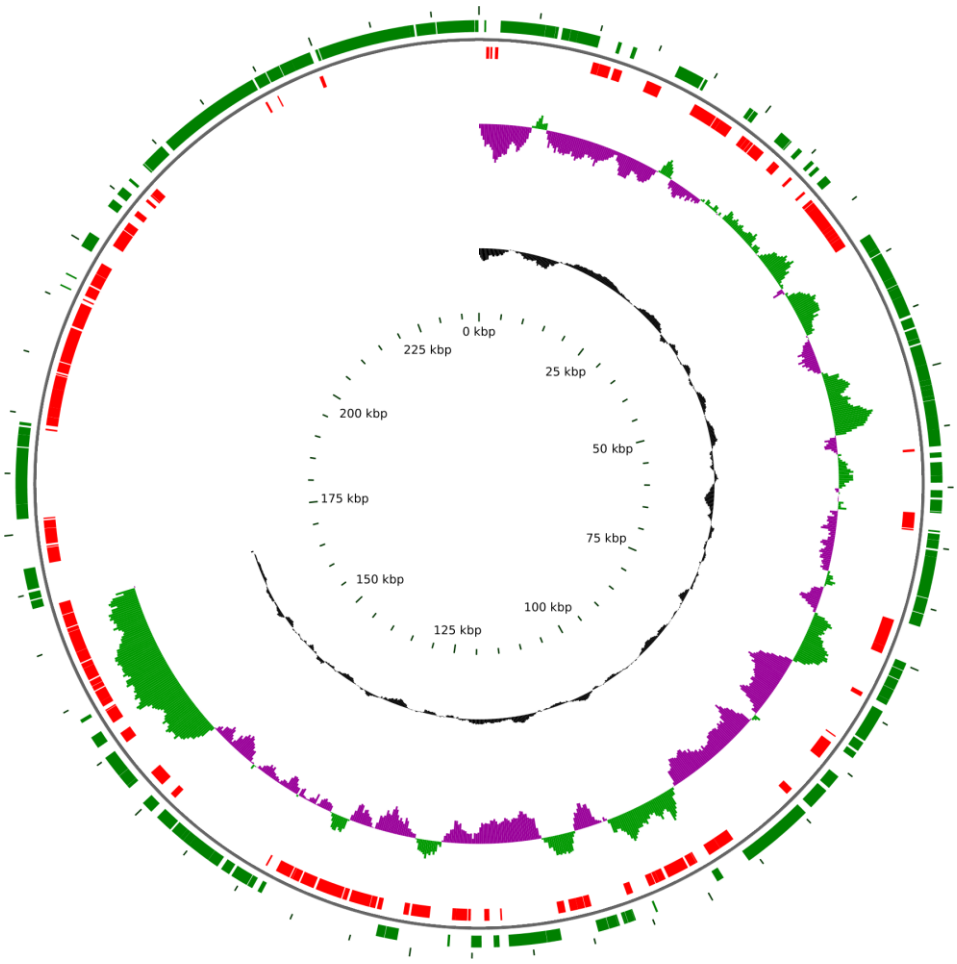

Figure 6: Genomic atlas. From the outer circle inward, coding regions are marked on the first two rings: outside the dividing line if encoded on the positive strand and inside the dividing line if encoded on the negative strand. The third ring shows the CG skew, with sharp changes in skew occurring at the origin and terminus of replication. The innermost graph shows local CG content measured in a sliding window as a black plot.

Genome annotation

Whole genome annotation is the process of identifying features of interest in a genomic sequence, and labelling them with useful information. *Annotator* annotates bacterial, archaeal and viral genomes and produce detailed output files. *GLIMMER* [9] prediction were used to identify the CDS location.

Feature	Count
tRNA	2
gene	249
CDS	244
misc RNA	3

Table 5: Details of each feature.

