Investigations into the efficacy of the slug and snail biological control *Phasmarhabditis hermaphrodita*

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A thesis submitted in partial fulfilment of the requirements of Liverpool John Moores University for the degree of Doctor of Philosophy

This research programme was carried out in collaboration with The Royal Horticultural Society

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Declaration

I hereby declare that this thesis has been composed by myself and that it has not been accepted in any previous application for a degree. The work of which it is a record was carried out by myself unless otherwise stated. All sources of information have been acknowledged by means of references and all quotations have been distinguished by quotation marks

Kerry McDonald-Howard

November 2022

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Chapter 1: Literature review of the history of *Phasmarhabditis hermaphrodita* and other methods of slug control

1.1 Introduction

Pestiferous slugs pose a significant global threat to sustainable agriculture, horticulture and floriculture (Barker, 2002). Slugs cause crop damage by eating seeds, stems, growing points and leaves, leading to a reduction in growth (Port, 1986; South, 1992). They can be a major pest throughout the lifecycle of field vegetables and in extreme cases, whole fields have to be re-sown resulting in economic losses (Willis *et al*., 2006). Contamination of the harvested crop also occurs from slug mucus and faeces, resulting in poor product quality (Port and Ester, 2002). It is estimated that a lack of slug control for crops such as oilseed rape and wheat would lead to £43.5 million a year in loss of product in the UK alone (Nicholls, 2014). In Europe, wheat and oilseed rape suffer greatly from slug damage (Ester and Wilson, 2005) and in 2010 it was reported that 22% of winter wheat crops suffered damage from slugs, and if left untreated by chemical molluscicides a 5% decrease in yield would be expected (ADAS, 2010). As well as causing damage in agriculture, slug feeding can affect plant community diversity and richness (Wilby and Brown, 2001) with preferential feeding on native species aiding in exotic plant growth (Joe and Daehler, 2008). Slug feeding reduces conservation efforts such as forest regeneration (Côté *et al*., 2005) and threatens endangered species such as lichens (Cameron, 2009). Slugs can also act as invasive pests, causing damage to native plants that may not have anti-herbivore defences due to limited predation pressure (Feeny, 1992) and alter species composition (Schreiner, 1997). Slugs can also be responsible for transmitting plant pathogens (Wester *et al*., 1964; Hasan and Vago, 1966) including *Phytophthora* (Telfer, 2015). They can also act as vectors of nematode parasites (South, 1992; Grewal *et al*, 2003a), such as the rat lungworm, *Angiostrongylus cantonensis*, the causal agent of eosinophilic meningitis, which is recognised as an emerging tropic and sub tropic zoonotic disease (Yong *et al*., 2015). Other veterinary important nematode parasites vectored by slugs include *Angiostrongylus vasorum*, *Muellerius capillaris* and *Protostrongylus tauricus* (Patel *et al*., 2014; Lange *et al*., 2018).

Slugs are commonly controlled by chemical bait pellets containing metaldehyde. In the past methiocarb was used, however it is toxic to beneficial invertebrates and other non-target organisms (Purvis and Bannon, 1992; Nicholls, 2014).The use of Metaldehyde in Great Britain was banned from the 1st April 2022 (Jones, 2022). Metaldehyde pellets were used

globally (Castle *et al.*, 2017). From 2008 to 2014 an estimated 1640 t of metaldehyde was used in the UK alone (Simms *et al*., 2006). Slugs feed on the pellets and exhibit symptoms such as increased levels of mucus secretion and paralysis, and die within several days from water loss (Booze and Oehme, 1986; Bailey, 2002). Though effective these bait pellets also cause harm to non-target organisms including canines and other vertebrates (Cope *et al*., 2006). Metaldehyde is also now considered an important emerging pollutant of concern due to leaching into watercourses (Stuart *et al.*, 2012) as it is highly mobile in soil (Kay and Grayson, 2014). Furthermore, in parts of the UK metaldehyde concentrations in water bodies have exceeded the European Union's regulatory drinking water standard for pesticides (Kay and Grayson, 2014). An alternative slug pellet (Ferramol®) is composed of iron III phosphate or ferric phosphate and is registered for use in many European countries (Speiser and Kistler, 2001). It has been used to control slugs e.g. *Arion ater*, however it has been shown that high doses can lead to mortality and reduced activity in earthworms (Langan and Shaw, 2006).

In agriculture: trapping; drilling at a greater depth; ploughing; crop rotation; increasing crop diversity and firm seedbed preparation can also help to limit slug damage (Glen *et al*., 2000) although some practices such as direct drilling and minimal tillage can result in an increase in pest slug populations (Barker, 2002). Drilling at depths of 25 – 45 mm has been shown to provide the most effective protection against slug damage (Glen *et al*., 1990). Ploughing and firm seedbed preparation reduces slug numbers by disrupting their normal surface activity patterns (Glen *et al.,* 2006). Brooks *et al.* (2006) investigated using alternative food sources to reduce slug damage in winter wheat, by planting red clover before planting winter wheat. Here the slugs continued to preferentially feed on the red clover even when wheat seeds were introduced, although this turned out to be a complex control strategy to implement.

In gardens and greenhouses, damage by gastropods can be limited by cultural control methods such as the use of copper tape, garlic and mulch, although they are inefficient for larger scale agricultural use (Schüder *et al.*, 2003). The use of copper tape or copper-impregnated matting has been shown to reduce the velocity of pest slugs, possibly due to irritation (Schüder *et al*., 2005). In choice experiments, copper was seen to repel slugs and they nearly always avoided mulch as it dries out quickly (Schüder *et al*., 2005). The above listed methods however, are time consuming and not always effective.

Due to these issues, the only realistic alternative for slug control is the gastropod parasitic nematode *Phasmarhabditis hermaphrodita* Schneider (Nematoda: Rhabditidae) (Fig 1). Key diagnostic features can be found in in Stock and Hunt (2005).The Nematode which has been formulated into a biological control agent (Nemaslug®) produced and sold by BASF Agricultural Specialities (Wilson *et al.,* 1993). *P. hermaphrodita* (strain DMG0001) is sold in 15 different European countries (Pieterse *et al.*, 2017a) and has been on the market since 1994. It has been successfully used to reduce slug damage in agriculture, floriculture and horticulture, sometimes to comparable levels as metaldehyde treatment (Rae *et al*., 2007). Here we describe the research that has been carried out on *P. hermaphrodita* over 25 years since its release as a biocontrol product and provide information on the *Phasmarhabditis* genus, the susceptibility of slugs and snails, host interactions, bacterial associations, results of field trials and suggestions for future research to enhance the use of *P. hermaphrodita* in the field.

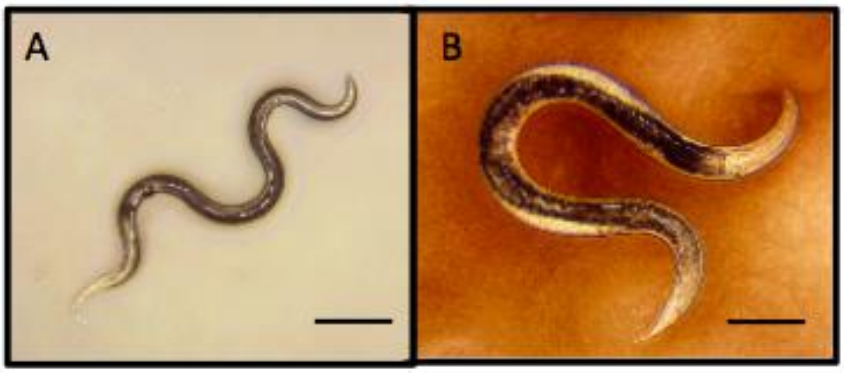

Fig 1.1 Dauer stage *P. hermaphrodita* (A) seek out slugs in soil and then penetrate inside. Once the slug dies the nematodes exit the dauer stage and grow to young adult nematodes (B) and reproduce on the cadaver. Bars represents 100 µm.

1.2 Slug parasitic nematodes and the genus *Phasmarhabditis*

There are 108 nematodes associated with slugs and snails (Grewal *et al.,* 2003a) used as either definitive, intermediate or necromenic hosts (Pieterse *et al.,* 2017a). Forty-seven species of nematode, belonging to 8 families, use molluscs as a definitive host (Grewal *et al*., 2003a; Pieterse *et al*., 2017a). However, the only nematodes that can kill slugs and snails are those from the genus *Phasmarhabditis* (Wilson *et al.,* 1993). There are some reports of mortality being caused by *Alloionema appendiculatum* towards *Arion vulgaris* but not at levels considered suitable for a biocontrol agent (Nermut' *et al*., 2019a).

Phasmarhabditis hermaphrodita is a Rhabditid clade V nematode (Ross *et al*., 2010a), and along with different *Phasmarhabditis* species, are easy to isolate using protocols described by Wilson *et al.* (1993; 2016), Wilson (2012) and Andrus and Rae (2019a), with many different species isolated from around the world. Identification can be accomplished using 18SrRNA primers (Blaxter *et al*., 1998), species-specific primers and qPCR methodologies for nematodes isolated from soil and hosts (Macmillan *et al.,* 2006; Jaffuel *et al.,* 2019). *P. hermaphrodita* was first described from Germany by Schneider (1859), and it has since been isolated in many different parts of the world. In 1900 Maupas isolated *P. hermaphrodita* in Normandy, France and 50 years later in 1953 it was re-isolated by Mengert in Germany. *P. hermaphrodita* was isolated in the UK in the early 1990s from diseased grey field slugs (*Deroceras reticulatum*) at Long Ashton Research Station, University of Bristol (Wilson *et al.,* 1993). For a personal account about its initial isolation in the early 1990s see Glen and Coupland (2017). Further research focused on finding suitable bacteria for mass production (Wilson *et al*., 1995a,b) and proof the nematode could be used to control slugs under field conditions (e.g. Wilson *et al*., 1994a; 1995c). Research carried out by Mike Wilson was used as a blueprint to produce *P. hermaphrodita* commercially first by MicroBio, then Becker Underwood and now BASF Agricultural Specialities. Subsequently, interest in the species grew with it being found in: France (Coupland, 1995); Chile (France and Gerding, 2000); Iran (Karimi *et al.,* 2003); Czech Republic (Nermut' *et al.,* 2010); Egypt (Genena *et al*., 2011); New Zealand (Wilson *et al*., 2012; 2016); Norway (Ross *et al*., 2016) and most recently in Belgium (Singh *et al*., 2019). One of the biggest markets for slug control is the USA, but for years *P. hermaphrodita* was never found despite several surveys (Grewal *et al*., 2000; Kaya and Mitani, 2000; Ross *et al*. 2010b). However, recently numerous strains of *P. hermaphrodita* and other *Phasmarhabditis* species have been found in North America (see Tandingan de Ley *et al*., 2017 for an overview; McDonnell *et al*., 2018a; Tandingan De Ley *et al*., 2014). The US strains of *P. hermaphrodita* have been shown to kill neonate *Lissachatina fulica* (the giant African snail) (McDonnell *et al*., 2018b), and several other *Phasmarhabditis* species can kill *D. reticulatum* (McDonnell *et al*. 2018c; Schurkman *et al*., 2022a), the snails *Succinea* spp. (Schurkman *et al*., 2022b) and *Theba pisana* (Tandingan De Ley *et al*., 2020; Schurkman *et al*., 2022c) and the subterranean slug *Testacella haliotidea* (McDonnell *et al.,* 2022). It seems therefore, virulence towards slugs is an evolutionary conserved trait across the genus *Phasmarhabditis*, indeed it has recently been shown another three species (*P. bohemica*, *P. bonaquanense* and *P. apuliae*) can infect and kill slugs (*D. reticulatum*) (Nermut' *et al*., 2022a). Interestingly, full mitochondrial analysis of *P.*

hermaphrodita from the U.K. and the U.S. implies the U.K. strain was introduced to the U.S. (Howe *et al*., 2020).

Nematodes from the genus *Phasmarhabditis* are problematic to classify as there are some poorly described species but currently 18 species have been isolated from terrestrial gastropods including *P. apuliae, P. bohemica, P. bonaquaense, P. californica, P. circassica, P. clausilliae, P. hermaphrodita, P. meridionalis P. neopapillosa, P. papillosa, P, safricana, P. akhaldaba, P. kenyaensis, P. thesamica, P. quinamensis, P. zhejiangensis* and *P. tawfiki* and 1 species (*P. huizhouensis*) from rotting leaf litter (Azzam, 2003; Tandingan de Ley *et al*., 2017; Nermut' *et al*., 2016a,b, 2017; Gorgadze *et al*., 2022; Huang *et al.*, 2015; Ivanova and Spiridonov, 2017, 2020, 2021; Ivanova *et al*., 2021; Pieterse *et al*., 2020; Ross *et al*., 2018; Zhang and Liu, 2020). There are another two *Phasmarhabditis* species, including *P. nidrosienses* (isolated from marine habitat) and *P. valida* (isolated from littoral detritus) (Andrássy, 1983), but after a recent revision they were moved to the genus *Buetschlinema* (Sudhaus, 2011).

It is clear from the numerous surveys carried out over the last 25 years that *Phasmarhabditis* nematodes are commonly found in many countries from diverse terrestrial gastropod hosts. For example, *P. tawfiki* was isolated from the snail *Eobania vermiculata* and the slug *Limax flavus* in Egypt (Azzam, 2003). *P. bonaquaense* was found in the slug *Malacolimax tenellus* in the Czech Republic (Nermut' *et al.*, 2016a) and *P. apuliae* was isolated from slugs *Milax sowerbyi* and *M. gagates* from Italy (Nermut' *et al*., 2016b). Nermut' *et al.* (2017) described a new species (*P. bohemica*) from the Czech Republic found in *D. reticulatum*. *P. papillosa* has been isolated from *D. panormitanum* and *Tandonia sowerbyi* from the UK and *D. reticulatum* in the USA (Ross *et al*., 2010b; Tandingan De Ley *et al*., 2016) and South Africa (Pieterse *et al.*, 2017b). *P. neopapillosa* has been isolated from *D. reticulatum, D. panormitanum, L. flavus, Arion ater* and *Arion distinctus* in Scotland and England (Ross *et al.*, 2010b; Andrus and Rae, 2019a). Ross *et al*. (2018) also found a new species (*P. safricana*) from the slug *D. reticulatum* in South Africa. *P. californica* has been isolated from the U.S.A. from numerous species including *D. reticulatum, D. laeve, Arion hortensis* and *L. valentiana* (Tandingan De Ley *et al*., 2016), as well as being found in *Geomalacus maculosus* in Ireland (Carnaghi *et al.*, 2017) and from the snail *Oxychilus draparnaudi* in Wales (Andrus and Rae, 2019a) and Germany (Keyte *et al*., 2022). There are other studies that have identified new *Phasmarhabditis* species specifically from snails (see Morand *et al*., 2004, for further details). Recently, *P. meridionalis* was isolated from snails

(*Quantula striata*) in Vietnam (Ivanova and Spiridonov, 2017) and in 2019, *P. circassica* and *P. clausiliiae* were found in snails *Oxychilus* sp. and *Clausiliidae* sp., respectively in Russia (Ivanova *et al*., 2020).

There are several *Phasmarhabditis* species still awaiting description. For example, Waki (2017) found two *Phasmarhabditis* species in Japan, but could not identify them to species. Also two species (called "*Phasmarhabditis* sp. SA3" and "*Phasmarhabditis* sp. SA4") were isolated from slugs in nurseries in South Africa (Pieterse *et al*., 2017c). A possible *Phasmarhabditis* species was also isolated from the earthworm *Lumbricus terrestris* by Zaborski *et al.* (2001); however, it was only identified morphologically and was described as being virulent towards *L. terrestris*, which is highly unusual for a gastropod-parasitic nematode. Finally, Kiontke *et al*. (2007) isolated *Phasmarhabditis* sp. EM434 from North America. There is limited information on this species and amounts to only a few DNA sequences in the National Centre for Biotechnology Information (NCBI) database.

Out of all the currently described species, *P. hermaphrodita* (Wilson *et al.*, 1993), *P. neopapillosa* (Hooper *et al*., 1999; Glen *et al.*, 1996), *P. tawfiki* (Azzam and Tawfiki, 2003), *P. papillosa* (Pieterse *et al*., 2017b), *P. safricana* (Ross *et al.*, 2018) and *P. californica* (Tandingan De Ley *et al*., 2020) have been shown to kill slugs and snails. There are preliminary reports that *P. bohemica*, *P. bonaquaense* and *P. apuliae* can also kill slugs (Nermut' *et al*., 2019b). Taken together, these results demonstrate pathogenicity towards terrestrial gastropods is not confined to one species and is an evolutionary conserved trait across the genus *Phasmarhabditis*.

1.3 Life cycle of *P. hermaphrodita*

Phasmarhabditis hermaphrodita is a facultative parasite, able to kill several species of terrestrial gastropods and grow and reproduce on a variety of organic matter (Maupas, 1900; Tan and Grewal, 2001a; Macmillan *et al*., 2009) (Fig. 2). It is also able to infect larger host species e.g. *A. ater* where it will remain until the host dies and reproduce on the cadaver, termed "necromeny" by Schulte (1989) (Fig. 2). *P. hermaphrodita* is a hermaphroditic nematode and the occurrence of males in extremely rare (Andrassy, 1983), with Maupas (1900) finding only 1 male in 14,888 hermaphrodites.

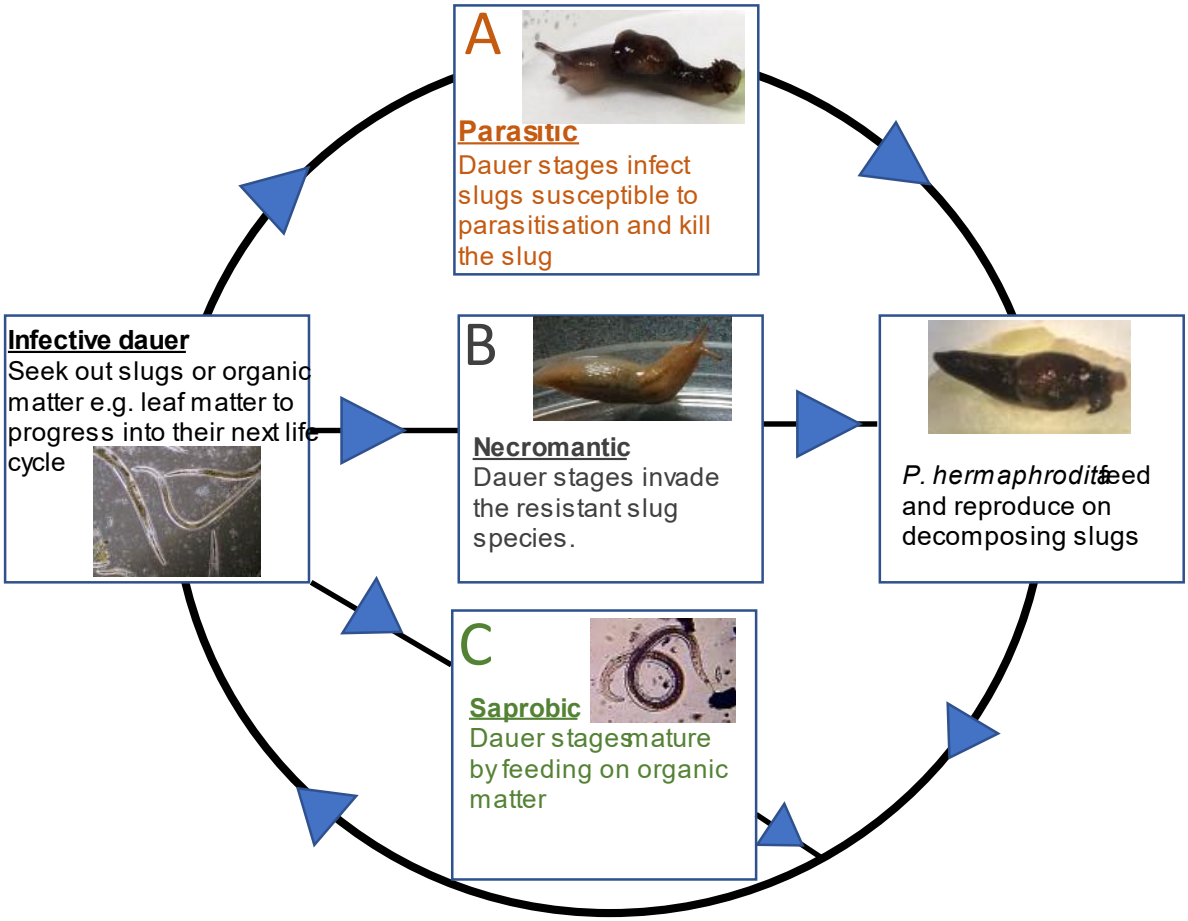

Fig 1.2. *P. hermaphrodita* (A) can complete its life cycle in three ways. It can parasitise and kill susceptible hosts such as *D. reticulatum* (B), infect resistant slug species such as *A. valentianus* (C) and wait for it to die (termed "necromeny"- Schulte, 1989) or reproduce on organic matter (C). In each case once the food supply has been depleted it will turn to the infective (dauer) stage and move through soil to find more hosts.

1.4 Chemoattraction of *P. hermaphrodita* to slug and snail host cues

In order to locate hosts *P. hermaphrodita* dauers seek out slugs in the soil by following mucus, faecal and volatile cues (Rae *et al.*, 2006, 2009a; Hapca *et al.*, 2007a-c; 2009; Nermut' *et al.*, 2012a). Nictation (where nematodes stand on their tail hoping to latch on to hosts passing by) and body waving has not been observed in *P. hermaphrodita*, potentially due to their long length (Kruitbos *et al*., 2009; Brown *et al*., 2011a). Alternatively these nematodes employ a "cruiser" based foraging strategy where they actively search for hosts following cues. In one of the first studies to investigate the chemotactic response of *P. hermaphrodita*, Rae *et al.* (2006) found that *P. hermaphrodita* was attracted to foot and mantle mucus of the pestiferous slug species *D. reticulatum* as well as slug faeces. As many

slug species display homing behaviour and return to the same location each night (Duval, 1972), faecal attraction of *P. hermaphrodita* may be beneficial for infecting new hosts. Volatile host cues such as CO_2 were found to be the least attractive cues to *P. hermaphrodita* (Rae *et al.*, 2006), potentially due to the vast quantities of CO_2 released by microorganisms in soil (Bradley and Wiel, 1999) but also due to *P. hermaphrodita* entering the slug host through the back of the mantle and not the respiratory pore (Wilson *et al.*, 1993). Hapca *et al.* (2007a,b) observed that there were significant differences in the speed of *P. hermaphrodita* movement, distribution of turning angles and the fractal dimension of nematode foraging trail when exposed to mucus from *D. reticulatum* on agar plates. Furthermore, research by Small and Bradford (2008) showed that *P. hermaphrodita* responded to mucus from an array of slug species. Specifically, they showed that when in contact with slug mucus, nematodes would show increased frequency and duration of head thrusting and head waving, but decreased duration and frequency of forward crawling. They found there was little difference between these behaviours when exposed to mucus from six susceptible or non-susceptible slug species. Rae *et al.* (2009a) found that *P. hermaphrodita* not only responded to mucus from *D. reticulatum* but was positively attracted to a wide range of diverse slug and snail species. Of these species tested, *P. hermaphrodita* showed a preference for slugs such as *Arion subfuscus*, *D. invadens* and the snail *Cornu aspersum*. These hosts represent a range of parasitic and necromenic life cycles. *P. hermaphrodita* was found to be more attracted to slugs opposed to earthworms (*L. terrestris* and *Eisenia hortensis*). Interestingly reproductive success of *P. hermaphrodita* was not greater on attractive species (Rae *et al.*, 2009a). In a similar experiment recently Laznik *et al.* (2022) recorded the chemotactic response of *P. papillosa* to slug mucus and found mucus from *L. maximus* and *C. aspersum* were particularly attractive compared to *A. vulgaris* and *D. reticulatum* (for reasons unknown).

All these studies have focused on using the commercial strain of *P. hermaphrodita* (strain DMG0001) that has been in culture since 1994. To gain more insight into how wild strains of *P. hermaphrodita* would behave Andrus and Rae (2019b) exposed several wild isolated strains of *Phasmarhabditis* species (*P. hermaphrodita*, *P. neopapillosa* and *P. californica*) to mucus from 7 different slug species. The wild strains differed in their preference to the slug species tested with *P. neopapillosa* preferring *Arion* sp. In a similar study Andrus *et al.* (2018) exposed *P. hermaphrodita*, *P. neopapillosa* and *P. californica* to mucus from snails. They found surprisingly, the commercial strain of *P. hermaphrodita* DMG0001 showed little chemotactic response and remained at the point of application

whereas a wild isolates of *P. hermaphrodita* and *P. californica* were attracted to mucus of *Cepaea nemoralis*, *Cepaea hortensis* and *Arianta arbustorum*. There is little information about what the exact compounds in slug and snail mucus *Phasmarhabditis* nematodes are attracted to; however, Andrus *et al*. (2018) exposed *P. hermaphrodita* to a range of metal ions (e.g., $MgCl_2$, $FeSO_4$) and hyaluronic acid and found that the nematodes showed strong attraction to 1% hyaluronic acid – an abundant component of slug mucus. Furthermore, Cutler and Rae (2021) demonstrated there is natural variation in the chemotactic response of wild strains of *P. hermaphrodita*, *P. californica* and *P. neopapillosa*.

The majority of chemotaxis experiments investigating the behaviour of *P. hermaphrodita* have been carried out on agar plates and therefore may not be applicable to their natural soil environment. Hapca *et al*. (2007b) used a more realistic experimental design and found speed, turning angle distribution, fractal dimension and mean square displacement were reduced when sand grains were placed on agar plates. Nermut' *et al.* (2012a) utilised both agar plates and sand filled olfactometers, and showed that *P. hermaphrodita* was attracted to dead slugs (*D. reticulatum*) but not faeces and mucus, and in soil olfactometers the nematodes were averted from dead slugs leading the authors to hypothesise that the large variety of decay gases caused *P. hermaphrodita* to suffer from a lack of oxygen and move away (Nermut' *et al.*, 2012a). Macmillan *et al.* (2009) used columns packed with different substrates and showed *P. hermaphrodita* moved best through organic matter, un-compacted soil and soil containing large aggregates. Dispersal of *P. hermaphrodita* was increased when placed in mineral soils with the earthworm *Lumbricus terrestris*. They also showed *P. hermaphrodita* (DMG0001) was unable to move through the soil column, but a wild isolated strain from Norway dispersed significantly more.

1.5 How *P. hermaphrodita* kills slugs - the questionable role of bacteria

Once *P. hermaphrodita* locates a slug host they enter through the back of the mantle and migrate to the shell cavity (Wilson *et al.*, 1993; Tan and Grewal, 2001a). Larvae then develop into self-fertilising hermaphrodites and start to reproduce (Wilson *et al.*, 1993; Tan and Grewal, 2001a). This produces characteristic signs of infection such as a swollen mantle and shell ejection (Fig. 3). Host death occurs 4-21 days after initial infection (Wilson *et al.*, 1993), and nematodes feed and reproduce on the cadaver. When the food source is depleted, dauer juveniles enter the soil to locate a new host.

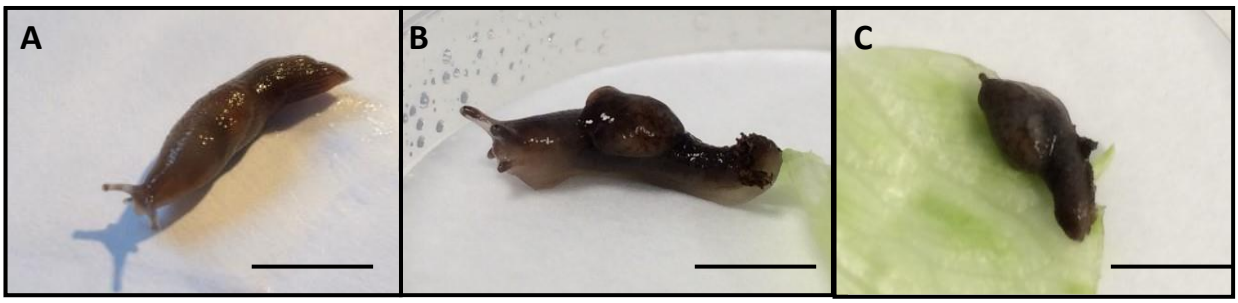

Fig 1.3. *P. hermaphrodita* produces characteristic signs of infection when parasitising pestiferous hosts such as *D. invadens* (A). Nematodes infect the slug through a pore in back of the mantle and reproduce, causing a swelling of the mantle area (B), this eventually leads to the internal shell being ejected from the mantle and death (C). Bars represent 0.5 cm in A and B and 1 cm in C.

It is currently unknown how *P. hermaphrodita* kills slugs. Early research focused on a paradigm similar to entomopathogenic nematodes (EPNs) and their symbiotic relationship with bacteria. EPNs of the families Steinernematidae and Heterorhabditidae associate with symbiotic bacteria (Steinernematidae with *Xenorhabdus* spp. and Heterorhabditidae with *Photorhabdus* spp.) that are responsible for killing host insects (Forst *et al.*, 1997). It was previously thought *P. hermaphrodita* functioned similarly to EPNs and acted as a vector for the bacterium *Moraxella osloensis*, and the host died due to septicaemia (Tan and Grewal, 2001b). When the first strain of *P. hermaphrodita* (DMG0001) was isolated an attempt was made to identify a bacterium that could be used for industrial production of these nematodes. Indeed, it is clear that bacterial diet, substrate and inoculation density can have dramatic effects on growth, lipid content and length of nematodes (Wilson *et al.*, 1995a,b; Nermut' *et al.*, 2012b, 2014). Initial studies focused on feeding *P. hermaphrodita* on bacteria from infected slugs and from *P. hermaphrodita* emerging from dead slugs (Wilson *et al.*, 1995a,b). Many different bacterial species were isolated and tested including: *Acinetobacter calcoaceticus*, *Aeromonas hydrophila*, *Aeromonas* sp., *Bacillus cereus*, *Flavobacterium breve*, *FIavobacterium odoratum*, *Moraxella osloensis*, *Providencia rettgeri*, *Pseudomonas fluorescens* (isolate no. 1a), *Pseudomonas fluorescens* (isolate no. 140), *Pseudomonas fluorescens* (isolate no. 141, *P. fluorescens* (pSG), *Pseudomonas paucimobilis*, *Serratia proteamaculans*, *Sphingobacterium spiritocorum* and *Xenorhabdus bovienii*. Successful feeding and growth of *P. hermaphrodita* has also been recorded on *Pseudomonas* sp.1, *Bacillus* sp. 1, *Escherichia coli* OP50 and *E. coli* BR (Andrus and Rae, 2019a). *Moraxella osloensis* was chosen as it produced consistently high yields of pathogenic nematodes

(Wilson *et al*. 1995a,b). It should be stressed that this bacterium was chosen for commercial production and does not reflect the natural tritrophic interactions that may be occurring between slugs, *P. hermaphrodita* and bacteria in the wild. A study by Rae *et al.* (2010) showed that *P. hermaphrodita*, when grown on rotting slugs or emerging after parasitising slugs (*D. reticulatum*), had no evidence of *M. osloensis* being present. Therefore, these nematodes do not vertically transmit this bacterium. Similarly, Nermut' *et al.* (2014) found that *P. hermaphrodita* strain (DMG0001) lost *M. osloensis* after repeated culturing. However, research has shown that injection of 40 and 60 hr cultures of *M. osloensis* into the haemocoel of *D. reticulatum* will kill slugs, with the 60 hr cultures being more pathogenic than the 40 hr cultures (Tan and Grewal 2001b). This is thought to be due to a lipopolysaccharide (LPS) which acts as an endotoxin (Tan and Grewal 2002, 2003) and *ubiS* and *dsbC* genes that are upregulated by *M. osloensis* when infecting *D. reticulatum* (An *et al.*, 2008). *M. osloensis* is only toxic to *D. reticulatum* when injected and showed no contact or oral toxicity to slugs (Tan and Grewal, 2003).

The relationship between *M. osloensis* and *P. hermaphrodita* has been categorised as 'symbiotic' yet there are compelling reasons why this may not be the case, which is out with the scope of this thesis (see Wilson and Rae, 2015 for further details). What is clear is *P. hermaphrodita* can grow on a multitude of different bacterial species and they can affect the numbers of offspring produced and the nematode's pathogenicity. Whether or not the nematode relies on a strict symbiotic relationship with one bacterium is a matter of debate. It would certainly be worth investigating what bacteria wild isolates of *P. hermaphrodita* associate within nature. Nermut' *et al*. (2014) found *P. hermaphrodita* DMG0001 and wild strains harboured a range of bacterial species including *Acinetobacter* sp., *Alcaligenes faecalis*, *Bacillus cereus* and *Stenotrophomonas* sp. were identified in both strains, while *Pseudomonas putida* was isolated from the wild strain only. Indeed Schurkman *et al*. (2022d) profiled the microbiome of wild *Phasmarhabditis* from California and found the most predominate bacterial species were *Shewanella*, *Clostridium perfringens*, Aeromonadaceae, Pseudomonadaceae and Actinetobacter. but they did not carry out any other experiments; therefore, it is difficult to come to any major conclusions about the role of bacteria in US strains of *Phasmarhabditis.* In contrast, a recent study by Sheehy *et al*. (2022) showed conclusively that *M. osloensis* is not present in infective juveniles of *P. hermaphrodita* (wild and commercial strains), *P. californica* and *P. neopapillosa* even though they had killed a slug. Furthermore, genotyping of the *M. osloensis* strains used by BASF Agricultural

Specialities used to grow *P. hermaphrodita* revealed the species was actually more closely related to *Psychrobacter faecaelis*, therefore there seems to be limited use of *M. osloensis* in the pathogencity process.

1.6 Reproduction

Upon host death, nematodes proliferate on the cadaver, and multiple factors can influence progeny dynamics. Rae *et al.* (2009a) found *P. hermaphrodita* grown on tissue from different species of slugs and snails yielded different numbers of offspring with *D. invadens* (*panormitanum*) producing the highest number of progeny followed by *L. marginatus, M. gagates, C. hortensis* and *D. reticulatum*. Nermut' *et al.* (2014) found development and quality of *P. hermaphrodita* was severely affected by growing substrate. *P. hermaphrodita* was able to successfully grow on multiple substrates including a mixture of homogenised pig kidney with different homogenised slug species (*Arion lusitanicus* and *D. reticulatum*) and homogenised moth (*Galleria mellonella*); *D. reticulatum* and *A. lusitanicus* faeces and leaf compost. The authors found the yield of *P. hermaphrodita* to be greater on invertebrate-based substrates; however, the quality of *P. hermaphrodita* produced remained stable based on body size and lipid size. Tan and Grewal (2001a) reported similar findings of dauer juveniles of *P. hermaphrodita* recovering and multiplying in slug and snail faeces homogenates but not soil samples. These results indicate that reproducing on an invertebrate substrate may have an evolutionary advantage, as it will produce higher numbers with the same quality of progeny as when it kills and reproduces on a host (Nermut' *et al.*, 2014). Similarly, Nermut' *et al*. (2022b) showed animal tissue based substrates enabled faster developmental time in *Phasmarhabditis* species such as *P. bohemica, P. bonaquaense* and *P. apuliae.*

Intraspecific competition for resources can also influence *P. hermaphrodita* development and quality (Nermut' *et al*., 2012b; 2014). Specifically, developmental time, lipid content, yield and body length can be affected by increasing numbers of conspecifics. However, nematodes may leave these areas of dense populations to find other resources (Nermut' *et al.*, 2012b). The time it takes for new infective juveniles can differ with species. For example, Pieterse *et al*. (2022) found *P. bohemica* had the shortest development cycle compared to *P. hermaphrodita, P. papillosa* and *P. kenyaensis* when grown on rotting slug (*D. invadens*) but it should be noted for industrial production *P. hermaphrodita* is best as it is the only hermaphrodite. As well as species, temperature can also severely affect the survival

and growth of *P. hermaphrodita*. Survival dramatically decreases at 25ºC and 35ºC but there is no difference at 5, 10 and 15ºC (Grewal and Grewal, 2003a) with the optimum growth temperature for *P. hermaphrodita* at 17°C (Wilson *et al*., 1993).

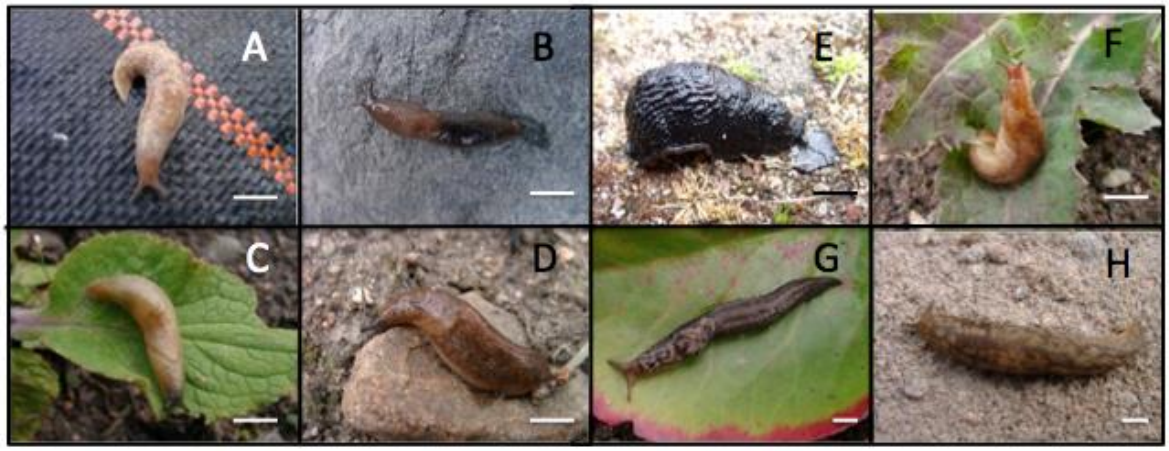

Fig 1.4. *P. hermaphrodita* can cause rapid mortality to the susceptible slugs *D. reticulatum* (A), *D. invadens* (B), *M. gagates* (C) and *T. sowerbyi* (D) but *A. ater* (E), *A. subfuscus* (F), *L. maximus* (G) and *L. flavus* (H) are resistant, for reasons unknown. Bars represent 0.5 cm.

1.7 Susceptibility of terrestrial gastropods to *P. hermaphrodita*

There are currently 21 species of slug and 18 species of snail that have been tested for their susceptibility to *P. hermaphrodita* under laboratory conditions (Fig 4, Table 1). To date, 13 slug species and 8 snail species can be killed by *P. hermaphrodita.* There is little research into understanding how *P. hermaphrodita* is able to kill terrestrial gastropods and very little information about why there is this difference in susceptibility of different species. Some studies have shown that younger stages of certain slug species are susceptible to *P. hermaphrodita* whereas adults are not including *A. lusitanicus* (Speiser *et al*., 2001; Grimm, 2002) (although it should be noted *P. papillosa* can supposedly kill this slug species according to Laznik *et al*. 2020); *A. ater* (Wilson *et al*., 1993, Rae *et al*., 2009a), *A. vulgaris* (Antzée-Hyliseth *et al*., 2020) and *C. aspersum* (Glen *et al*., 1996). It has also been recorded that *P. californica* can kill neonate *C. aspersum* but not adults (Grannell *et al*., 2021). Confusingly, studies that have carried out the same experiment have reported different results. For example, the Giant African snail (*Lissachatina fulica*), has been shown to be killed by a wild strain of *P. hermaphrodita* from the US (Mc Donnell *et al*., 2018b) but the commercial strain *P. hermaphrodita* DMG0001 has no negative effect on these snails

(Williams and Rae, 2015). Also Morley and Morritt (2006) reported the freshwater snail *Lymnaea stagnalis* was killed by *P. hermaphrodita* but Whitaker and Rae (2015) observed no mortality when repeating the same experiment. These differences could be due to whether the commercial strain of *P. hermaphrodita* or wild isolates were used in experiments or whether host snails were collected or cultured. For example, Morley and Morritt (2006) used a lab strain of *L. stagnalis* whilst Whitaker and Rae (2015) used wild collected *L. stagnalis*. Similarly, Williams and Rae (2015) used the commercial strain of *P. hermaphrodita* and exposed them to *L. fulica* whilst Mc Donnell *et al*. (2018b) used a wild strain of *P. hermaphrodita*. It is interesting to speculate why there are such differences, perhaps it could be due to continuous lab culturing, which can have severe effects on the phenotype of laboratory animals (Huey and Rosenzweig, 2009). Nematodes are no different. For example, traits such as heat, UV light and desiccation tolerance and reproductive potential have been shown to be reduced in *H. bacteriophora* through continuous culturing in *Galleria mellonella* (Wang and Grewal, 2002). The effect of continuous lab culturing in nematodes and hosts could therefore play a role in the differences found in these experiments

One common symptom of *P. hermaphrodita* infection is host feeding inhibition, which is strongly observed in slugs such as *D. reticulatum* and *D. invadens* but has also been observed in slug species it cannot kill (Wilson *et al*., 1993; Rae *et al*., 2009a). It has been suggested that rapid reduction in slug control in field trials is probably from host feeding inhibition as opposed to slug mortality (Glen *et al*., 1994, 2000; Wilson *et al*., 1994a). Feeding inhibition may be a defensive behaviour of slugs to contract and reduce the numbers of nematodes penetrating inside (Grewal *et al*., 2003b). Some species however, are not killed by *P. hermaphrodita* and their feeding is not inhibited e.g. *L. pseudoflavus* (Rae *et al*., 2008).

In contrast to slugs, the effect *P. hermaphrodita* has on snails has not been investigated in detail (although these nematodes have been isolated regularly from snails, see Morand *et al.*, 2004). *P. hermaphrodita* has been shown to cause high levels of mortality to snails (*Theba pisana, Trochoidea elegans* and *Monacha cantiana*) (Coupland *et al*., 1995; Tandingan de Ley *et al*., 2020; Genena and Mostafa, 2010). There are, however, many snail species resistant to infection by *P. hermaphrodita*. One reason for this may be due to the snail shell. An observation during an infection experiment using *P. hermaphrodita* and *L. fulica* found nematodes permanently fixed in the shell (Williams and Rae, 2015). Evidence of this process has also been shown in live *C. nemoralis* (Williams and Rae 2016) (Fig 5), *A. arbustorum* (Rae, 2018) and in museum collections of *C. aspersum* and *H. pomatia*

(Cowlishaw *et al*., 2019). This process is remarkably well conserved across the Stylommatophora and has been thought to be present when the two major clades diverged 80-130 MYA (Rae, 2017a) and nematodes have even been observed in the vestigial shell of the slug *L. pseudoflavus* (Rae *et al*., 2008). Nematodes have been infecting gastropods since they evolved in the late Cambrian (Grewal *et al*., 2003a) and this evolutionary arms race has resulted in slugs and snails co-opting their shell to encapsulate and encase parasitic nematodes instead of just using the shell for shelter (Rae, 2017a). Interestingly, dark morphs of the snail *Cernuella virgata* were found to be more resistant to *P. hermaphrodita* than light morphs but this was not due to phenoloxidase levels (Scheil *et al*., 2014). These authors did not dissect the snails or examine the shells for nematodes but perhaps this difference in susceptibility was due to the effectiveness of the shell morphs to encase invading nematodes?

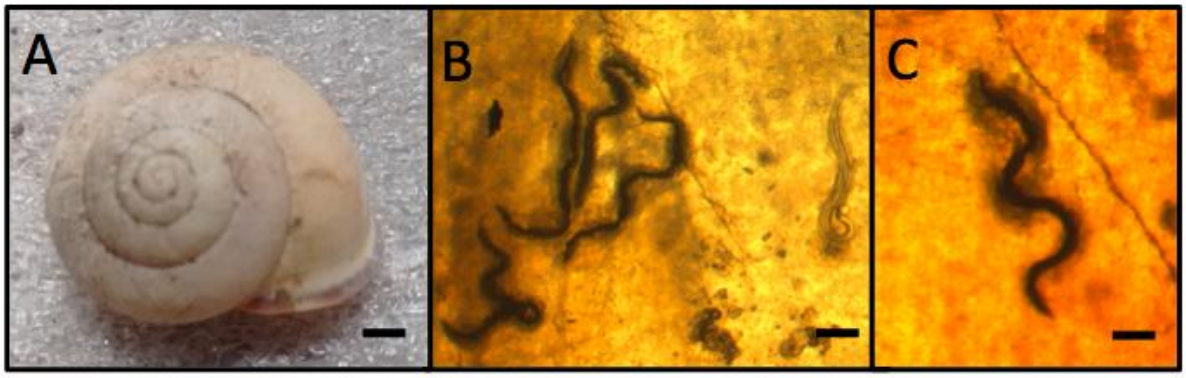

Fig 1.5: Snails such as *Cepaea nemoralis* (A) can be infected with *P. hermaphrodita* under lab and field conditions and are trapped, encased and killed in the shell (B and C). Scale bars in A represent 2 mm and 100 micrometers in B and C.