Sequence similarity search with Gene Ontology

A search was performed using the BLASTp [12] program against the **uniprot_sprot_bacteria** database. The **BLOSUM62** scoring matrix was used with genetic code **11**, a gap opening of **11** and extension of **2**, an expect-value cut off of **1e-1**. The minimum percentage of identify threshold was **80** and the minimum alignment length threshold was **150**. The output was limited to **5** alignments. Number of sequences that resulted in BLAST hits: **33**.

The top hit for each report was recorded and the organism name and protein name for each report noted. The accession number in the top hit for each BLAST report was submitted to the Gene Ontology (GO) database [13] and each term identified was recorded. Presented below are summary charts and tables for all the top hits with matching terms in the GO database.

Most likely species: *Photorhabdus luminescens*

Closest sub-species/strain: laumondii strain TT01

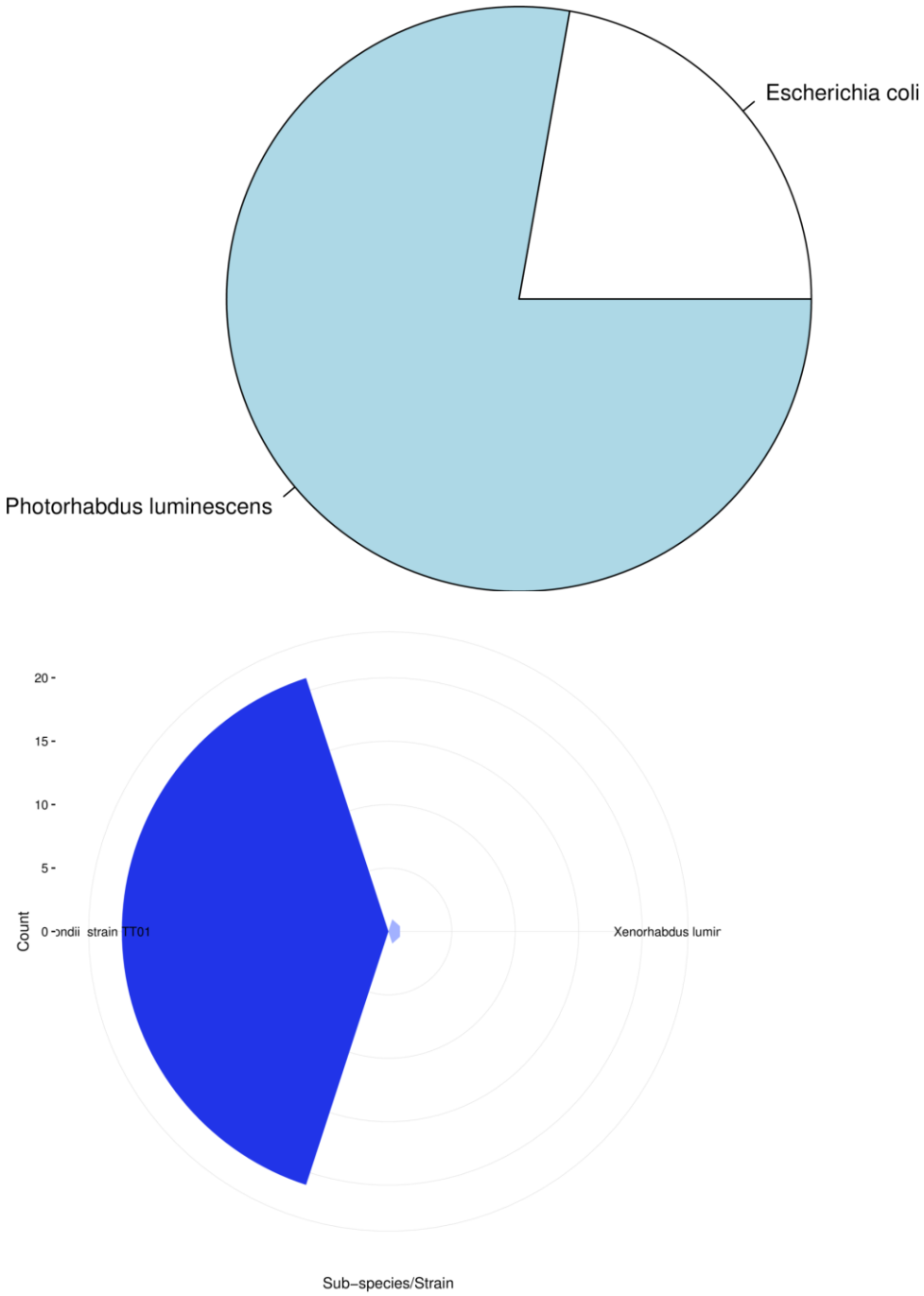

Figure 7: (left) Pie chart summary of top organism hits and (right) Bar chart summary of top sub-species/strain hits.

Gene Ontology Function	Count
cytoplasm	16
integral component of membrane	4
membrane	4
plasma membrane	4
cytosol	3
cytochrome o ubiquinol oxidase complex	1

integral component of plasma membrane	1
respiratory chain	1
riboflavin synthase complex	1
type III protein secretion system complex	1

Figure 8: Gene Ontology terms for C associated with the top hits.

Gene Ontology Function	Count
metal ion binding	11
transferase activity	11
ATP binding	10
nucleotide binding	10
hydrolase activity	8
catalytic activity	4
isomerase activity	4
zinc ion binding	4
kinase activity	3
oxidoreductase activity	3

Figure 9: Gene Ontology terms for F associated with the top hits.

Gene Ontology Function	Count
metabolic process	4
protein folding	4
protein transport	4
oxidation-reduction process	3
pathogenesis	3
phosphorylation	3
queuosine biosynthetic process	3
transport	3
AMP salvage	2
glycolytic process	2

Figure 10: Gene Ontology terms for P associated with the top hits.

Virulence factors database