As well as use in agriculture *P. hermaphrodita* could be used to reduce snail populations that vector medically important parasites. Specifically, application of the nematode has been shown to negatively affect freshwater snails *Biomphalaria alexandrina* and *B. pfeifferi* (under lab conditions), which could potentially result in a diminished transmission of Schistosomiasis (Abou-Elnour *et al*., 2015; Okonjo *et al*., 2015). The potential of these nematodes to control *Biomphalaria* snails warrants significant attention and could be highly promising.

1.8 Host avoidance and behavioural manipulation

In order to reduce parasitism by *P. hermaphrodita* slugs avoid areas where nematodes are present. Avoidance behaviour is the first strategy an organism can employ to reduce the threat of parasitism (Curtis, 2014). Slugs such as *D. invadens* and *A. ater* are able to detect and avoid areas where *P. hermaphrodita* is present, and spend less time feeding and resting in such areas (Wilson *et al.*, 1999). Wynne *et al.* (2016) showed that several diverse slugs from three different families will avoid *P. hermaphrodita* but snails do not avoid the nematodes. They also showed that slugs avoided *P. hermaphrodita* specifically and would not avoid EPNs (*Steinernema kraussei*) or the vinegar eelworm (*Turbatrix aceti*) - both of which are not parasites of molluscs. Resistant slug species *A. subfuscus, A. hortensis* and *L. valentiana* avoid *P. hermaphrodita*, although *L. flavus* is also resistant to *P. hermaphrodita* infection and does not avoid the nematode (Morris *et al.*, 2018; Wynne *et al.*, 2016). Slugs do not avoid areas treated with the supernatant of a liquid suspension of *P. hermaphrodita* suggesting that the slugs are avoiding the mechanical stimulus of the nematodes probing the slug's body, rather than a chemical cue (Wynne *et al.*, 2016). However, when a slug is infected with *P. hermaphrodita* the usual avoidance behaviour is abrogated and slugs are more likely to be found on areas where *P. hermaphrodita* is present (Morris *et al.*, 2018). The exact reason why the nematodes are influencing slug behaviour is unclear, but it could allow for more successful reproduction, survival or dispersal (Morris *et al.*, 2018). It is unclear how *P. hermaphrodita* is able to manipulate slug behaviour; however, Morris *et al.* (2018) and subsequently Cutler *et al.* (2019) found that it could be linked to neurotransmitter signalling as slugs (*D. invadens*) fed fluoxetine or sertraline.were driven towards nematodes whereas infected slugs treated with cyproheptadine, which suppresses serotonin levels were not attracted to the nematodes. Uninfected slugs treated with apomorphine, which stimulates dopamine receptors, failed to avoid *P. hermaphrodita*, and infected slugs treated with a dopamine antagonist (haloperidol) no longer moved towards *P. hermaphrodita* (Cutler *et al.*, 2019). This suggests that *P. hermaphrodita* is somehow able to influence levels of biogenic amines to alter slug behaviour (Morris *et al.*, 2018; Cutler, *et al*., 2019).

Interestingly, *P. hermaphrodita* has been reported to have extreme effects on slug behaviour. For example, infected slugs eat less (Glen *et al*., 2000), are slower (Bailey *et al*., 2003), are more likely to be found under refuge traps (Wilson *et al*., 1994a), move underground to die (Pechova and Foltan, 2008), and infected freshwater snails are more likely to be found outside of the water (Morley and Morritt, 2006). Not only does *P.*

hermaphrodita influence host behaviour, it has been suggested they exhibit an anti-feeding effect on scavenging beetles (*Carabus nemoralis* and *Pterostichus melanarius*) by deterring them from dead, infected slugs where the nematodes are reproducing (Foltan and Puza, 2009). Whether the nematode is actively manipulating the behaviour of the slugs or this is a by-product of infection of sick slugs warrants further investigation.

1.9 The effect of *P. hermaphrodita* on non-target organisms

For its use as a biological control agent, the commercial strain of *P. hermaphrodita* has been tested against non-target beneficial invertebrates. As expected for a parasite of gastropods, *P. hermaphrodita* has been shown not to harm several insect species including *Tenebrio molitor* (Wilson *et al*., 1994b), *G. mellonella* (Wilson, M.J. unpublished) or *Pterostichus melanarius* (Wilson *et al*., 1993). The earthworms *L. terrestris, Eisenia fetida, E. hortensis, E. fetida, E. andrei* and *Dendrodrilus rubidus* are also unaffected by the nematode as well as the platyhelminth *Arthurdendyus triangulatus* (Grewal and Grewal, 2003b; DeNardo *et al*., 2004; Rae *et al*., 2005). Zaborski *et al.* (2001) reported a *Phasmarhabditis*-like nematode that potentially killed earthworms (e.g. *L. terrestris*) but there has been no subsequent research. This nematode was only identified morphologically and causing earthworm mortality would be highly unusual for a gastropod parasitic nematode. Another *Phasmarhabditis* species (*P. californica*) has also been exposed to earthworms (*L. terrestris* and *E. fetida*) as well as the insect larvae *T. molitor* and *G. mellonella* with no mortality of any species observed (Cutler and Rae, 2022).

The effect of *P. hermaphrodita* on non-target molluscs has also been investigated under lab conditions and in the field. From seven snail species commonly found in hedgerows, Wilson *et al.* (2000a) found that high doses of *P. hermaphrodita* caused mortality to just two (*M. cantiana* and *C. hortensis*). Also, over a two-year field trial Iglesias *et al*. (2003) found no effect of *P. hermaphrodita* on the snail species *Ponentina ponentina* and *Oxychilus helveticus* or on acarids, collembolans or earthworm populations. Therefore, the effect of *P. hermaphrodita* on non-target organisms seems limited.

1.10 Production and field application of *P. hermaphrodita*

1.10.1 Production

Consistent and efficacious control as well as cost, storage, delivery, handling and marketing are required for any worthwhile commercial nematode biocontrol product (Georgis *et al*., 2006). *P. hermaphrodita* has successfully been in production since 1994 by MicroBio, then Becker Underwood and now BASF Agricultural Specialities. *P. hermaphrodita* is grown in *in vitro* liquid culture with the bacterium *M. osloensis* (Wilson *et al*., 1995a,b) with upwards of 100,000 dauers per ml being produced (Glen *et al*., 1994). Monoxenic liquid culture of nematodes for mass production allows for more predictable and high virulent yields (Wilson *et al.,* 1995a,b, Ehlers and Shapiro-Ilan, 2005). After monoxenic fermentation, dauers are harvested. Young *et al*. (2002) investigated the most effective dauer recovery methodology and concluded using a combination of continuous phase density and flotation by adjustment was best. The same authors also found that the introduction of an air supply to break apart and clear insoluble spent media was recommended. To separate dauers and other life stages, the product can be sieved at an aperture size of 75-106 µl (Wilson *et al*., 2001) or by using vibrating membrane filtration (Wilson *et al.,* 2003). Centrifugation and repeated washing can also be used (Rae *et al*., 2007). After extraction, dauers are mixed with an inert gel polymer and packaged (Pieterse *et al*., 2017a).

1.10.2 Field use and application

Phasmarhabditis hermaphrodita is formulated into a water-dispersible formulation that can be suspended in water and applied to soil at a rate of 3 x 10^9 third stage infective juveniles (dauer larvae) per hectare (Rae *et al.,* 2007), via spraying equipment (Glen *et al*., 1994). *P. hermaphrodita* dauers have been applied successfully to crops via backpack sprayers and irrigation lines (Wilson and Gaugler, 2000). As well as being applied to the soil surface *P. hermaphrodita* can be incorporated into soil through cultivation to kill subterranean slugs though this has mixed results in terms of efficacy at reducing slug damage and slug numbers (Wilson *et al*., 1996). *P. hermaphrodita* has been used to successfully control slug damage in an array of plants including lettuce (Wilson *et al*., 1995c; Grubišić *et al.,* 2018), winter wheat (Wilson *et al*., 1994a; Wilson *et al.*,1996), oilseed rape (Speiser and

Andermatt, 1996; Kozolowski *et al*., 2012), cabbage (Grubišić *et al*., 2003, 2018; Kozolowski *et al*., 2012), asparagus (Ester *et al*., 2003a), Brussels sprouts (Ester *et al*., 2003b), glasshouse orchids (Ester *et al*., 2003c) and sugar beet (Ester and Wilson, 2005).

There have been few field trials using *P. hermaphrodita* since 2009 but many before: for a complete list of field trials and results see Rae *et al*. (2007). Failure to reduce slug damage has also been observed. For example, Wilson *et al.* (1995c) reported no reduction in slug damage in lettuce. Also, Iglesias *et al.* (2003) found *P. hermaphrodita* treated areas did not affect slug numbers contaminating the harvested crop (but did reduce slug damage). This is similar to Rae *et al*. (2009b) who also reported a lack of reduction in slug numbers in lettuce.

Other factors may influence the efficacy of *P. hermaphrodita* such as watering regime and earthworm activity, which was investigated in comparison to chemical controls by Dörler *et al.* (2019). No effect on slug feeding or mortality was seen, however this could be due to the use of the slug *A. vulgaris*, which is known to be resistant to *P. hermaphrodita* (Speiser *et al*., 2001; Grimm, 2002). It has however been suggested that failures could be avoided by following recommended protocols (Wilson *et al*., 2000).

The effect of treatments of crops before nematode application has also been investigated. Iglesias *et al*. (2001) found that when manure was applied prior to *P. hermaphrodita* dauer juveniles, they were rendered ineffective, possibly due to poor dauer survival, manure interfering with chemoreception or the manure attracting more slugs. In contrast, Vernavá *et al*. (2004) and France *et al*. (2002) found no effect of cover crops or lupin on the ability of nematodes to control slugs in the next crop planted.

Novel application strategies that improve efficiency and economic use of nematode biological control products will improve their attractiveness (Grewal *et al*., 2005) and have been investigated with *P. hermaphrodita*. Ester and Geelen (1996) found that the best control of slugs in sugar beet utilised nematode application and methiocarb pellets in furrow treatment. However Wilson *et al.* (2000) found methiocarb reduced nematode survival, but not infectivity. In spite of this, there is limited scope for this combination as methiocarb has since been banned (Jones, 2014; Nicholls, 2014). Multiple lower rate applications of *P. hermaphrodita* can sometimes offer better control (Ester and Wilson, 2005), or the same level of control as standard recommended broadcast rate (Ester *et al*., 2003a,b; Grewal *et al*., 2001; Rae *et al*., 2009b) but they require more time to achieve a reduction in slug damage (Hass *et*

al., 1999a,b; Iglesias *et al*., 2001). Lower application rates and concentration could be beneficial for larger areas of crop, as *P. hermaphrodita* can be applied via irrigation lines (Brown *et al*., 2011b), instead of broadcast application. Nematodes have also been applied in bands but offered no economic advantage over recommended broadcast application at the standard rate, possibly due to too few nematodes being applied (Hass *et al*., 1999a,b). Other application strategies such as dipping root plugs in a nematode/carboxymethyl cellulose solution have also been found to be successful therefore providing protection against slugs using a lower number of nematodes and reducing the cost (Rae *et al*., 2009b; Kozlowski *et al*., 2014). More targeted application methods have been proposed by Brown *et al*. (2011b) who described nematode application machinery (Wroot water Nemaslug xtra applicator) that injects nematodes onto irrigation water and aerates and agitates the nematode solution allowing nematodes to be applied over a longer time scale. In plots of hostas, targeted application of *P. hermaphrodita* to slug shelters at a reduced application rate provided similar protection to that of uniform broadcast application (Grewal *et al*., 2001). Similarly, damage to oilseed rape by *A. lusitanicus* was reduced for 25 days by spraying *P. hermaphrodita* on the plants at a rate of 2 x 10 nematodes/cm^2 (Jaskulska and Kozlowski, 2012). In order to optimise the numbers of *P. hermaphrodita* used for slug control several models have been developed (Glen *et al*. 2000; Hass *et al*. 1999b; Wilson *et al*. 2004; Schley and Bees, 2006).

1.11 Persistence and environmental factors affecting the success of *P. hermaphrodita* in the field

In order for *P. hermaphrodita* to be successfully used as a biological control agent, it must persist in soil after application. There is little research on this. However, soil type can affect the movement and persistence of *P. hermaphrodita* (MacMillan *et al*., 2006, 2009). The persistence of *P. hermaphrodita* in soil has been monitored using real time qPCR techniques (Macmillan *et al*., 2006) showing that the *P. hermaphrodita* population declines sharply after two weeks (Hatteland *et al*., 2013). However, in other studies survival of *P. hermaphrodita* has been recorded much longer. Nermut' (2012) found *P. hermaphrodita* could survive up to 5 months in wet sand, and even 8 months in garden soil and organic horticultural substrate. In field trials *P. hermaphrodita* can survive up to 6 weeks in soil (Kozlowska *et al*., 2014) and even up to 99 days (Vernavá *et al*., 2004). Under lab conditions, Grewal and Grewal (2003b) showed survival of *P. hermaphrodita* was best at 5, 10 and 15ºC

and osmotic desiccation in 10% glycerol could increase survival of the nematodes at temperature extremes.

Unfavourable abiotic and biotic conditions including UV light, temperature and desiccation affects nematode survival and persistence (Wilson and Gaugler, 2004). This can be reduced by cultivating the land immediately after nematode application (Wilson *et al.*, 1996; Hass *et al.,* 1999a). Nematodes are predated upon by mites, collembolans and fungi (Strong, 2002; Wilson and Gaugler, 2004). DNA analysis has shown mites and collembola e.g. *Heteromurus nitidus* eat *P. hermaphrodita* under lab conditions and in the field (Read *et al*., 2006; Heidemann *et al*., 2011; Fiera, 2014) and fungi have been speculated to affect the survival of these nematodes (Nermut', 2012).

With temperature increasing due to climate change the efficacy of *P. hermaphrodita* to control slugs may be affected. Wilson *et al.* (2015a) found evidence of this as slug feeding was not reduced in infected slugs as temperatures increased from 14°C to 24°C. It is thought *P. hermaphrodita* is well adjusted to the cooler climate of northern Europe (Glen and Wilson, 1997). However, Iglesias *et al.* (2001) found *P. hermaphrodita* could be used to reduce slug damage in warmer conditions in Spain, where the mean air temperature was 19.8 ± 2.6°C. The impact of temperature on the efficacy of *P. hermaphrodita* was also investigated through field trials using predicted winter warming conditions by El-Danasoury and Iglesias-Piñeiro (2017). They found damage to plants and slug survival was much lower in the predicted wintering conditions than under normal wintering conditions. Therefore, *P. hermaphrodita* may perform better at controlling slug damage under winter warming conditions.

1.12 Combining chemical and biological control methods with *P. hermaphrodita*

There is evidence to show *P. hermaphrodita* combined with other methods could enhance slug control. In 2007 the efficacy of combining infection of *P. hermaphrodita* with exposure to cadmium and *Bacillus thuringiensis* (BT) to the snail *H. aspersa* was investigated (Kramarz *et al*., 2007). The growth rate of *H. aspersa* was reduced by both BT and cadmium and increasing doses of *P. hermaphrodita* (Kramarz *et al*., 2007). Also, the snails exposed to the highest doses of *P. hermaphrodita* accumulated the highest level of cadmium (Kramarz *et al*., 2007).

The repellent effect of Birch tar oil (BTO) has been investigated and suggested for possible complementary use with *P. hermaphrodita* to control *A. arbustorum* and *A. lusitanicus* (Lindqvist *et al*., 2010). The authors found that BTO repels *A. arbustorum* and *A.*

lusitanicus in confined heavily nematode infested areas and repeated application of BTO over several weeks was required to deter *A. lusitanicus* with weekly treatments offering the best slug control.

Other more novel strategies have been investigated. *P. hermaphrodita* has been used in combination with wasp venom from *Pimpla hypochondriaca* to kill and inhibit feeding of *D. reticulatum* (Richards *et al*., 2008). The authours concluded that together with *P. hermaphrodita* the venom can be more effective than *P. hermaphrodita* on its own and is more successful at causing slug fatality and significantly reducing slug feeding. One of the suggested strategies for future studies is by releasing the venom into the slug haemocoel by genetically engineering *P. hermaphrodita* to express individual venom factors (Richards *et al*., 2008).

More recently the behaviour and feeding of *Tetanocera elata* fly larval, (a parasitoid and predator of slugs) has been explored and its potential for use with *P. hermaphrodita* (Ahmed *et al*., 2019). The results demonstrate *T. elata* larvae suffer in development and pupariation if feeding from an infected slug with only 20% pupating. Oddly, the larvae did however show a preference for slugs previously infected with *P. hermaphrodita.* Ultimately further work is needed to examine if they can provide a consistently efficient synergistic level of slug control.

1.13 Aims of thesis

The overall aim of the thesis was to investigate what abiotic and biotic factors could influence the efficacy of the *P. hermaphrodita* in its ability to reduce slug damage in the field. As there are reports in the literature of *P. hermaphrodita* failing to provide protection against slugs (e.g. Wilson *et al*. 1995c; Iglesias *et al.*, 2003; Rae *et al*., 2009b) and anecdotal reports from gardeners associated with the Royal Horticultural Society (Jones, pers. comm.) it is imperative to try and understand what the reasons for these failures actually are. To do this the primary aims of the thesis were to:

1. Examine the effect of 6 different soil types on the survival, movement and efficiency at killing slugs. These soils were sourced from two different RHS gardens (Wisley and Harlow Carr) and two soils were used (turf and garden loam). This aim was explored in Chapters 2 and 3.

I also recorded whether nematodes could be used in conjunction with other methods of slug control, which focused on essential oils – a novel and effective method, that has received little attention for use in controlling terrestrial gastropods, with the aim:

2. To discover whether *P. hermaphrodita* could be used in conjunction with essential oils to provide superior control against slugs in the lab, under semi-field conditions and in a field trial. This aim consisted of Chapters 4, 5 and 6.

In a final experiment I looked at the influence of the microbiome in the grey field slug *D. reticulatum*, a highly pestiferous species. The aim consisted of Chapter 7:

3. To elucidate the microbiome of the grey field slug and discover whether it is manipulated by *P. hermaphrodita*

Ultimately, these experiments have highlighted the previously unknown influence of soil on the ability of *P. hermaphrodita* to control slugs, as well as suggesting a new essential oil, which could be used in slug control as well as giving an overview of the mechanistic insight of the microbiome of slugs and how it is shaped by *P. hermaphrodita.* The far-reaching conclusions of this research are summarised in my discussion in Chapter 8.

This research has already resulted in published papers in peer reviewed journals. I contributed to Barua *et al*. (2020) (see Appendix 1), where I helped test whether 13 essential oils were able to kill beneficial nematodes. Also, I developed a slug culturing protocol (Chapter 4), which was recently published (MacDonald-Howard *et al*., 2021) (see Appendix 2). The microbiome study (Chapter 7) is currently under review in Microbiology Open and the results of Chapter 2 and 5 are in preparation, soon to be submitted as papers.

Chapter 2: The effect of different soil types and temperature on the survival of *P. hermaphrodita*

2.1 Introduction

In field studies using various crops *P. hermaphrodita* has been shown to provide protection against slugs such as asparagus (Ester *et al*., 2003a), lettuce (Wilson *et al*., 1995c) and Chinese cabbage (Wilson *et al*., 1999); however, there are some studies that have recorded failure of *P. hermaphrodit*a in providing slug protection. Size and presence of slugs that are not susceptible to the nematode are the main contributors for the lack of protection. Weight and age of slugs can cause varying success of infection in different species (Speiser *et al*., 2001; Grimm, 2001; Glen *et al*., 1996), for example only small *Arion lusitanicus* (<0.15g) are killed by *P. hermaphrodit*a, but larger specimens are not (Grimm, 2001).

As well as non-susceptible slug species present in field trials, abiotic and biotic factors (e.g., soil type, temperature and moisture) can severely affect the success of nematode biological control agents in controlling pests (Campos-Herrera, 2015). There is remarkably little information about how soil type and temperature can affect the survival of *P. hermaphrodita* after application. A study by Nermut' (2012) found *P. hermaphrodita* could persist in wet sand for at least five months and organic horticultural substrate and garden soil for up to 8 months. However, there is no information about how different soils affect the survival of *P. hermaphrodita*.

Here I assessed the survival of *P. hermaphrodita* when added to different soil types (compost with and without peat, clay loam, loam, sandy loam and sandy soil), at three different temperatures (5, 10 and 15°C) over 48 days. These results could assist in explaining why there are variable reports on the use of *P. hermaphrodita* in the field.

2.2 Materials and methods

2.2.1 Source of soils and nematodes

Six different soil types were used in the experiment (compost with and without peat, clay loam, loam, sandy loam and sandy soil). Soil was collected from two Royal Horticultural Society (RHS) gardens (Harlow Carr in Harrogate and Wisley in Woking). At each RHS garden there were two sample areas, one from an established garden bed and one collected from under an area of turf. At Harlow Carr RHS Gardens, sandy clay loam soil was collected from a garden bed and a sandy clay loam soil was collected from under turf. At Wisley RHS

Gardens, sandy loam soil was collected from a garden bed and a sandy soil from under an area under turf. Soil analysis was conducted on the four soils and can be seen in Appendix 3A,B. For the final two soil types (compost with and without peat) were purchased from local garden centres (there was no soil analysis carried out on either compost). To prevent seasonal variance of soil structure and composition, the samples were collected mid-November 2018 and again in mid-November 2019. Fresh samples were used for each repetition of the experiment.

P. hermaphrodita (Nemaslug®, strain DMG0001) were purchased from an online retailer and stored at 10°C until use. Nematodes were used within one month of arrival.

2.2.2 Assessing the effect of different soils and temperatures on the survival of *P. hermaphrodita*

Soils were either autoclaved at 121°C for 15 mins with a cooling rate of 40 minutes at 80°C or frozen at -20°C for 5 days to eliminate any resident nematodes. Both approaches were used as autoclaving can affect the physical structure of soil (Berns *et al*., 2008; Tanaka *et al*., 2003) and freezing soils represented more realistic conditions. After autoclaving and freezing, the soils were rewetted to 10-15% water content which was monitored using the Xiaomi Flower Care monitoring system throughout the entire experiment. The system monitors the temperature and soil humidity. Five cm Petri dishes were filled to the lip with each soil. To each Petri dish 2,000 *P. hermaphrodita* infective juveniles were applied and then incubated at 5, 10 or 15°C. After 3, 6, 12, 24 and 48 days the nematodes were extracted and the number of live infective stage and non-infective stage nematodes were quantified. Three replicate plates were used for each time point.

To quantify the number of live nematodes they were extracted by adding the soil from individual Petri dishes into 50 ml Falcon tubes and then half filling with tap water. The Falcon tubes were then shaken vigorously for 2 min and three 1 ml subsamples were pipetted into a 5 cm Petri dishes with a grid on the bottom. This technique uses a similar method as Circular Estimate Method developed as a simple method to estimate *Caenorhabditis elegans* culture densities in liquid medium (Josende *et al*., 2019). This process was repeated for each of the three Petri dishes used on each time point over 48 hrs.

2.2.3 Data analysis

A Generalised Linear Model (GLM) with Turkey's post hoc test was used to compare the number of alive infective stage (and non-infective stage) *P. hermaphrodita* exposed to the six different soils (compost with and without peat, clay loam, loam, sandy loam and sandy soil), that were either frozen or autoclaved, at three temperatures (5, 10 or 15°C), over five time points (3, 6, 12, 24 and 48 days).

2.3 Results

2.3.1 Survival of infective dauer stage *P. hermaphrodita* in autoclaved soils incubated at 5, 10 and 15°C over 48 days

In autoclaved soils at 5°C there was a significant interaction between the effects of time and soil type on the survival of infective stage *P. hermaphrodita* ($F(20, 240) = 19.649$, $P < 0.001$) (Fig 6A). *P. hermaphrodita* survival was highest in sandy clay loam soil from Harlow Carr (from under a garden bed) and compost (without peat) compared to the other soils ($P < 0.001$) (Fig 6A). The number of surviving nematodes was lowest in the sandy loam soil from Wisley with only approximately 100 *P. hermaphrodita* alive after 12 days, but their mean survival was not significantly different from sandy soil from Wisley ($P = 0.091$) or sandy clay loam from Harlow Carr (from under turf) ($P = 0.232$) but was significantly different from sandy clay loam soil from Harlow Carr (from under garden bed), compost with or without peat ($P < 0.001$).

At 10°C there was a statistically significant interaction between the effects of time and soil on the survival of infective stage *P. hermaphrodita* ($F(20, 240) = 9.123$, $P < 0.001$, Fig 6B). Similar to the survival of nematodes at 5°C, the survival of the nematodes was significantly higher in compost without peat than all other soils ($P < 0.001$) (Fig 6B). For the other soils, the survival of the nematodes was largely similar. For example, there was no difference between the survival of *P. hermaphrodita* in sandy loam soil and sandy soil (both from Wisley) ($P = 0.474$), sandy clay loam soil from a garden bed ($P = 0.623$) and sandy clay loam soil from turf (both from Harlow Carr) ($P = 0.488$). Similarly, for sandy soil from Wisley there was no difference among it and the survival of *P. hermaphrodita* in sandy loam soil from Wisley ($P = 0.474$), sandy clay loam from Harlow Carr (under turf) ($P = 1$) and compost (with peat) ($P = 0.119$).

At 15°C there was a statistically significant interaction between the effects of time and soil on the survival of nematodes ($F(20, 240) = 24.175$, $P < 0.001$, Fig 6C). Like the

previous results for the survival of nematodes at 5°C and 10°C, the compost (without peat) provided the most successful substrate for nematode survival with significant difference between it and all other soils ($P < 0.001$) (Fig 6C). There was no difference in the survival of nematodes added to the sandy loam soil from Wisley, sandy soil from Wisley, sandy clay loam soil from Harlow Carr (both from under turf and garden bed and compost (with peat) 48 days ($P > 0.05$) (Fig 6C).

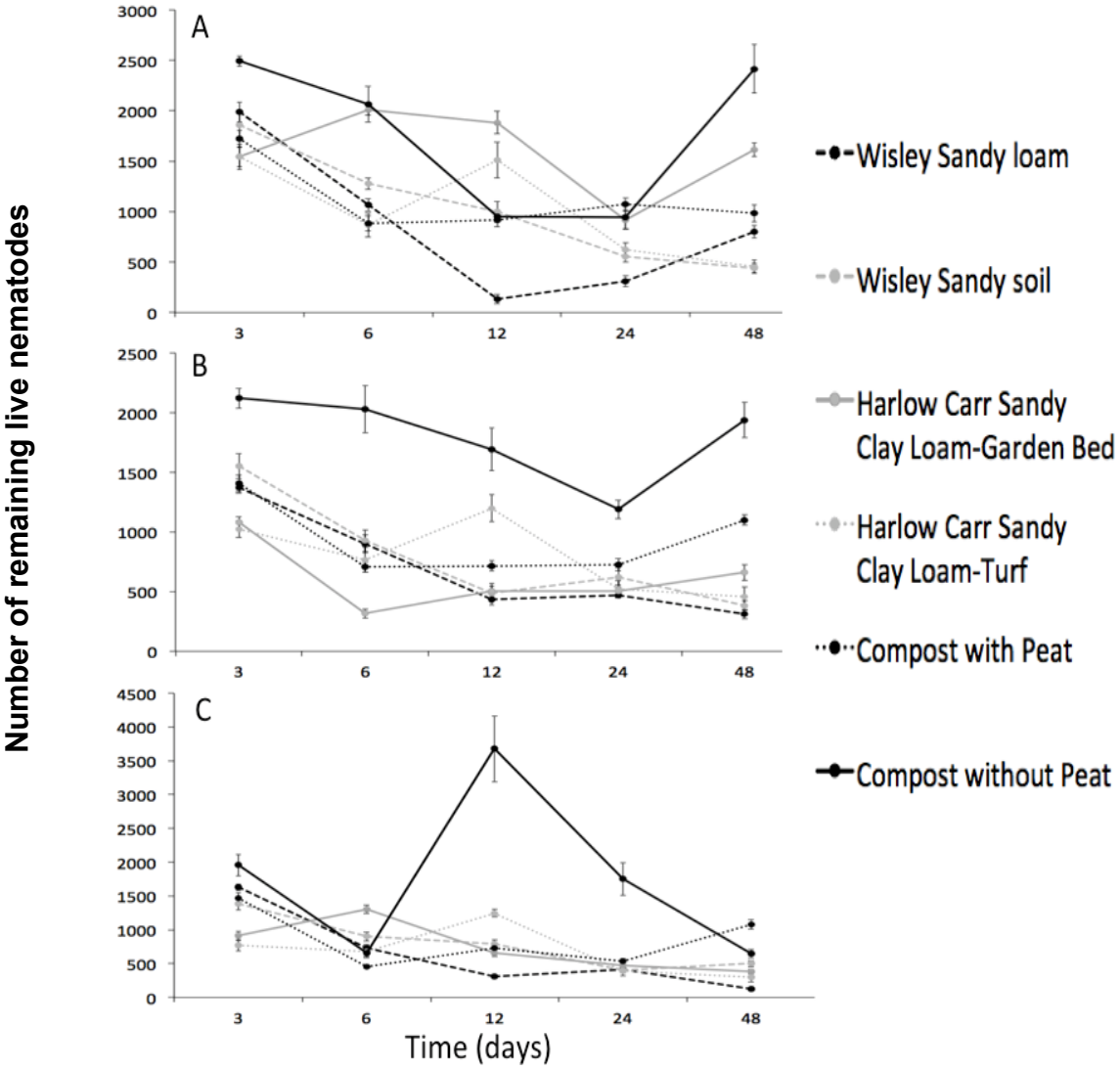

Fig 2.1: The mean number of infective stage *P. hermaphrodita* present in six different previously autoclaved soils including Wisley garden bed (long dash black line), Wisley turf (long dash grey line), Harlow Carr garden bed (solid grey line), Harlow Carr (short dash grey line), compost with peat (short dash black line) and compost without peat (solid black line) at 5°C (A), 10°C (B) and 15°C (C) over 48 days. N=3, n=9 for each soil type. Bars represent ± one standard error.

2.3.2 Number of non-infective stage *P. hermaphrodita* in autoclaved soils incubated at 5, 10 and 15°C over 48 days

Surprisingly, when counting the number of nematodes at each time point in some of the soils at 5°C it was clear the nematodes had molted, exited the infective juvenile stage and had begun to reproduce, as numerous other life stages were present in the autoclaved soils. The number of nematodes in these stages differed with the soil and over time and there was a significant interaction with each variable ($F(20, 240) = 111.037$, $P < 0.001$, Fig 7A). Specifically, in soils such as sandy soil from Wisley, compost (with peat), sandy clay loam (from turf and garden bed) from Harlow Carr there was no significant difference between the numbers of non-infective stage nematodes growing in each soil. The maximum number found was in sandy loam from Harlow Carr on day 12 (266.67 ± 60.1) and the lowest was in day 24 in sandy soil from Wisley (11.11 ± 7.35). This soil was poor for nematodes exiting the infective juvenile stage with 0 nematodes in other life stages found on all time points. In contrast the nematodes were able to exit the infective juvenile stage and reproduce prolifically, especially in the case with peat free compost where nematode numbers increased exponentially over time with the number of non-infective stage nematodes being significantly different from all soils ($P < 0.001$) (Fig 7A).

The experiment carried out at 10°C yielded similar results. There was a statistically significant interaction between time and soil on the number of non-infective stage nematodes ($F(20, 240) = 129.533$, $P <0.001$, Fig 7B). The number of non-infective stage nematodes was highest in peat free compost and was significantly different to all other soils ($P < 0.001$, Fig 7B). Also the number of non-infective nematodes growing in sandy loam soil from Wisley was significantly different to sandy soil from Wisley ($P < 0.001$), sandy clay loam from under turf from Harlow Carr ($P = 0.009$), compost (with peat) ($P = 0.002$) and compost (without peat) ($P < 0.001$) but not sandy clay loam soil (from a garden bed in Harlow Carr) ($P = 0.541$). The lowest number of nematodes produced was in sandy soil from Wisley, but the numbers did not differ from sandy clay loam from turf in Harlow Carr ($P = 0.216$) or compost (with peat) ($P = 0.5$).

At the highest temperature of 15°C there was a statistically significant interaction between the effects of time and soil on the survival of nematodes ($F(20, 240) = 334.769$, $P < 0.001$, Fig. 7C). The number of non-infective stage nematodes was highest in peat free compost and was significantly different from all other soils ($P < 0.001$, Fig. 2C). The number of nematodes reached 9622.22 ± 344.4 in 12 days. In contrast, sandy soil from Wisley

produced the lowest number of non-infective stage nematodes as there were significant differences between it and sandy loam soil (also from Wisley) (P = 0.004), sandy clay loam soil from Harlow Carr from a garden (P < 0.001), and from turf (P = 0.034), compost (with peat) (P = 0.010) and compost (without peat) (P < 0.001).

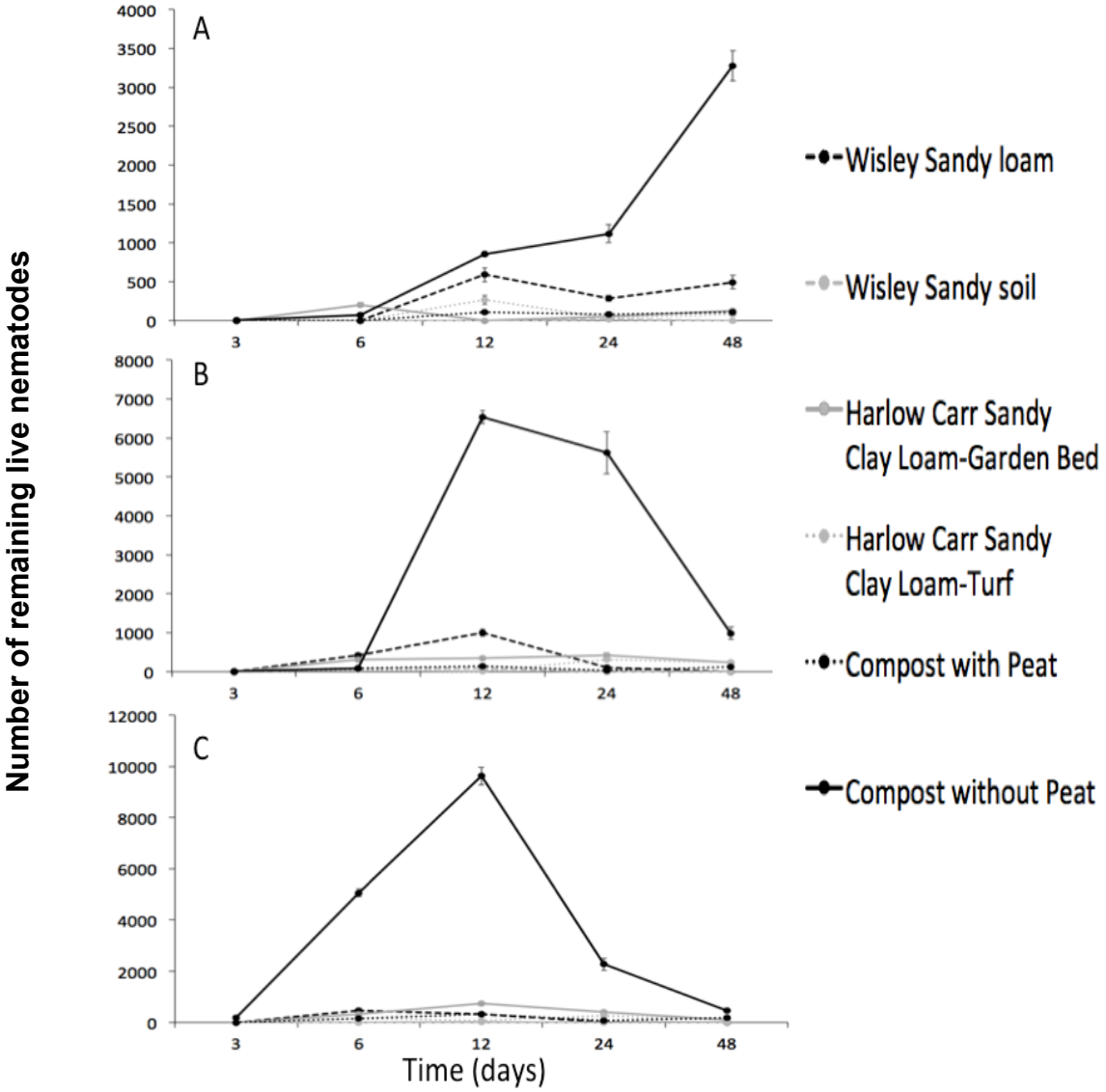

Fig 2.2: The mean number of non-infective stage *P. hermaphrodita* added to six different previously autoclaved soils including Wisley garden bed (long dash black line), Wisley turf (long dash grey line), Harlow Carr garden bed (solid grey line), Harlow Carr (short dash grey line), compost with peat (short dash black line) and compost without peat (solid black line) at 5°C (A), 10°C (B) and 15°C (C) over 48 days. N=3, n=9 for each soil. Bars represent ± one standard error.

2.3.3 Survival of infective dauer stage *P. hermaphrodita* in frozen soils incubated at 5, 10 and 15°C over 48 days

At 5°C there was a statistically significant interaction between the effects of time and soil type on the survival of infective stage nematodes exposed to the previously frozen soils ($F(20, 240) = 7.012$, $P < 0.001$, Fig 8A). The nematodes survived best in compost with and without peat and there was a significant difference between both substrates and all the other soils tested ($P < 0.001$) (Fig 8A). Out of the two, compost (without peat) proved to the best substrate for nematode survival with the mean number of surviving *P. hermphrodita* being significantly higher than compost (with peat) ($P < 0.001$) (Fig 8A).

At 10°C there was a statistically significant interaction between the effects of time and soil on the survival of nematodes ($F(20, 240) = 13.106$, $P < 0.001$, Fig 8B). Similar to the results above, compost (without peat) and compost (with peat) were best for nematode survival and there were significantly higher number of nematodes compared to the other soils ($P < 0.001$). Of these two, compost without peat was best, and was significantly different from compost with peat ($P < 0.001$). Sandy soil from Wisley was the poorest performing soil with significantly lower number of *P. hermaphrodita* surviving compared to all other soils ($P < 0.001$). Sandy loam soil from Wisley and sandy clay loam from under turf from Harlow Carr performed to a moderate level and there was no significant difference between them ($P = 0.906$) but there was between them and the other soils ($P < 0.001$).

At 15°C there was a statistically significant interaction between the effects of time and soil on the survival of nematodes ($F(20, 240) = 10.263$, $P < 0.001$, Fig 8C). Similar to the results for 5°C and 10°C compost (without peat) was the best substrate for nematode survival as there was a significant difference between the number of surviving *P. hermaphrodita* and all other soils ($P < 0.001$). There was no significant difference in the number of surviving nematodes applied to sandy soil from Wisley, sandy clay loam soil (from turf and garden bed) from Harlow Carr ($P > 0.05$) apart from sandy loam soil from Wisley ($P < 0.05$).

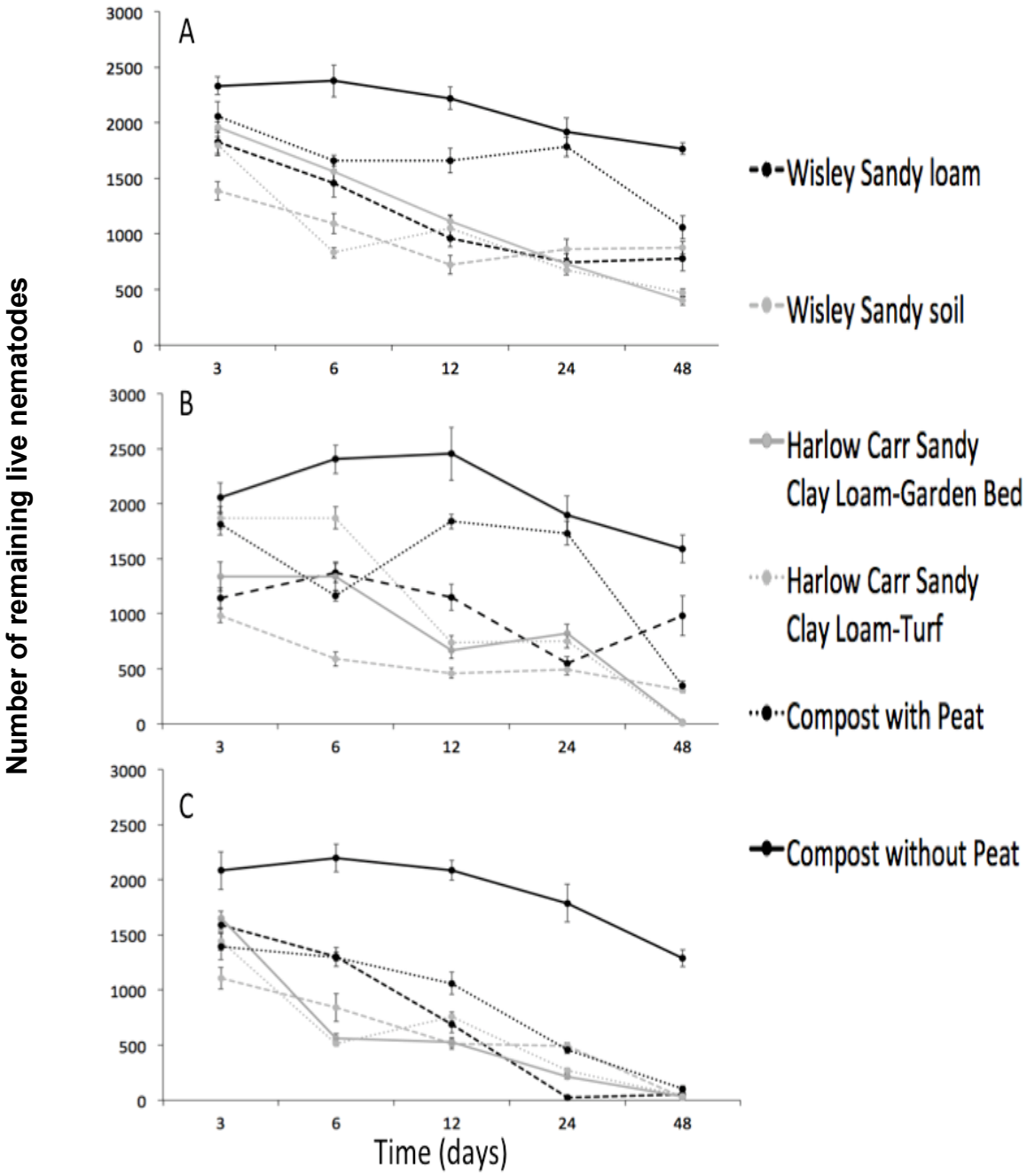

Fig 2.3: The mean number of infective stage *P. hermaphrodita* added to six different previously frozen soils including Wisley garden bed (long dash black line), Wisley turf (long dash grey line), Harlow Carr garden bed (solid grey line), Harlow Carr (short dash grey line), compost with peat (short dash black line) and compost without peat (solid black line) at 5°C (A), 10°C (B) and 15°C (C) over 48 days. N=3, n=9 for each soil. Bars represent ± one standard error.

2.3.4 Number of non-infective stage *P. hermaphrodita* in frozen soils incubated at 5, 10 and 15°C over 48 days

At 5°C there was a statistically significant interaction between the effects of time and soil type on the number of non-infective stage *P. hermaphrodita* added to previously frozen soil ($F(20, 240) = 40.651$, $P < 0.001$, Fig 9A). The number of non-infective stage nematodes fluctuated dramatically and differed significantly with soil type ($P < 0.001$). For example, the number of non-infective nematodes was highest in sandy clay loam soil from a garden bed in Harlow Carr (compared to all other soils) after 6 days reaching a pinnacle of 1022.2 ± 64.1 ($P < 0.001$). However, on day 12 the number of nematodes was highest for those in sandy soil from Wisley (1011.1 ± 69.1) and there were significant differences between it and all soils ($P < 0.05$). On day 24 the number of non-infective nematodes was highest in sandy loam soil from Wisley and differed significantly from all other soils ($P < 0.05$). Unlike in autoclaved soils, the number of non-infective stage nematodes in peat free compost was negligible and produced the lowest number of nematodes (55.6 ± 17.6 on day 12).