VFDB [14] is an integrated and comprehensive database of virulence factors for bacterial pathogens (also including Chlamydia and Mycoplasma). A search was performed using the BLASTp [12] program against the **VFs**. database. The **BLOSUM62** scoring matrix was used with genetic code **11**, a gap opening of **11** and extension of **2**, an expect-value cut off of **1e-1**. The minimum percentage of identify threshold was **80** and the minimum alignment length threshold was **150**. The output was limited to **5** alignments.

Number of sequences that resulted in BLAST hits: **15**.

ORF	% Identity	Query Len	Hit Len	Entry
00064 [35680 - 33563]	96	705	705	VFG041828(gi:37527623) (sctV) Type III secretion protein SctV [T3SS (SS035)] [Photorhabdus luminescens subsp. laumondii TTO1]
00069 [37879 - 37007]	87	290	290	VFG041820(gi:37527628) (sctW) Type III secretion control protein SctW [T3SS (SS035)] [Photorhabdus luminescens subsp. laumondii TTO1]
00070 [38100 - 39389]	97	429	441	VFG041657(gi:253988533) (sctN) type III secretion system ATPase [T3SS-1 (SS033)] [Photorhabdus asymbiotica subsp. asymbiotica ATCC 43949]
00076 [39934 - 41217]	85	427	442	VFG041662(gi:253988531) (sctP) type III secretion component protein sctp [T3SS-1 (SS033)] [Photorhabdus asymbiotica subsp. asymbiotica ATCC 43949]
00078 [41199 - 42143]	89	314	309	VFG041661(gi:253988530) (sctQ) type III secretion system protein [T3SS-1 (SS033)] [Photorhabdus asymbiotica subsp. asymbiotica ATCC 43949]
00080 [42140 - 42793]	96	217	217	VFG041660(gi:253988529) (sctR) type III secretion system protein [T3SS-1 (SS033)] [Photorhabdus asymbiotica subsp. asymbiotica ATCC 43949]
00082 [43059 - 43847]	96	262	262	VFG041668(gi:253988527) (sctT) type III secretion component protein sctt [T3SS-1 (SS033)] [Photorhabdus asymbiotica subsp. asymbiotica ATCC 43949]
00084 [43844 - 45523]	96	559	350	VFG041667(gi:253988526) (sctU) type III secretion component protein sctu [T3SS-1 (SS033)] [Photorhabdus asymbiotica subsp. asymbiotica ATCC 43949]
00086 [45750 - 46568]	94	272	272	VFG041670(gi:253988521) (rhaS) transcriptional activator proteinExsA/virf [T3SS-1 (SS033)] [Photorhabdus asymbiotica subsp. asymbiotica ATCC 43949]
00094 [48097 - 49782]	93	561	618	VFG041673(gi:253988518) (sctC) type III secretion outer membrane protein pscc [T3SS-1 (SS033)] [Photorhabdus asymbiotica subsp. asymbiotica ATCC 43949]
00095 [49779 -	88	423	424	VFG041675(gi:253988517) (sctD) type III secretion component protein sctd [T3SS-1 (SS033)] [Photorhabdus asymbiotica subsp.