At 10°C there was a statistically significant interaction between the effects of time and soil on the number of non-infective stage *P. hermaphrodita* ($F(20, 240) = 24.397$, $P <0.001$, Fig 9B). The highest number of non-infective nematodes was in sandy clay loam from turf from Harlow Carr, which was significantly different to all other soils ($P < 0.001$). The number of nematodes in sandy loam soil from Wisley was also high with 444.44 ± 53 nematodes produced on day 24, which was significantly different from sandy soil from Wisley ($P < 0.001$) and sandy clay loam from Harlow Carr from turf ($P < 0.001$) but not clay loam soil from a garden bed in Harlow Carr ($P = 0.982$), compost (with peat) ($P = 0.367$) or compost (without peat) ($P = 0.933$). The soil that produced the lowest number of nematodes was sandy soil from Wisley. The nematodes did not exit the infective stage and the number of non-infective stage nematodes was significantly different from all other soils ($P < 0.001$).

At 15°C there was a statistically significant interaction between the effects of time and soil on the number of non-infective stage *P. hermaphrodita* ($F(20, 240) = 72.501$, $P < 0.001$, Fig 9C). At the highest experimental temperature both sandy clay loam from turf and garden bed from Harlow Carr produced the highest number of non-infective stage nematodes, which each being significantly different to all other soils ($P < 0.001$). Similar to the results of the experiments with lower temperatures the number of non-infective stage nematodes was lowest in Wisley sandy soil but this did not differ from sandy loam from Wisley ($P > 0.05$).

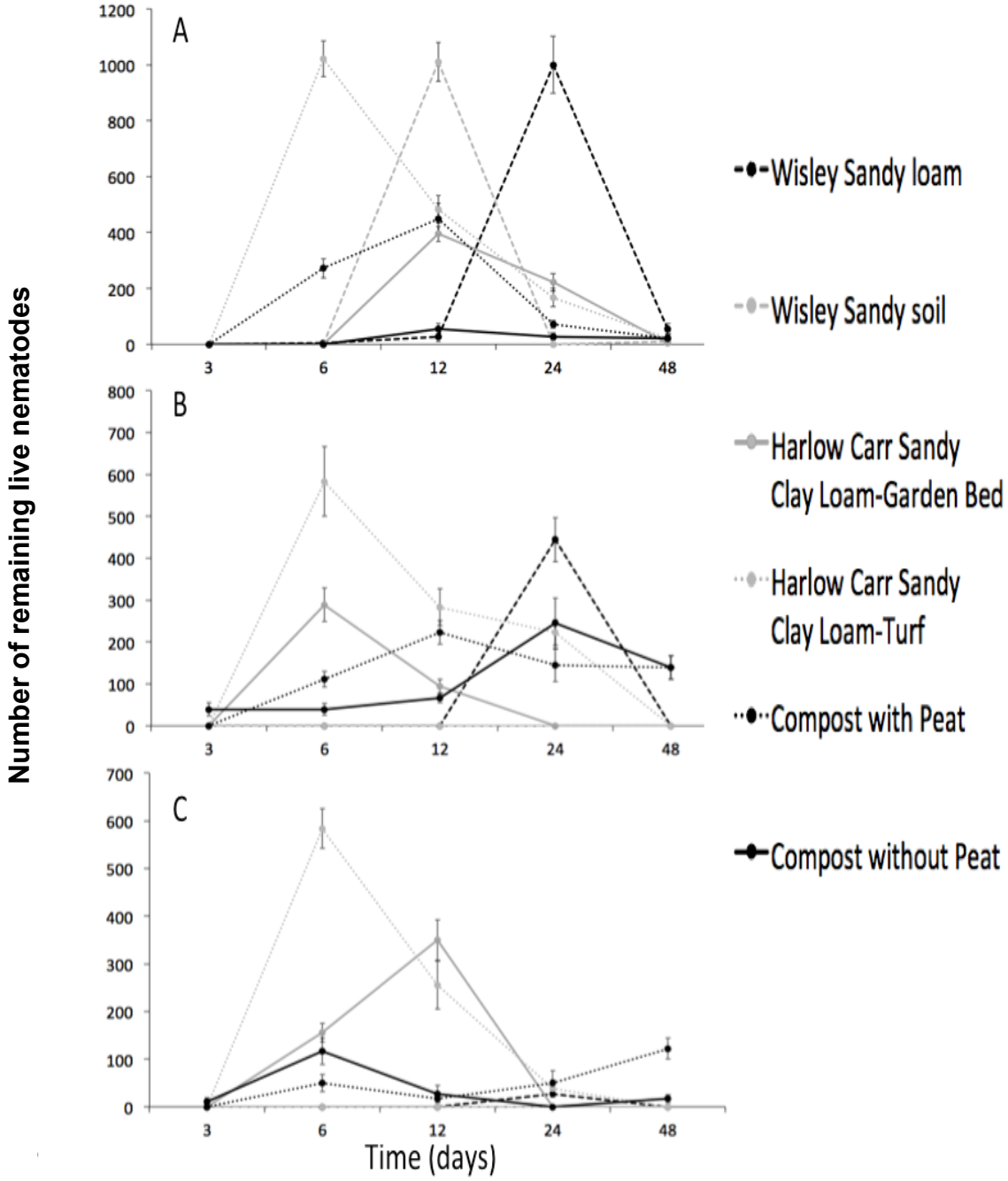

Fig 2.4: The mean number of non-infective stage *P. hermaphrodita* added to six different previously frozen soils including Wisely garden bed (long dash black line), Wisley turf (long dash grey line), Harlow Carr garden bed (solid grey line), Harlow Carr (short dash grey line), compost with peat (short dash black line) and compost without (solid black line) at 5°C (A), 10°C (B) and 15°C (C) over 48 days. N=3, n=9 for each soil. .. Bars represent ± one standard error.

2.4 Discussion

Upon application of nematodes in the field, Smit (1996) proposed a model whereby nematodes experience quick decline (40 to 90% within hours or days of application), then a more steady decrease then the population is maintained at low levels due to infection and reproduction in hosts. The reasons for the rapid decrease in population is due to exposure to UV light, desiccation, exposure to parasites and pathogens as well as the physical properties of soil e.g. temperature, oxygen, moisture retention and texture (Griffin, 2015; Smits, 1996; Wilson and Gaugler, 2004). For example, *S. riobrave* and *Heterorhabditis bacteriophora* persisted longer in high silt and clay soil (80 and 15%, respectively) compared to sandy soils (>93% sand) (Shapiro-Ilan *et al*., 2000). Kung *et al*. (1990) showed survival of *S. glaseri* and *S. carpocapsae* was lowest in clay then silty clay, sand or sandy silt. In a field experiment, Toepfer *et al*. (2004) found the efficiency of *H. bacteriophora*, *H. megidis* and *S. feltiae* to kill western corn rootworm (*Diabrotica virgifera virgifera*) was best in heavy clay or silty clay soil rather than sandy soils. Kung *et al*. (1990) showed survival in soil differed with soil type. Specifically, survival of *S. carpocapsae* was best in sandy loam, while *S. glaseri* was best in sand. Increasing amounts of clay had a detrimental effect on nematode survival of both species. Other than clay and silt content, physical characteristics of soil (e.g. bulk density) can also affect the survival of nematodes. For example, Portillo-Aguilar *et al*. (1999) showed survival of *H. bacteriophora*, *S. carpocapsae* and *S. glaseri* was severely affected by increasing bulk densities of sandy loam soil. Williams *et al*. (2013), in a meta-analysis of studies on *Heterorhabditis downesi* and *S. carpocapsae* to control the large pine weevil (*Hylobius abietis*) - a cryptic pest that breeds under the bark of stumps – showed that neither weevil population density nor species of stump, but rather soil type (peat versus mineral) was the most important (and only significant) factor determining efficacy; with peat soils being more efficacious than mineral.

MacMillan *et al*. (2006, 2009) showed soil type could affect the movement and persistence of *P. hermaphrodita.* In general, once applied to soil the persistence of *P. hermaphrodita* population declines sharply after two weeks (Hatteland *et al*., 2013). However, Nermut' (2012) found *P. hermaphrodita* could survive up to 5 months in wet sand, and even 8 months in garden soil and organic horticultural substrate. In field trials *P. hermaphrodita* can survive up to 6 weeks in soil (Kozlowska *et al.*, 2014) and even up to 99 days (Vernavá *et al*., 2004). Under lab conditions we showed *P. hermaphrodita* could survive

for 48 days in a selection of soils but *P. hermaphrodita* survived best in compost (without peat) compared to the other soils. This is in agreement with other studies, for example, Herren *et al*. (2018) discovered the more mature the compost, the better the survival of EPNs. They even suggested EPNs could be applied in infected cadavers in compost as an environmentally friendly method, which could be more beneficial than applying nematodes via water. Also, Khumalo *et al*. (2021) showed the addition of organic soil amendments e.g. mulch; compost or potting mix was beneficial for EPN survival (*H. bacteriophora*) as it prevented moisture loss. Compost without peat also resulted in prolific reproduction of *P. hermaphrodita*. However compost with peat did not have such an effect, with survival similar to the other soils and the nematodes did not exit the infective stage. There is little research on the effect of peat on nematode survival, growth and reproduction but fulvic acids from a peat bog inhibited reproduction of *Caenorhabditis elegans* (Steinberg *et al*., 2002). Perhaps this inhibitory effect is due to such acids.

In this study I have shown *P. hermaphrodita* will exit the infective juvenile stage and reproduce in substrates such as compost without peat. This may seem surprising but the nematode has been show to reproduce in leaf litter (MacMillan *et al*., 2009), earthworms (Rae *et al*., 2009), and slug faeces (Tan and Grewal, 2001b). This is an important difference between EPNs and *P. hermaphrodita* in terms of lifestyle that needs to be addressed. Principally, when added to soil EPNs will never exit the infective juvenile stage when added to a medium such as compost as they can only reproduce on their symbiotic bacteria harboured in their intestine. However, *P. hermaphrodita* is a facultative parasite able to reproduce on an array of bacteria (Wilson *et al*., 1995a,b; Andrus *et al*., 2018). Non-infective stage *P. hermaphrodita* do not infect slugs (Tan and Grewal, 2001a), therefore would be unable to reduce slug populations however, it is promising the nematodes managed to reproduce to effectively the subsequent generations developed into infective juveniles.

Temperature can also severely affect the survival of nematodes in soil (Stuart *et al*., 2015) and *P. hermaphrodita* is no different. It was previously known that survival of *P. hermaphrodita* dramatically decreases at 25ºC and 35ºC (Andrus and Rae, 2019a) but there is no difference at 5, 10 and 15ºC (Grewal and Grewal, 2003b) with the optimum growth temperature for *P. hermaphrodita* at 17°C (Wilson *et al*., 1993). However, we found regardless of temperature (5, 10 and 15ºC) or whether the soils had been autoclaved or frozen, the substrate that was best for nematode survival was compost without the addition of peat. The nematodes were able to exit the infective juvenile stage in this substrate too and

proliferate. Surprisingly, when the soils were frozen and we counted the number of non-infective stage nematodes this was not the case and sandy clay loam and sandy soils, sandy soil as well as compost (with peat) allowed nematodes to reproduce. The reasons behind this different in nematode growth and reproduction depending on sterilisation regime are unknown.

I concentrated on using the commercial strain of *P. hermaphrodita* (called DMG0001), which has been in commercial production for 25+ years. There is nothing known about the survival of other *Phasmarhabditis* species in soil or if different strains of *P. hermaphrodita* may survive for longer. This is worthwhile investigating as in intra and inter species variation in survival in soil has been reported in EPNs. Shapiro-Ilan *et al*. (2006) found *S. diaprepesi* and *S. carpocapsae* survived well in a loamy sand soil compared to *H. mexicana* and *H. indica*. They also observed considerable variation in persistence between strains of *S. carpocapsae*. Also, Hass *et al*. (2002) found differences in survival of three *Heterorhabditis* isolates (*H. bacteriophora*, *H. megidis* and *H. downesi*) over 141 days added to a loam soil, and these differences were attributed to lipid content. Also, though we examined the number of surviving *P. hermaphrodita* over time, we have no information about how infective or pathogenic these nematodes are, which is commonly monitored in persistence studies with EPNs (e.g. Khumalo *et al*., 2021). Similarly, it would be worthwhile examining the survival of *P. hermaphrodita* that had killed slugs, instead of the Nemaslug® product as these nematodes would be important in recycling in slug hosts.

In summary, survival of *P. hermaphrodita* is dependant on soil type, with peat-free compost being the best soil. Surprisingly, the nematodes readily exited the infective juvenile stage in many soils, which could prove problematic for slug control. Therefore, farmers and gardeners that use *P. hermaphrodita* should check soil type before application, if not, suboptimal slug control may occur.

Chapter 3: The effect of soils on movement and lethality of *P. hermaphrodita*

3.1 Introduction

The efficacy of nematodes to be able to kill hosts (as well their survival) can be severely affected by soil properties e.g. temperature, oxygen, moisture retention and texture (Griffin, 2015; Smits, 1996; Wilson and Gaugler, 2004). This has been poorly researched for *Phasmarhabditis* nematodes but is well known for EPNs. For example, Toepfer *et al*. (2010) showed the efficiency of *H. bacteriophora*, *H. megidis* and *S. feltiae* to kill western corn rootworm (*D. virgifera*) was best in heavy clay or silty clay soil rather than sandy soils (of reasons unknown). The only research to look at the properties of soils and how this affects *Phasmarhabditis* nematodes was by Macmillan *et al*. (2006), who did not look at efficacy of the nematodes to kill slugs *per se*, but did find *P. hermaphrodita* was capable of growth on leaf litter, moved poorly in mineral soils and seemed to disperse best in clay loam. Interestingly, results in Chapter 2 show *P. hermaphrodita* infective juveniles added to compost (without peat) developed into non-infective stage nematodes, but whether or not this affects the efficacy of these nematodes to kill slugs remains to be discovered. Thus, the aim of this chapter was to investigate whether different soils (used in the previous chapter) would affect the ability of *P. hermaphrodita* to kill slugs.

3.2 Materials and methods

3.2.1 Source of invertebrates

D. invadens and *D. reticulatum* were chosen as suitable slug hosts to test as they are highly pestiferous species, common in the Merseyside area with a worldwide distribution (Hutchinson, 2014). *D. invadens* were collected from a garden in Maghull, Merseyside (grid reference SD370011), and *D. reticulatum* were collected from field and woodland near the *D. invadens* site. Slugs were stored in non-airtight containers and fed lettuce or carrot *ad libitum*. Slugs were examined before the experiment for any signs of nematode infection e.g. swollen mantle, lesions on the cuticle and if they did display such symptoms they were discarded.

As the commercial strain of *P. hermaphrodita* (DMG0001) sometimes shows variable efficacy at infecting and killing slugs "*P. hermaphrodita* MG2" (a wild strain collected by Andrus and Rae (2019a), which has been kept in culture at LJMU since 2014) was used. The nematodes were grown up using standard liquid media (Wilson *et al*. 1995a,b) supplied by Dr. James Cutler.

3.2.2 Bioassay used to monitor the survival of *D. invadens* exposed to *P. hermaphrodita* MG2 added to six different soils

The infection bioassay used to test whether the lethality of *P. hermaphrodita* MG2 differs with different soils was based on Cutler *et al*. (2020) and Sheehy *et al*. (2022). Briefly, 30 ml universal tubes were filled up to a level of 3.5 g with each soil type. The soil types varied in composition and weight therefore the level of 3.5 cm was used to enable controlled comparisons. Eighteen universal bottles were used for each soil and were split into 9 used for studying the survival of slugs exposed to nematodes and the other 9 were used as untreated controls, with slugs added but with no nematodes, just water. To half of the universal bottles 1000 *P. hermaphrodita* MG2 were added to the soil, then two *D. invadens* were added (mean weight 0.20 g ± 0.031, n =144) and a piece of moist cotton wool was added on top and the lid loosely placed on top and stored at 10°C for 5 days. After this, slugs were removed and individually placed on 5 cm Petri dishes with pre-moistened filter paper and a disc of lettuce (3.5 cm in diameter). The survival of the slugs was monitored and after 10 days the amount the slugs had eaten was quantified by tracing the remnants of the lettuce onto 1 x 1 mm^2 graph paper (Rae *et al*., 2009).

3.2.3 Movement of *P. hermaphrodita* and *P. neopapillosa* through six different soils with *D. reticulatum* as an attractant

Plastic 50 ml Falcon tubes were cut into three sections (0 to 3.5 cm, 3.5 to 7 cm and 7 to 9.5 cm) and half filled with one of six soils used in the previous experiment to a height of 1.5 cm. Soils were placed in the -80°C for 24 hours to kill any resident nematodes. To the first section 2,000 infective stage nematodes (either *P. californica* or *P. neopapillosa*) were added in 1 ml of water to the top of the soil. Two slugs (*D. reticulatum*) were added to the third section as an attractant for the nematodes and a disc of lettuce and carrot was also added. A layer of fine netting was added to prevent the slugs from moving into the other sections. The controls for the experiment included the same set-up with lettuce and carrot discs added but no slugs were placed in the tube. All sections were securely fitted back together using Parafilm®. The tubes were stored in an incubator set at 15°C for 7 days.

Soil moisture was monitored using a Xiaomi Flower Care monitoring system. After 7 days, the sections were separated and the soil from each section was placed in individual 50 ml Falcon tubes. Fifty mls of tap water added, the mixture was homogenised using a vortexer and three 1 ml samples were removed and the numbers of nematodes was quantified using a

dissecting microscope. Counts of nematodes were calculated as the total number of nematodes per 50 ml (by multiplying the average in 3 mls by 50). There were 6 tubes for each of the 6 soils (3 with nematodes, 3 without) and the whole experiment was repeated 3 times.

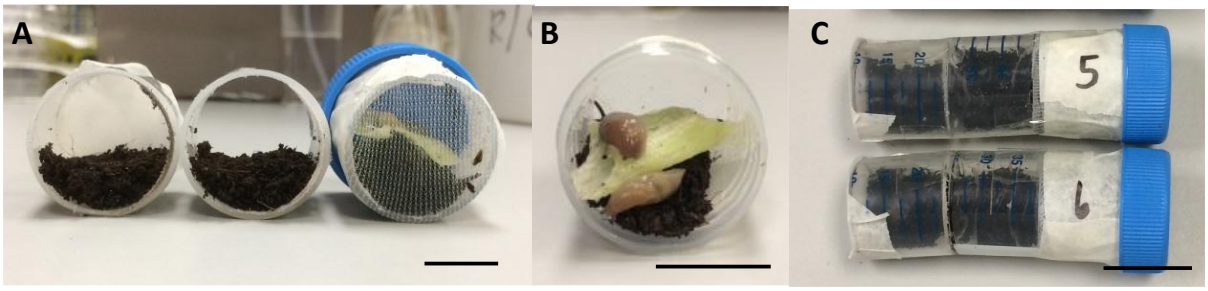

Fig 3.1. The three-divide sections each with compost substrate 1. Initial application point of nematodes. 2. Middle section. 3 attractant section including 2x *D.invadens* and a disc of lettuce contained behind a net to prevent slugs moving. Scale bar represents 1.5cm (A). Slug in section 3 (B) Scale bar represents 1.5cm. Sections 1,2,3, fixed together after nematode application Scale bar represents 3.5cm (C).

3.2.4 Data analysis

The survival of *D. invadens* exposed to *P. hermaphrodita* MG2 added to the six different soils was compared using a Log Rank test in OASIS (Yang *et al*., 2012). The number of 1 x 1 mm^2 squares of lettuce the slugs ate in each soil was compared using a One Way ANOVA and Tukey's test. A One Way ANOVA and Tukey's test was also used to compare the numbers of *P. hermaphrodita* and *P. neopapillosa* found in sections 0 to 3.5 cm, 3.5 to 7 cm and 7 to 9.5 cm in each of the six soils with and without a slug added.

3.3 Results

3.3.1 Survival of *D. invadens* exposed to *P. hermaphrodita* MG2 applied to different soils

The addition of *P. hermaphrodita* MG2 to each soil resulted in significantly more slugs dying (Fig 10A) compared to the untreated control (Fig 10B) over 15 days ($P<0.05$). Two soils resulted in slugs dying faster. *P. hermaphrodita* MG2 added to compost with peat resulted in *D. invadens* dying faster than turf from Wisley and also turf from Harlow Carr ($P<0.05$; Fig 10A). There was no difference in the survival of *D. invadens* exposed to water (untreated control) in the different soils over 15 days (Fig 10B).

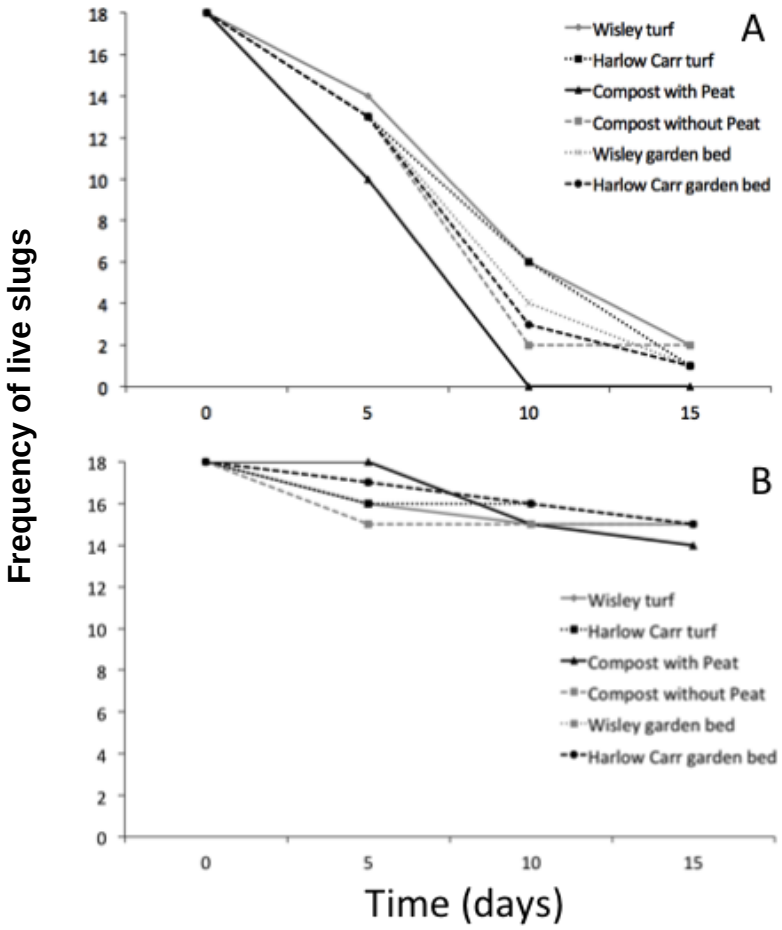

Fig 3.2: Frequency of live slugs exposed to *P. hermaphrodita* MG2 applied to six different soils over 15 days (A) or exposed to water (untreated control) (B), n=54.

3.3.2 Feeding behaviour of *D. invadens* exposed to *P. hermaphrodita* MG2 applied to different soils

Exposure of *P. hermaphrodita* MG2 to *D. invadens* resulted in severe feeding inhibition with the number of 1 x 1 mm^2 squares of lettuce eaten being significantly different from the untreated control for each soil ($P<0.05$; Fig 11). There was no difference in the number of 1 x 1 mm^2 squares eaten by *D. invadens* added to the nematode exposed soils ($P>0.05$; Fig 11).

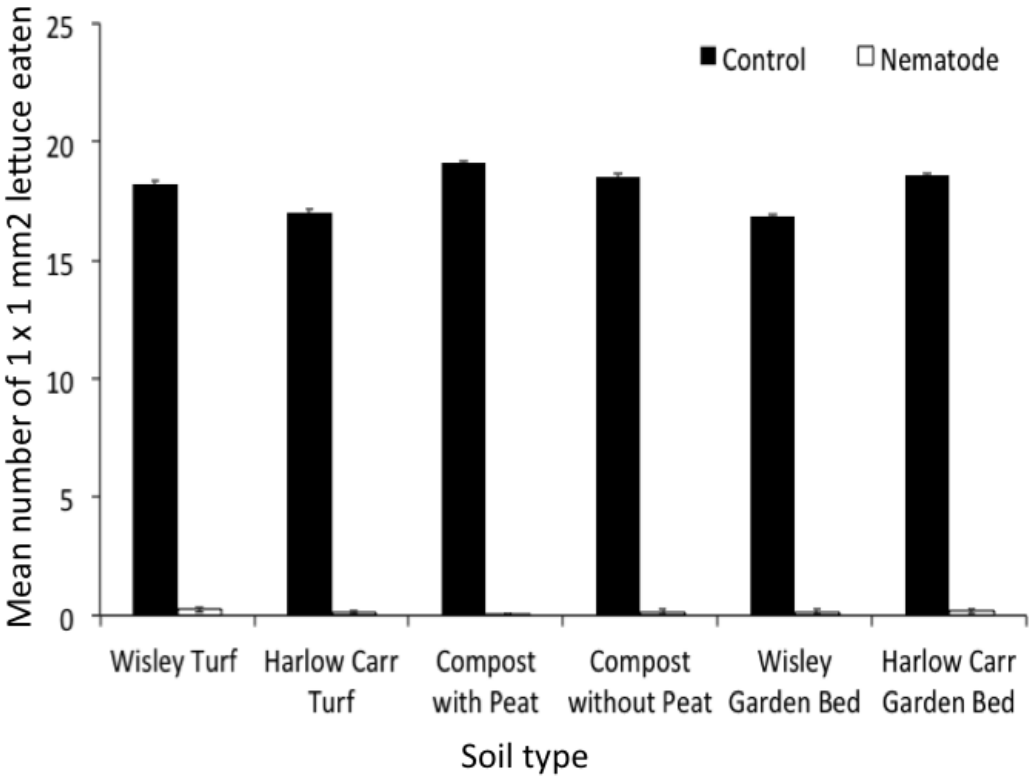

Fig 3.3: Mean number of 1 x 1 mm^2 lettuce eaten by *D. invadens* exposed to *P. hermaphrodita* MG2 or water (untreated control). Bars represent ± one standard error. n=54.

3.3.3 Movement of *P. hermaphrodita* and *P. neopapillosa* through six different soils with *D. reticulatum* as an attractant

There was a highly significant difference between the numbers of *P. hermaphrodita* (DMG0001) found in section 0 to 3.5 cm, 3.5 to 7 cm and 7 to 9.5 cm when applied to each of the six soils with all nematodes remaining at the point of application ($P<0.001$; Fig 12). The presence of a slug in the 7 to 9.5 cm section did not encourage *P. hermaphrodita* to move towards it (Fig 12A). This was also the case for *P. neopapillosa* (Fig 13A,B).

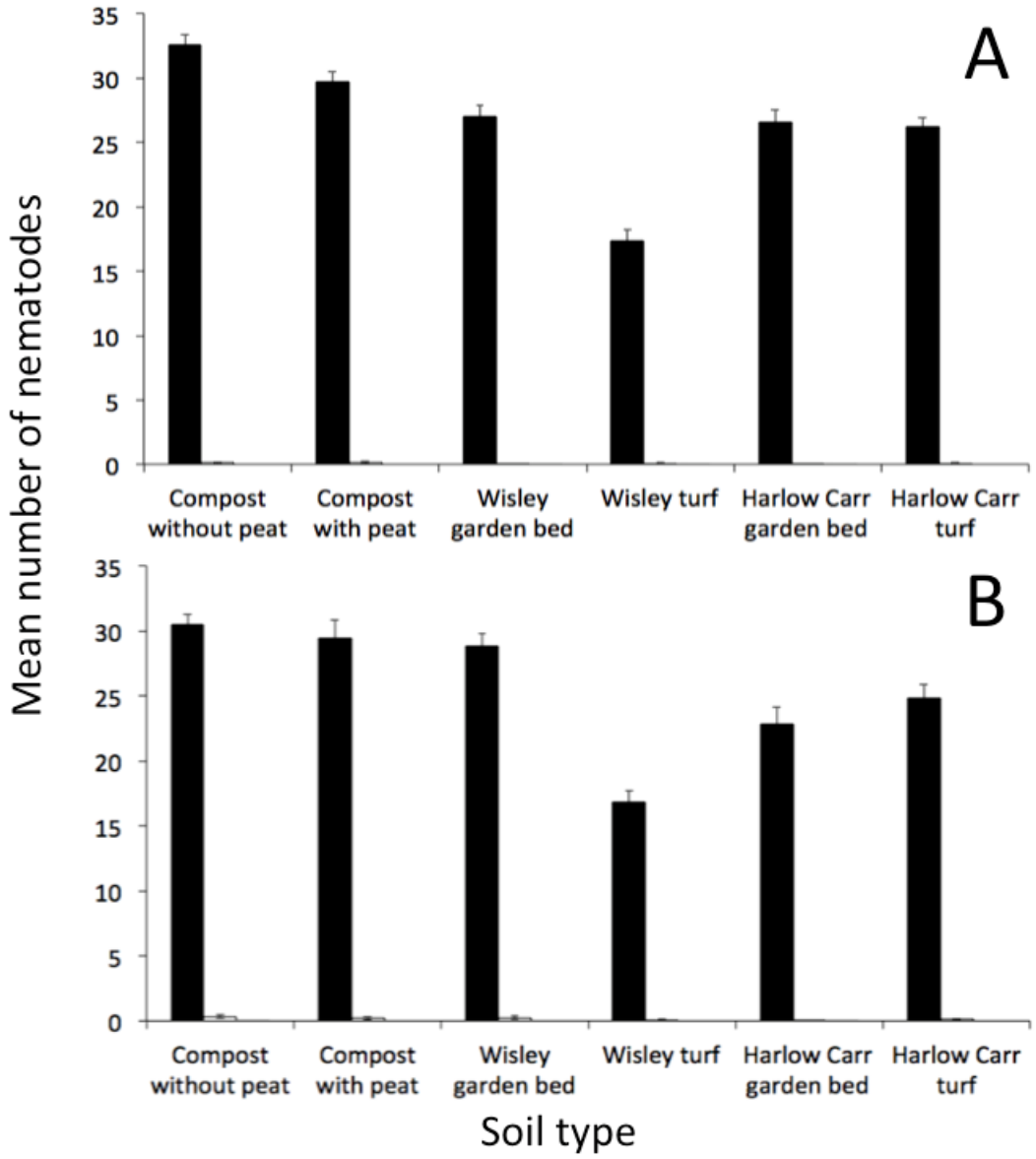

Fig 3.4: Mean number of *P. hermaphrodita* (DMG0001) found at application point 0 to 3.5 cm (black bars), 3.5 to 7 cm (white bars) and 7 to 9.5 cm (grey bars) with slug present (A) or absent (B) in six different soils, N=3, n18 for each soil type.Bars represent ± one standard error.

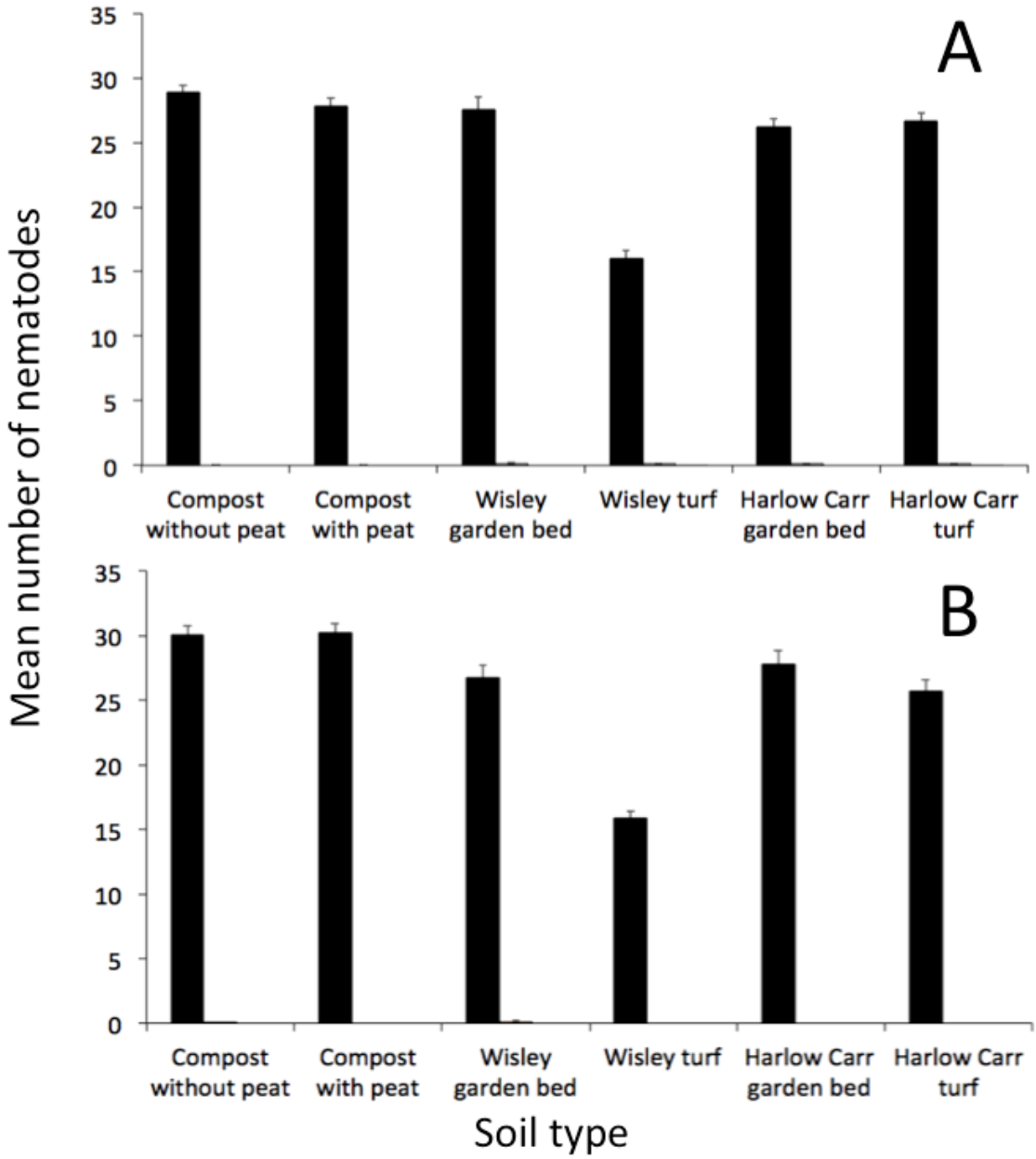

Fig 3.5: Mean number of *P. neopapillosa* found at application point 0 to 3.5 cm (black bars), 3.5 to 7 cm (white bars) and 7 to 9.5 cm (grey bars) with slug present (A) or absent (B) in six different soils, N=3, n18 for each soil. Bars represent ± one standard error.

3.4 Discussion

P. hermaphrodita MG2 was lethal to *D. invadens* when used in all six soils, though death of the slugs was fastest in slugs exposed to *P. hermaphrodita* MG2 added to compost with peat compared to turf from Wisley and Harlow Carr. The reasons for this are unknown, but differences in the efficacy of nematodes to control pests has been reported in the literature (using EPNs). For example, Campos-Herrera and Gutiérrez (2009) examined the effects of soils on the virulence of 17 strains of *S. feltiae* to several insects and found increasing clay

content had a dramatic effect on the virulence of the nematode. This conclusion is similar to work conducted by Toepfer *et al*. (2010), who showed EPNs (*H. bacteriophora*, *H. megidis* and *S. feltiae*) killed western corn rootworm (*D. virgifera*) best in heavy clay or silty clay soil compared to sandy soils (of reasons unknown). Also Kruitbos *et al.* (2010) showed *S. carpocapsae* dispersed well in peat and located hosts but remained at the point of application when added to sand. Perhaps these differences are due to the ability of cues dispersing through the soil matrix more efficiently in compost (with peat) compared to turf soil. Compost is a granular matrix with bigger pore space compared to turf, which is tightly bound causing smaller pores and could therefore inhibit the cues getting through. *P. hermaphrodita* relies on mucal and faecal cues (Rae *et al*., 2006; 2009), which are soluble and are needed to trigger the response of the nematode to find slugs. If these do not penetrate or exude through the soil then it will be difficult for the nematodes to find slugs (though it must be noted all soils where nematodes were applied, did result in dead slugs).

In our movement experiment *P. hermaphrodita* (DMG0001) and *P. neopapillosa* stayed at the point of application when added to six different soils. This is in agreement with previous research by Wilson *et al*. (1999), although they only looked at one type of soil. In terms of strategies for EPNs to infect hosts they are broadly split into 'cruisers' or 'ambushers (Lewis *et al*., 1992). Hunters actively roam through the soil looking for hosts, but ambushers wait for their hosts to pass then latch on. A crucial point about ambushers is they nictate (stand on tail) (Campbell and Gaugler, 1993), but *Phasmarhabditis* nematodes do not, therefore, these nematodes do not seem to fit with the behavioural ecology paradigm for EPNs.

Macmillan *et al*. (2009) looked at how dispersal of *P. hermaphrodita* (DMG0001) and a wild isolate of *P. hermaphrodita* from Norway could be affected by soil type. They found in general, nematode movement was reduced in sandy loam soils compared to clay loam, and both strains moved readily through leaf litter compared to peat (and they recorded *P. hermaphrodita* also reproduced in leaf litter). The dispersal of the nematodes was also enhanced by the presence of earthworms (*Lumbricus terrestris*), showing phoretic hosts are utilised by these nematodes to gain access to lower soil layers where slugs may be present. The results from this experiment were in agreement with Wilson *et al*. (2000) and Macmillan *et al*. (2009), as the nematodes did not move from the point of application, even though the nematodes were in relatively close proximity to the slug host.

These results suggest knowledge of soil type would be beneficial before application of *Phasmarhabditis* nematodes as not only can it affect the ability of nematodes to reproduce in soil (as found in Chapter 2), and the ability to kill slugs (but has little effect on movement through soil).

Chapter 4: A method for culturing and mating slugs and its utility for screening essential oils for toxic effects on juvenile slugs

4.1 Introduction

There is very little information about how to culture slugs. There are at least 36 species of slug in the U.K (Anderson, 2005) and as many as 44 (Rowson *et al*., 2014), many of which are non-native (Cameron, 2016). Working with slugs can be difficult as experiments can only be carried out with slugs collected from the wild. There is little information on how to rear them in the lab. Therefore, the aim of this chapter was to discover whether three species of slug (*Deroceras invadens*; *Arion hortensis* and *Ambigolimax valentianus*) could be cultured and mated to produce offspring and whether these offspring would be viable enough to produce more progeny. This could then allow extensive experiments using *P. hermaphrodita* or other novel molluscicides in a high throughput manner. The later is important as several large, highly pestiferous slug species are resistant to *P. hermaphrodita* (e.g. *A. subfuscus* and *A. valentianus*, Rae *et al*., 2009; Ester *et al*., 2003), and new molluscicides are needed.

One such alternative is the use of essential oils to kill pests, which offer an attractive alternative to chemical-based pellets as they are non-toxic to humans and exempt from registration e.g. in the US they "are exempt from pesticide registration and residue tolerance requirements under US federal law (Sect. 25(b) of the Federal Insecticide, Fungicide and Rodenticide Act)" (Klein *et al*., 2020). Some essential oils, have been shown to kill slugs and snails. Klein *et al*. (2020) showed thyme, spearmint and pine oil were lethal to *D. reticulatum* and could be used in glasshouse trials to control slugs, to a level comparable to chemical control (metaldehyde). Also, Mc Donnell *et al*. (2016) showed essential oils such clove bud oil could be used to kill the eggs and juvenile stage of the pestiferous snail and invasive pest *Cornu aspersum*. However, there is no information if these essential oils could be used to kill other pestiferous species of slug and whether there is a superior essential oil that could be used to target slug eggs.

Therefore, the aim of this chapter was to 1) discover whether slugs could be bred under laboratory conditions 2) investigate whether slug eggs or juveniles could be used to investigate if a selection of essential oils could be toxic to two species of pest slug.

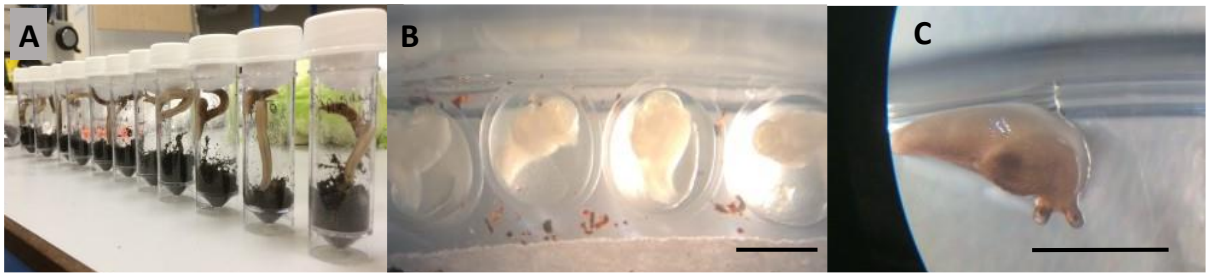

Fig 4.1. *A. valentianus* exhibiting circling courtship behaviour aided by optimal moist environment and temperature (A). 6-day aged embryos of *D.invadens* observe under dissecting microscope (B). 1-day old *D.invadens* observed under dissecting microscope (C). Scale bars represent 1mm.

4.2 Materials and methods

4.2.1 Slug culture methodology

Preliminary observations in the laboratory showed that *D. invadens* and *A. hortensis* preferred to lay eggs in peat free compost (SylvaGrow®) compared to garden bed or turf soil. Observations also demonstrated differences in egg development for the three species at different temperatures. Specifically, *D. invadens* at 10°C, and *A. hortensis* and *A. valentianus* at 15°C produced the highest level of viable eggs, presumably due to the differences in ecological niches they inhabit. The slugs were collected from a garden in Maghull, Liverpool (OS grid reference SD373027). They were kept in non-airtight plastic boxes (35 x 23 x 22 cm) at 5°C with moistened paper and fed lettuce *ad libitum* for 1 week before use to check for any signs of ill health. To sixty 50 ml universal bottles, compost (10-15% moisture content) was added to a height of 3.5 cm. Two adult *D. invadens, A. hortensis* (all animals collected were >0.10 g and >2.5cm) or *A. valentianus* (>0.2 g and >3.5cm) were added to each tube (twenty universal bottles with two slugs in each were used for each slug species). Tap water (0.5 ml) was added to each tube by spray, a ball of cotton wool was then added, and the lid was loosely closed. *Deroceras invadens* were stored in an incubator at 10°C and *A. hortensis* and *A. valentianus* were incubated at 15°C. After 8 days each clutch of eggs per tube was weighed and the slug eggs were counted, these were then transferred to 10 cm Petri dishes (approx. 60 eggs per dish) with pre-moistened filter paper with a small amount of compost substrate and sealed with Parafilm®. They were incubated for up to 20 days at 15°C.

Egg hatching occurred generally between day 14 and 20. After hatching, approximately 60 neonate slugs were transferred to compost (previously frozen at -80°C overnight to kill any metazoan parasites) in a container (30 x 10 x 10 cm). The container was kept at 50% humidity monitored using a Tinytag and the soil kept between 10 and 20% water content, monitored using a Xiaomi 4 in 1 plant monitor. The container was misted with distilled water once a week. Slugs were fed a mixture of iceberg lettuce, carrots and calcium tablets every two weeks. Any rotten food left was removed every 3 days. Once the slugs had reached approx. 5 mm they were transferred to containers containing 30 slugs of the same species and kept under the same conditions. After four months most adults of *D. invadens* and *A. hortensis* had reached a weight of >0.10 g and *A. valentianus* >0.20 g, and reached sexual maturity. They were mated using the same protocol previously mentioned. This process was repeated for the second and third generation for all three species.

4.2.2 Sourcing and Screening essential oils for lethal effects on slug eggs and slug juveniles

13 essential oils (Birch tar, Cedarwood, Bitter Orange, Clove, Garlic, Cinnamon, Eucalyptus, Lemongrass, Spearmint, Peppermint, Pine, Rosemary and Thyme. were used in the screening. These were sourced from the online supplier of essential oils: https://www.gb.pipingrock.com/.

Combining the use of the protocols outlined above to propagate slug eggs, I aimed to discover whether the eggs of *A. valentianus* and the juveniles of *A. subfuscus* and *A. valentianus*) could be killed with a selection of essential oils. The eggs of *A. valentianus* were collected using laboratory protocols and the juveniles of A. subfuscus were collected en masse on a dewy morning. The juveniles tended to congregate on grass bales just after hatching from the eggs in the leaf litter on the side of the lawn.