51050]				asymbiotica ATCC 43949]
00099 [51855 - 52421]	87	188	188	VFG041681(gi:253988514) (sctH) type III secretion component protein scth [T3SS-1 (SS033)] [Photorhabdus asymbiotica subsp. asymbiotica ATCC 43949]
00101 [52793 - 53503]	98	236	245	VFG041679(gi:253988512) (sctJ) type III secretion component protein sctj [T3SS-1 (SS033)] [Photorhabdus asymbiotica subsp. asymbiotica ATCC 43949]
00103 [53479 - 54129]	88	216	208	VFG041678(gi:253988511) (sctK) type III secretion component protein sctk [T3SS-1 (SS033)] [Photorhabdus asymbiotica subsp. asymbiotica ATCC 43949]
00104 [54108 - 55205]	97	365	205	VFG041677(gi:253988510) (sctL) type III secretion system protein [T3SS-1 (SS033)] [Photorhabdus asymbiotica subsp. asymbiotica ATCC 43949]

Table 6: Sequences that had BLASTphits.

16S Phylogeny

The sequence of 16S rRNA gene was retrieved using Metaxa [15] and the **SILVA SSU** database [16].

No full size 16S sequence was identified.

Versions

Module	Application	Version
full_gview		2.1
	gview	1.7
blast		2.2
	blastn	
	blastp	
	transeq	
genome_mapper		2.2
	bowtie	
	bowtie2	
	bowtie-build	
	bowtie2-build	
	samtools	1.2
	bcftools	1.2
	vcfutils.pl	1.2
	seqtk	

filtering		2.4
	prinseq-lite.pl	0.20.4
	prinseq-graphs.pl	0.20.4
clipping		2.5
	tagcleaner.pl	0.16
	fastq-mcf	r823
metaxa		2.2
	metaxa	1.1.2
	raxml	
	clustalo	
	plot	1.4
blast_GO		2.2
	blastp	
glimmer		2.1
	tigr-glimmer	
	transeq	
cusp		2.1
	cusp	
contiguator		2.1
	contiguator	2.7
annotator		1.1
quast		2.1
	quast	3.0
readqc		2.1
	fastqc	0.11.3

Table 7: List of the scripts and tools used to generate this results.

Database	Release
uniprot_trembl	2016-02-10
VFs	2016-02-09

Table 8: List of the databases used to generate this results.

References

1. NSilico LifeScience Ltd: ***Simplicity*™, Version 1.5**. Cork, Ireland; 2015.

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4. Schmieder R, Lim YW, Rohwer F, Edwards R: **TagCleaner: Identification and Removal of Tag Sequences from Genomic and Metagenomic Datasets**. *BMC Bioinformatics* 2010, **11**:341.

5. Bolger AM, Lohse M, Usadel B: **Trimmomatic: a Flexible Trimmer for Illumina Sequence Data**. *Bioinformatics* 2014, **30**:2114–20.

6. Langmead B, Salzberg SL: **Fast Gapped-Read Alignment with Bowtie 2**. *Nat Methods* 2012, **9**:357–9.

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10. Rice P, Longden I, Bleasby A: **EMBOSS: the European Molecular Biology Open Software Suite**. *Trends Genet* 2000, **16**:276–7.

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