Briefly, 5 cm Petri dishes (for 10 slug eggs) and 10 cm Petri dishes (for 5 juveniles) were lined with 5 or 10 cm diameter Whatman filter paper. To each 5 cm Petri dish 1 ml of tap water, Tween80, or 13 essential oils (pines, lemongrass, eucalyptus, birch tar, rosemary, cedarwood, garlic, thyme, peppermint, bitter orange, spearmint, clove, cinnamon) at a concentration of 0.5% was added (2 ml at 1% concentration was added to the 10 cm Petri dishes). Each oil was mixed 1:2 with Tween80, hence Tween80 was used as a control on its own.

Five *A. subfuscus* juveniles were gently placed into each Petri dish and they were sealed with Parafilm® and stored in a non-airtight plastic box with moist, saturated kitchen roll at 15°C. Their survival was monitored for 72 hours. This process was repeated four more times for *A. subfuscus*, which meant 20 juveniles were tested in total for each treatment.

Ten *A. valentianus* eggs were placed carefully into the 5 cm Petri dish and the viability was checked using a dissecting microscope. *A. valentianus* eggs tend to hatch at around 20 days (unpublished observation), therefore, the number of viable and hatched eggs were assessed on day 20. This process was repeated 3 times for *A. valentianus* but as there was a large founder population of these slugs more replicates could be set up and run in parallel. Therefore, a total of 90 eggs were used for each treatment.

4.2.3 Data analysis

A One Way ANOVA was used to analyse whether there were significant differences between the numbers of eggs and the weight of eggs laid in generation 1, 2 and 3 for each of the three slug species and between the numbers of eggs laid by the three different slug species in generation 1, 2 and 3. The numbers of slugs that hatched from the water control treatment as well as being exposed to Tween80, and the essential oils were analysed using a One Way ANOVA.

4.3 Results

4.3.1 Slug culturing method

Using the developed protocol, all three species of slug were able to be mated and lay eggs. There was no significant difference between the numbers of eggs or the weight of eggs produced in the first, second or third generation by *D. invadens*, *A. hortensis* or *A. valentianus* ($P>0.05$) (Table 2). All slug species produced similar numbers of eggs and there was no significant difference in the numbers of eggs produced by *D. invadens*, *A. hortensis* and *A. valentianus* in generation 1 ($P=0.80$), 2 ($P=0.70$) or 3 ($P=0.447$) (Table 2).

Table 1. Reproductive output of the three species over three generations, n=20.

D. invadens	pairs	clutch	Mean eggs per pair	SE	range	Mean Clutch Weight (g)	SE	Mean weight of individual eggs (g)	SE
P	20	19	26.5	2.7	5-50	0.10	0.01	0.004	0.0001
F1	20	18	28.6	2.8	10-54	0.16	0.05	0.006	0.002
F2	20	18	25.4	2.4	11-50	0.09	0.01	0.004	0.0001
A. hortensis									
P	20	17	21.1	2.4	4-36	0.14	0.02	0.007	0.0002
F1	20	17	24.3	2.9	3-56	0.14	0.02	0.006	0.0003
F2	20	16	21.7	2.7	3-40	0.14	0.02	0.006	0.0005
A. valentianus									
P	20	18	18.4	2.1	2-33	0.19	0.02	0.01	0.0003
F1	20	18	19.7	2.2	5-38	0.21	0.02	0.01	0.0001
F2	20	18	20.9	2.9	2-55	0.19	0.02	0.01	0.0005

4.3.2 Hatching of *A. valentianus* exposed to essential oils and the survival of *A. subfuscus* juveniles exposed to essential oils

Eggs of *A. valentianus* and juvenile *A. subfuscus* were exposed to 13 different essential oils, as well as a water and Tween control to assess molluscicidal effects. Over 72 hrs there was a strong molluscicidal effect of 11 essential oils on the survival of *A. subfuscus* juveniles compared to water and Tween (Fig 14A-C; 15A; $P<0.05$). The only oils that did not significantly affect the survival of *A. subfuscus* juveniles were birch tar oil and rosemary oil ($P>0.05$). In contrast, all 13 essential oils killed the eggs of *A. valentianus* (Fig 15B; $P>0.05$).

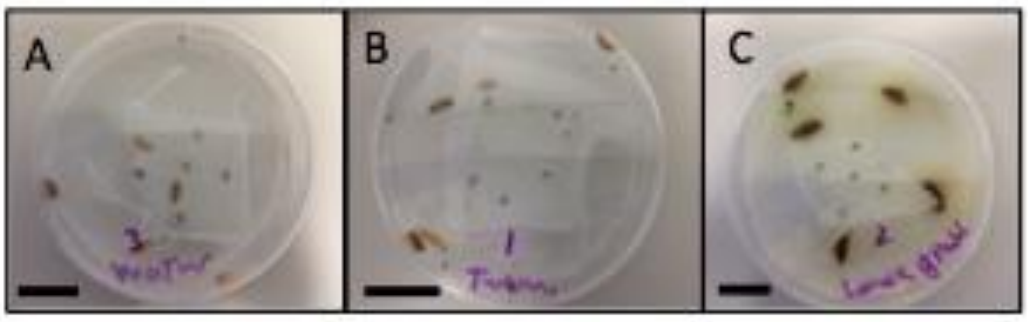

Fig 4.2: After 24 hrs *A. subfuscus* juveniles survived in Petri dishes with water (A), Tween80 (B) but all died in lemongrass (C). Scale bars represent 1 cm.

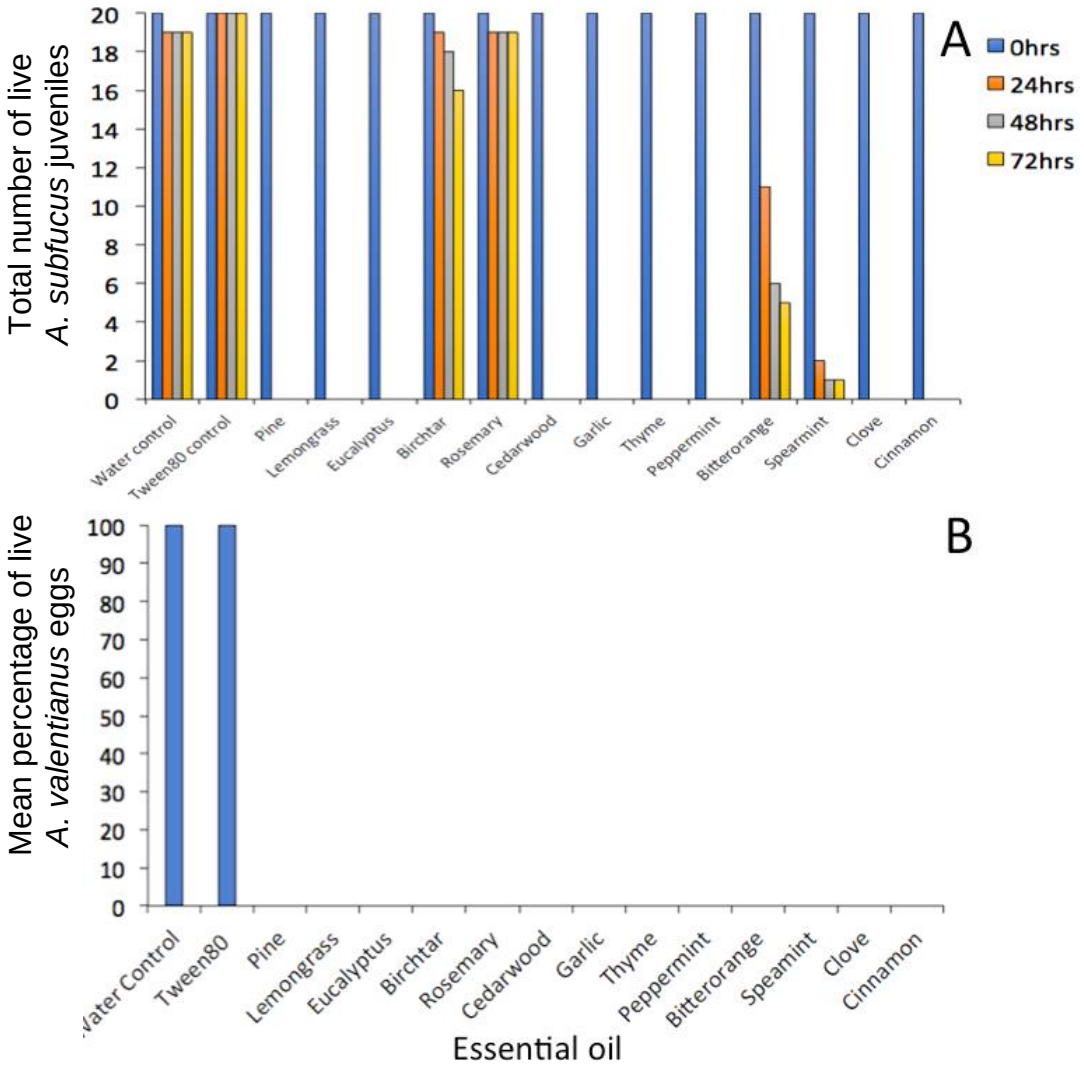

Fig 4.3: Total number of live *A. subfuscus* juveniles over 72 hrs. N=5, n=4 for each experiment. (A) and mean percentage of live *A. valentianus* eggs that have hatched and grown to juvenile

slugs,(B) exposed to water control, Tween80 control and 13 different essential oils. N=10, n=9. Bars represent ± one standard error.

4.4 Discussion

Using the protocol developed slugs can be routinely mated to consistently produce clutches of eggs over several generations using three different species from three genera. An important question to consider is whether continuous culturing may affect the health of the slugs due to several generations of potential inbreeding. However, there was no reduction in egg number or health of the slugs over 3 generations. Specifically, the numbers of eggs did not vary over time with *D. invadens* producing between 25 to 28 eggs, *A. hortensis* between 21 to 24 and *A. valentianus* between 18 to 20 over three generations. Although it has been reported that numbers of eggs varies with species (South, 1992) we did not observe any differences between the three species used. The numbers of eggs laid in our study by each species is similar to other studies. For example, Carrick (1938) found *D. reticulatum* could lay up to 500 eggs a year with a mean of 22 eggs per batch (range 9-49 eggs). However, that estimation is based on field studies; in captivity, Davies (1977) found the numbers of eggs per batch laid by *A. hortensis* ranged from 10 to 30.

Other studies that have attempted to rear slugs focused on using an array of different substrates. For example, Sivik (1954) used wooden trays with gauze and soil. Stephenson (1962) used a combination of loam soil, peat and sand in screw capped jars and Kingston (1966) used fine gravel or moistened filter paper and blackboard chalk for calcium. Vermiculite has also been used (Gray *et al*., 1985). In this study, slugs were housed in non-airtight plastic boxes with ample moisture and a thin layer of compost. When used for mating they were placed in 50 ml universal bottles with compost, which gave consistent results of similar numbers of eggs with no reduction in viability.

Maintaining consistent and correct moisture content is an important factor in slug rearing. Arias and Crowell (1963) found that *D. reticulatum* produced maximum number of eggs in soils at 75% saturation and no eggs in soils at 10%. However, Willis *et al*. (2008) showed *D. reticulatum* laid the most eggs in soils at 53% saturation. Compost used in this study was initially at a moisture content of 10-15% with a further 0.5 ml of water applied later through spraying directly into tubes increasing the compost moisture saturations to between 15 and 20%.

Another important factor to take into account in rearing slugs is diet. There are many diets that have been used to rear slugs including breakfast cereal, leaf litter and fungi (Cook and Radford, 1988), oat bran (Howlett *et al*., 2009), dog food and fresh fruit (Hamilton *et al*., 2020). Synthetic diets of calcium alginate beads have been used to rear slugs; however' reproductive output was poor (Wright, 1973). As *D. invadens, A. hortensis* and *A. valentianus* are all generalist herbivores (South, 1992) we fed them a mixture of lettuce, carrot and calcium tablets and showed they were able to grow and mature quickly with no reduction in reproductive output. We gave the slugs a choice of foods as previous research has shown they will choose the food type, which contained nutrients that they were lacking the most (Cook *et al*., 1999). Carrot is a particularly good choice compared to potato, lettuce, apple and bran, which decay quickly causing microbial contamination that may affect the slugs state of health (Stephenson, 1962).

A continuous culture of slugs could aid in further studies looking at slug genomics (Chen *et al*., 2020), transcriptomics (Ahn *et al*., 2017), behaviour (Kozlowski *et al*., 2016), microbiome analysis (Reich *et al*., 2018), feeding experiments (Barone and Frank, 2008) (to name but a few) but we concentrated on investigating whether this process could be used to screen new molluscicides, namely essential oils. The results show that all 13 essential oils, severely affected the hatching of eggs of *A. valentianus* and the growth and survival *A. subfuscus* juveniles. There was no 'stand out' oil, and nearly all were equally lethal to both slug species. Birch tar oil did not have the same level of lethality on the *A. subfuscus* juveniles as the other oils. Lindqvist *et al*. (2020) also found birch tar oil worked as a repellent rather than a toxic pesticide. As both slug species are resistant to *P. hermaphrodita* (Rae *et al*., 2009; Ester *et al*., 2003), urgent new molluscicides are needed. Klein *et al*. (2020) tested the same 13 essential oils but on adult *D. reticulatum*, and found thyme, spearmint and pine oil to be the most toxic and would work well at reducing slug numbers and damage when applied to potted plants containing annual ryegrass infested with slugs. Furthermore, research by Mc Donnell *et al*. (2016) screened 11 essential oils and found clove bud oil was most toxic to eggs and juvenile stages of the snail *C. aspersum*. Undoubtedly, these essential oils (and the ones I tested) show promise at controlling slugs and snails at the juvenile, egg and adult stage but it is unknown whether these oils could be combined with other biological control agents, such as *P. hermaphrodita*, to provide superior protection against slugs.

Chapter 5: An investigation into the potential of synergistic control of pest slugs (*Deroceras reticulatum* and *Arion ater*) using *P. hermaphrodita* and cedarwood oil

5.1 Introduction

The cost of nematodes can be prohibitively expensive compared to chemical control pellets such as metaldehyde but novel application strategies that improve efficiency and economic use of nematode biological control products will improve their attractiveness (Grewal *et al*., 2005). These include using novel application strategies of nematodes (e.g. applying several applications of nematodes at much lower rates compared to one broadcast application). For example, as slugs (*D. reticulatum* and *A. ater*) have been shown to avoid areas where nematodes have been applied (e.g. Wilson *et al*., 1999; Wynne *et al*., 2016) it has been suggested that *P. hermaphrodita* could be applied in smaller doses to deter slugs, which would reduce the number of nematodes applied as well as the cost. However, there has been mixed results taking this approach. Hass *et al*. (1999a,b) showed application of the nematodes in bands had little effect, but Grewal *et al*. (2001) showed application of nematodes to slug shelters reduced slug numbers.

Alternatively, nematodes could be mixed with chemicals, or other compounds that could enhance slug control and reduce cost. However, there has been little research on this. Wilson *et al*. (2000b), showed metaldehyde had no effect on the survival of *P. hermaphrodita,* but as it is being banned in the U.K. there is little reason to continue this line of research. A promising alternative is the use of essential oils, which were shown to severely affect the survival of slugs in Chapter 4. Also, it should be noted that Barua *et al*. (2020) showed that essential oils have the ability to kill beneficial nematodes in soil. When testing the survival and thrashing behaviour of the 13 essential oils tested Chapter 4, several were highly toxic (e.g. thyme, cinnamon, cloves and garlic) to *P. hermaphrodita*, *H. bacteriophora* and *S. feltiae*, however crucially cedarwood oil, was shown to not affect the survival and behavior of *P. hermaphrodita* (Barua *et al*. 2020). It is therefore, a promising candidate to use for further experiments.

The aim of this chapter was to investigate whether *P. hermaphrodita* could be combined with cedarwood oil to reduce slug damage in two pest species (*D. reticulatum* and *A. vulgaris*) in propagator trails carried out across the year in July, September and October. Ultimately, this research will show if application of *P. hermaphrodita* can be enhanced or

reduced using a combination of cedarwood oil and may offer a more effective slug control treatment.

5.2 Materials and methods

5.2.1 Source of invertebrates and essential oils

P. hermaphrodita strain DMG0001 (Nemaslug®) was supplied by Becker Underwood, BASF Agricultural Specialities, U.K. and stored at 15°C for 14 days until use.

Two species of slugs were used (*D. reticulatum* and *A. vulgaris*). *Arion vulgaris* (also known as non-topotype *A. lusitanicus*) is a serious pest of agriculture and since the 1950s has invaded many European countries (Zemanova *et al*. 2017). It is difficult to treat, as it is not killed by nematodes (Grimm, 2002; Speiser *et al*., 2001). Slugs were collected from local grassland with woodland on one side and wheat fields on the other (grid reference OSSD 362014). *Deroceras reticulatum* were collected from the long grass from under foliage on the sides of the path, during dusk and dawn in damp periods and stored in non-airtight containers and fed lettuce *ad libitum* and stored at 10°C until use. *Arion vulgaris* were collected directly from the grass paths during damp periods at various times of the day. Each slug was weighed before the experiment. The weight range used for *D. reticulatum* was between 0.20 and 0.70 g. The weight range for *A. vulgaris* was between 10 g and 20 g. Any slugs that showed any signs of infection were not used in the trial.**5.2.2 Investigating the efficacy of nematodes and cedarwood oil under semi-natural conditions**

A polytunnel was divided into two sections of 3 m x 90 cm. Within this, eight propagators (240 cm x 380 cm) were added and each propagator tray was filled to the top with peat-free Sylva compost and three 14 day old little gem lettuce seedlings were planted into each. Each propagator was set up 5 days prior to the start of the experiment to let the lettuce seedlings establish into the soil before adding the slugs and treatments. Air vents in the lids were covered in mesh netting to prevent the slugs from escaping, but enabled airflow. The top of the trays were taped with copper tape to stop the slug from traveling onto the lid. There were five treatments consisting of 1. control – where only water was applied, 2. *P. hermaphrodita* applied at the recommended rate of 30 nematodes per cm^2 (approx. 28,000 nematodes per propagator), 3. cedarwood oil (*Juniperus virginiana*) (purchased from Pipping rock) applied at 0.25% concentration and dissolved in Tween 80 (Tween 2:1 oil ratio) 4. cedarwood oil and *P. hermaphrodita* (30 nematodes per cm^2) and 5. Tween 80 (as the Cedarwood was mixed with Tween 80 a control of just the latter is needed); the Tween80 was

mixed with the oil at 2:1 ratio. A total volume of 560 ml of each treatment was applied to the trays. Ten *D. reticulatum* were added to four propagators and five *A. vulgaris* were placed the others. Slug shelters were placed in each tray using small plant pot saucers and each propagator was sealed using masking tape to prevent slugs from escaping. Temperature and moisture monitors were placed into random propagators for the experimental period.

The experiment was carried out over 14 days. On days 1, 2,3,7, and 14 the damage of the plants was recorded by taking photographs of the leaves and visually estimated of the percentage of damage on each leaf (Rae *et al*., 2009). The number of dead and alive slugs was recorded at the end of the experiment. It was noted during the experiment with *D. reticulatum* that the slugs laid eggs in the soil and on leaves. This was quantified by counting the eggs that were found in each tray on day 14. Also, on day 14 the plants were uprooted, measured for length from roots to tip of the leaves, weighed and photographed.

The experiment with *D. reticulatum* was repeated three times but it must be noted the first replicate consisted of four trays with ten slugs and the second and third replicate consisted of three trays with ten slugs in each. The first replicate took place on the 23rd of July 2020 and ran till the 6th of August. The second replicate began on the 16th of September till the 30th of September 2020 and the third replicate began on the 13th of October and ended on the 27th of October 2020.

5.2.3 Data analysis

The percentage of lettuce eaten by each slug species on days 1, 2, 4, 7 and 14 was Arcsine transformed and then analysed using a One way ANOVA with Tukey's post hoc test. The weight of lettuce was quantified and compared using a One way ANOVA with Tukey's post hoc test. The number of surviving slugs and the numbers of eggs laid by the slugs was quantified on day 14. These data were combined across all three replicates and analysed using a One way ANOVA with Tukey's post hoc test.

5.3 Results

5.3.1 Monitoring systems moisture and temperature data

There were three replicates of the mini-plot trial with two slug species (*D. reticulatum* and *A. vulgaris*) carried out at three different times of the year. Temperature varied throughout the year and the differences can be seen in Fig 16.

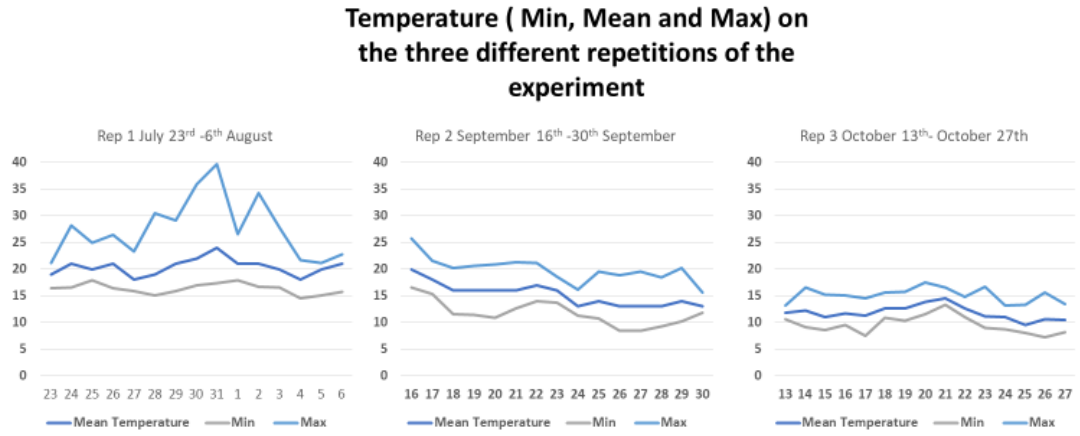

Fig 5.1: Temperature (min, mean and max) during three replicates of the propagator experiment.

5.3.2 The effect of nematodes and cedarwood oil on the percentage of plant damage caused by *D. reticulatum*

In replicate one, the application of *P. hermaphrodita* or Tween 80 had no effect on the amount of slug damage caused by *D. reticulatum* over 14 days (Fig 17A; $P>0.05$). The only treatments that had a significant effect in reducing the amount of slug damage compared to the untreated control was cedarwood oil and cedarwood oil and *P. hermaphrodita*. Both treatments significantly reduced slug damage on days 1, 2, 4, 7 and 14 compared to the untreated control (Fig 17A; $P<0.05$). There was no significant difference between the effectiveness of cedarwood oil and cedarwood oil and *P. hermaphrodita* in reducing slug damage over days 1, 2, 4, 7 and 14 (Fig 17A; $P>0.05$).

In replicate two, similar results were reported as in replicate 1. Specifically, cedarwood oil and cedarwood oil and *P. hermaphrodita* provided significant protection against slug damage by *D. reticulatum* on days 1, 2, 4, 7 and 14 compared to the untreated control (Fig 17B; $P<0.05$). There was no significant difference between the amount of slug damage eaten in both treatments (Fig 17B; $P>0.05$). The application of *P. hermaphrodita* on

its own did reduce damage to the plants significantly, but this was only on days 7 and 14 (Fig 17B; $P<0.05$). Though slug damage was reduced by the nematodes, this was significantly greater than both cedarwood oil and cedarwood oil with nematodes (Fig 17B; $P<0.05$).

As in replicate 1 and 2, cedarwood oil and the combination of cedarwood oil and *P. hermaphrodita* significantly in replicate 3 reduced slug damage by *D. reticulatum* on days 1, 2, 4, 7 and 14 compared to the untreated control (Fig 17C; $P<0.05$). There was no difference in the amount of damage on plants exposed to cedarwood oil and the combination of cedarwood oil and *P. hermaphrodita* (Fig 17C; $P>0.05$). Single application of *P. hermaphrodita* did significantly reduce slug damage but only on days 7 and 14 (Fig 17C; $P<0.05$) and the amount of damage reduced by the nematodes was comparable with cedarwood oil and the combination of cedarwood oil and *P. hermaphrodita* as there was no significant difference between all three treatments throughout the experiment (Fig 17C; $P>0.05$).

It should be noted that throughout the experiment and in the three replicates of the experiment, Tween80 did not have a phytotoxic effect on the plants or affected slug behaviour or feeding as there was no significant difference between the amount of lettuce eaten in the untreated and Tween treatment on any day or in any replicate (Fig 17; $P>0.05$). The highly efficient effect of cedarwood oil must therefore be due to the essential oil and not the emulsifier it is mixed with (Tween80).

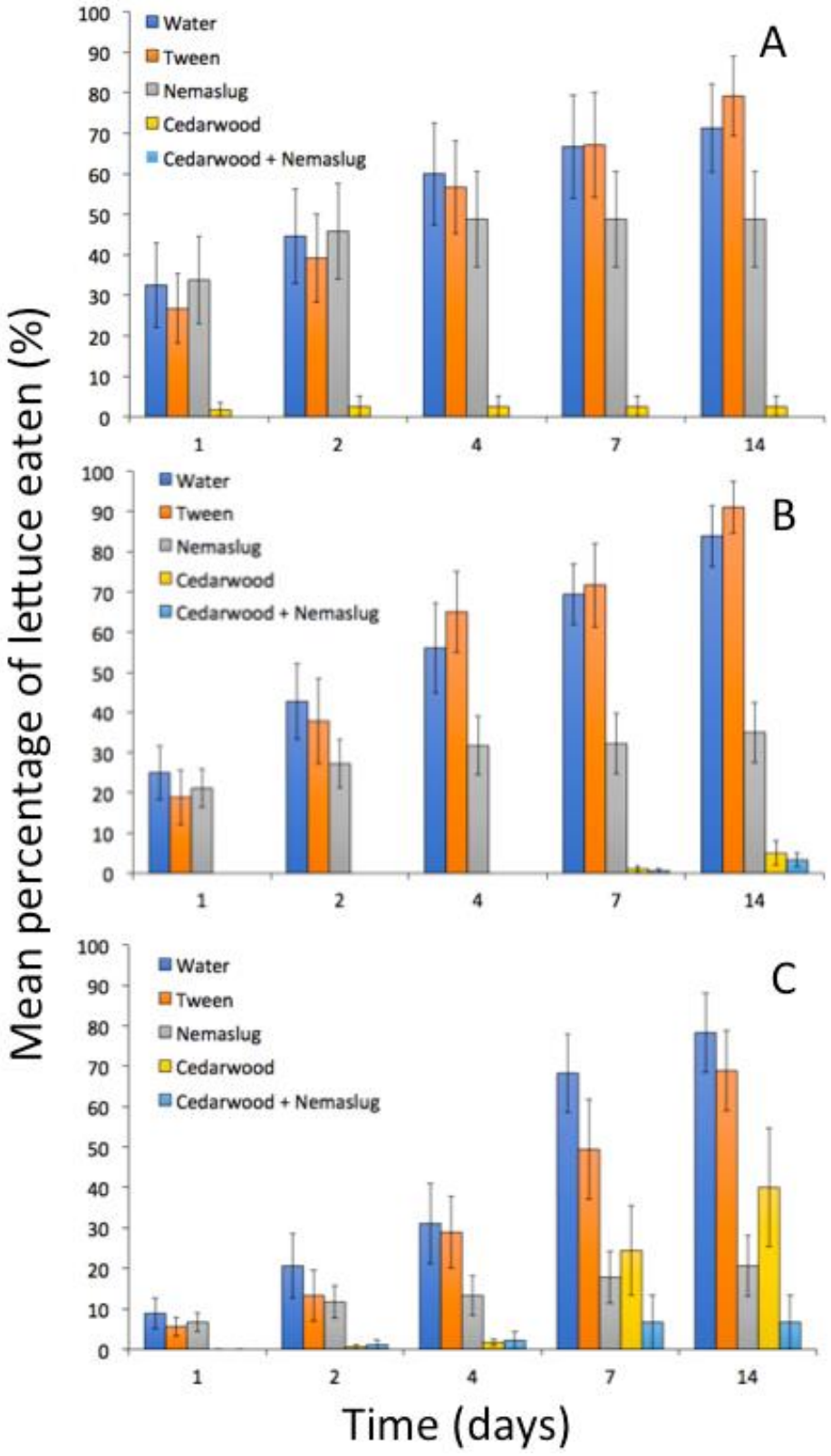

Fig 5.2: Mean percentage of lettuce plants (g) eaten over 14 days exposed to *D. reticulatum* and treated with water, Tween 80, nematodes, cedarwood or nematodes and cedarwood used in the first (A), second (B) and third (C) replicate. N=3, n=9for each treatment. Bars represent plus or minus one standard error.

5.3.3 The effect of nematodes and cedarwood oil on the weight of lettuce exposed to *D. reticulatum*

On day 14 individual lettuces were weighed (g) from each treatment (Fig 18). In replicate one, lettuces grown with an application of cedarwood or the cedarwood and nematodes were significantly heavier than those grown in the untreated control (Fig 18; $P<0.05$). There was no significant difference between the weight of lettuces grown with cedarwood or the cedarwood and nematodes (Fig 18; $P>0.05$). Lettuces grown with a single application of nematodes and the untreated control were of a similar weight (Fig 18; $P>0.05$).

In replicate two, lettuces grown with a single application of nematodes, cedarwood or the combination of nematodes and cedarwood were significantly heavier than the lettuces grown in the untreated control (Fig 18; $P<0.05$). There was no difference in the weight of lettuces grown with either cedarwood or the combination of nematodes and cedarwood (Fig 18; $P>0.05$). The lettuces grown with a single application of nematodes were significantly heavier than the lettuces grown in the untreated control but they significantly differed in weight compared to cedarwood and nematodes (Fig 18; $P<0.05$), but were not significantly different in weight compared to the single dose of cedarwood (Fig 18; $P>0.05$).

Surprisingly, in replicate three, the application of cedarwood and the combination of cedarwood and nematodes did not affect the weight of the lettuces and their mean weight did not significantly differ from the untreated control (Fig 18; $P>0.05$). The only treatment that had a significant effect on lettuce weight was the nematodes (Fig 18; $P<0.05$).

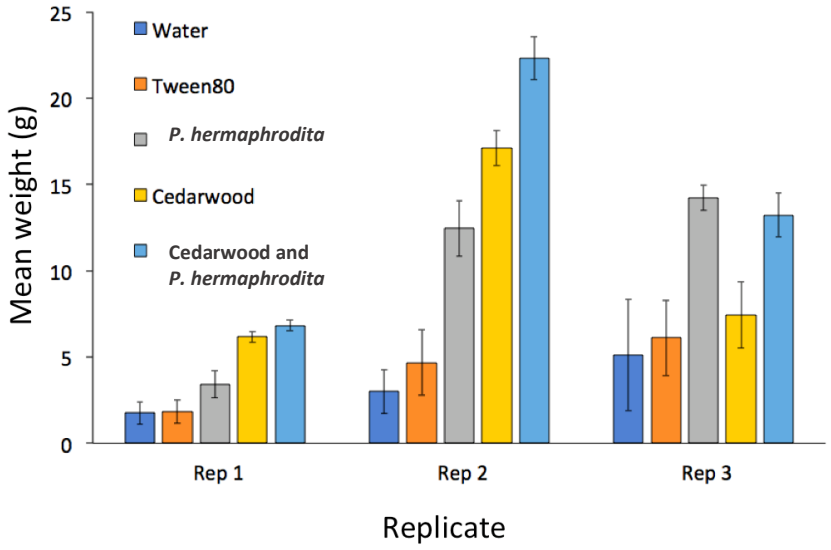

Fig 5.3: Mean weight of lettuce plants (g) on day 14 after being exposed to *D. reticulatum* and treated with water, Tween 80, *P. hermaphrodita*, cedarwood or *P. hermaphrodita* and cedarwood in three replicate experiments. N=3, n=9 for each treatment. Bars represent plus or minus one standard error.

5.3.4 The survival and number of eggs produced by *D. reticulatum* exposed to nematodes and cedarwood oil

On day 14 of the experiment, as well as the weight of the lettuces being recorded, the number of alive slugs was quantified as well as the number of slug eggs that could be found in the soil (Table 3). As individual replicates, statistical analysis (One Way ANOVA) cannot be carried out, as there are only three numbers from each treatment (corresponding to the number of alive slugs or eggs in each propagator). So I combined the data from the three replicates and carried out the statistical analysis.

When comparing the number of *D. reticulatum* found alive at the end of the experiment, there was a significant difference between the treatments (Table 2, $P<0.001$). A single application of nematodes, cedarwood and the combination of cedarwood and nematodes significantly reduced the number of *D. reticulatum* compared to the untreated control (Table 3; $P<0.05$). There was no difference between the number of alive slugs found in the propagators treated with nematodes, cedarwood and cedarwood and nematodes (Table 3; $P>0.05$).

The number of slug eggs found in the propagators in each treatment differed significantly (Table 3, $P<0.001$), however, the only treatments that were shown to have a

significant effect reducing the numbers of slug eggs in the soil was cedarwood and cedarwood and nematodes, compared to the untreated control (Table 3, P<0.05). These two treatments did not differ significantly in reducing slug numbers (Table 3, P>0.05). Nematodes on their own failed to reduce the number of slug eggs found in the soil compared to the untreated control (Table 3, P>0.05).

Replicate	Number of propagators	Treatment	Mean number of alive slugs ± standard error	Mean number of slug eggs ± standard error
1	4	Water	5.3 ± 1.1	63.5 ± 4.1
		Tween	4 ± 1.1	67.8 ± 4.9
		Nemaslug	0.3± 0.3	66.8 ± 10.2
		Cedarwood	0.8 ± 0.5	15.8 ± 3.5
		Nemaslug and Cedarwood	0 ± 0	0 ± 0
2	3	Water	9.7 ± 0.3	63.3 ± 21.9
		Tween	8 ± 0.6	61.3 ± 39.5
		Nemaslug	1.7 ± 1.7	27 ± 3
		Cedarwood	0.3 ± 0.3	0 ± 0
		Nemaslug and Cedarwood	0.3 ± 0.3	0 ± 0
3	3	Water	9 ± 0.6	124.3 ± 7.1
		Tween	9.3 ± 0.3	112.7 ± 3.4
		Nemaslug	4.7 ± 2	76.7 ± 37
		Cedarwood	9.3 ± 0.3	54 ± 24.6
		Nemaslug and Cedarwood	4 ± 0.6	45 ± 4

Table 2: The mean number of live *D. reticulatum* and mean number of slug eggs found in propagators exposed to five treatments on day 14. N=10, n9 for each treatment.

5.3.5 The effect of nematodes and cedarwood oil on the percentage of plant damage caused by *A. vulgaris*

Slugs (*A. vulgaris*) ate the lettuce voraciously in all three replicate experiments, and by day 4, 80%+ of the lettuce was gone (Fig 19). On day 7 and 14, it should be noted that the results are based on the remnants of plants that had regrown and as the percentage of the plant is estimated, a smaller plant, with smaller leaves, can produce a large percentage of leaf eaten, and these results after 7 and 14, should be viewed with scepticism. From the results on day 1, 2 and 4, it is clear the slugs ate all the lettuce and the treatments had little or no effect.

In replicate one, on days 1, 2 and 4, all the lettuce was eaten and the nematodes, cedarwood and the combination of cedarwood and nematodes did not reduce the amount of lettuce eaten by *A. vulgaris* compared to the untreated control (Fig 19, P>0.05). This was also the case with replicate two and three (Fig 19, P>0.05).

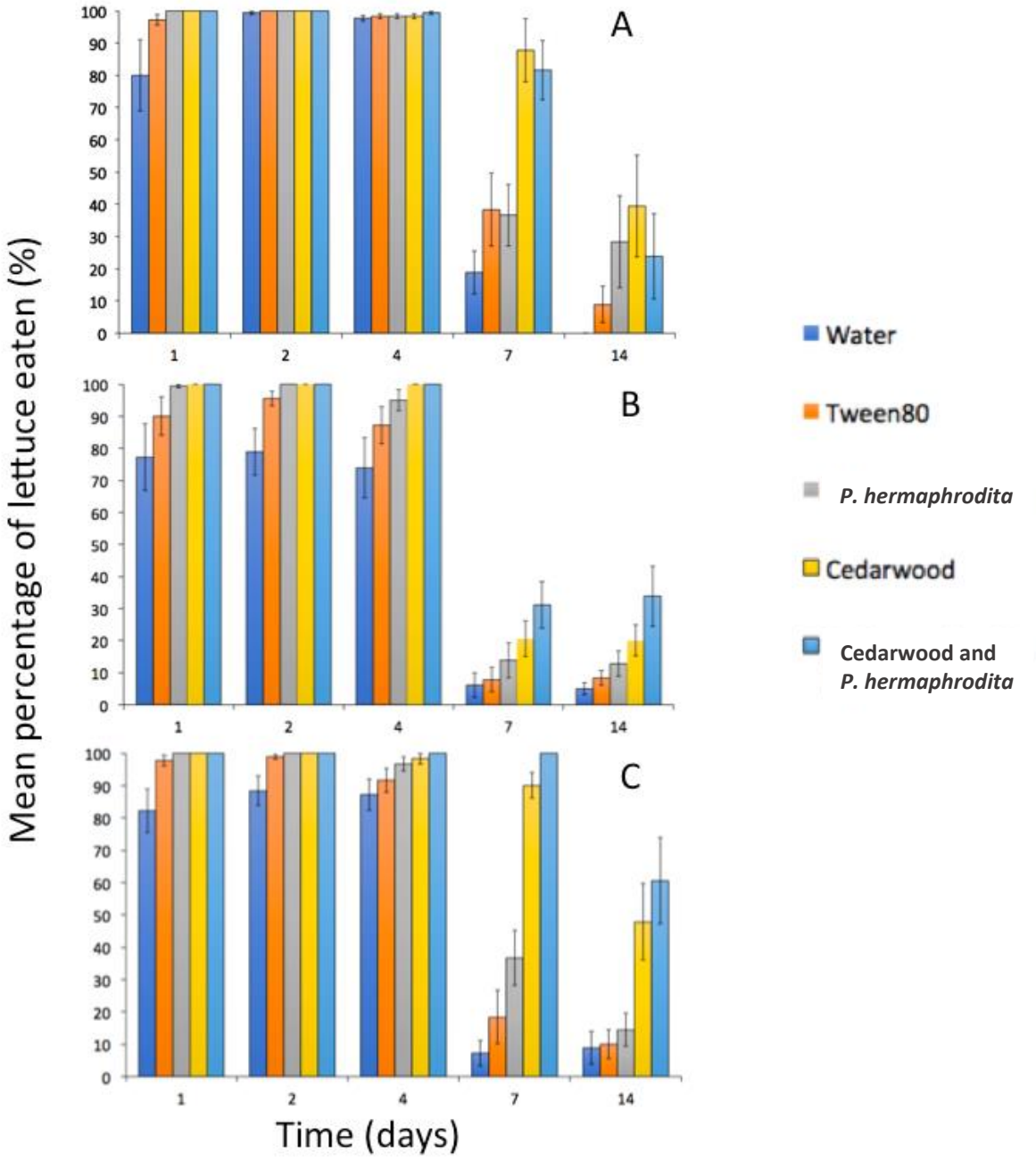

Fig 5.4: Mean percentage of lettuce plants (g) eaten over 14 days exposed to *A. vulgaris* and treated with water, Tween 80, nematodes, cedarwood or nematodes and cedarwood used in the first (A), second (B) and third (C) replicate. N=3, n=9 for each treatment. Bars represent plus or minus one standard error.

5.3.6 The effect of nematodes and cedarwood oil on the weight of lettuce exposed to *A. vulgaris*

A. vulgaris ate all the lettuce in four days, across all treatments but after this new lettuce began to grow from the stumps and the percentage of lettuce eaten was recorded (see above), as well as the weight of each plant. In replicate one, there was a significant difference between all treatments (Fig 20; $P<0.001$) but the only treatment to have a significant effect on lettuce weight was cedarwood and nematodes compared to the untreated control (Fig 20; $P<0.001$). In replicate two the only treatments to have a significant effect were cedarwood and the combination of cedarwood and nematodes, which had significantly heavier lettuce than the untreated control (Fig 20; $P<0.001$). Similar to replicate 1 and 2, the only treatment to have a significant effect on the weight of lettuces was cedarwood oil and nematodes, which were significantly heavier than the lettuces grown in the untreated control (Fig 20; $P<0.001$).

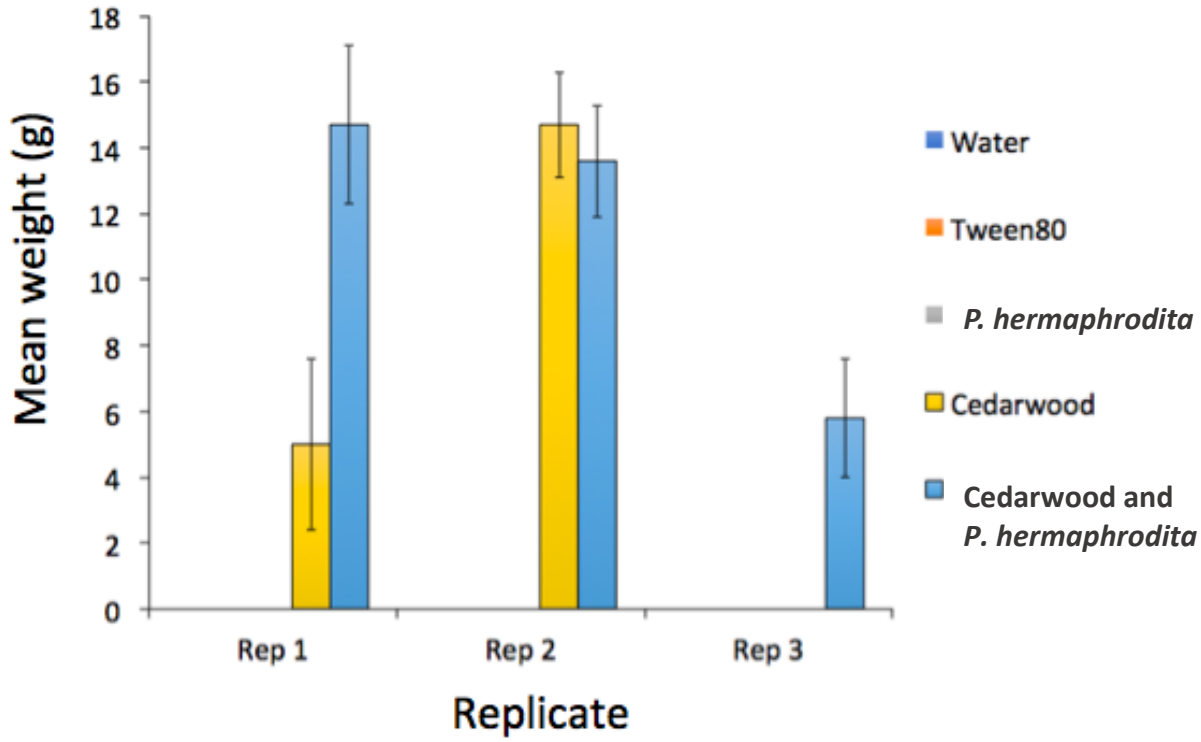

Fig 5.5: Mean weight of lettuce plants (g) on day 14 after being exposed to *A. vulgaris* and treated with water, Tween 80, *P. hermaphrodita*, cedarwood or *P. hermaphrodita* and cedarwood in three replicate experiments. N=3, n=9 for each treatment. Bars represent plus or minus one standard error.

5.3.7 The survival and number of eggs produced by *A. vulgaris* exposed to nematodes and cedarwood oil

As previously stated in the results section describing the results of the effect of different treatments on survival and egg numbers produced by *D. reticulatum*, the data from all three replicates for *A. vulgaris* was combined. Across all replicates, the only treatment to have an effect on slug survival was the combination of cedarwood and nematodes compared to the untreated control (Table 4; $P<0.05$). Application of nematodes or cedarwood oil had no significant effect on slug numbers compared to the untreated control (Table 4; $P>0.05$). There was no significant difference between the numbers of *A. vulgaris* eggs laid in any of the five treatments across all three replicates (Table 4; $P>0.05$).

Replicate	Number of propagators	Treatment	Mean number of alive slugs ± standard error	Mean number of slug eggs ± standard error
1	3	Water	2.7 ± 0.3	15.7 ± 15.7
		Tween	5 ± 0	0 ± 0
		Nemaslug	1 ± 0.6	2.3 ± 2.3
		Cedarwood	1 ± 0.6	43.3 ± 23.3
		Nemaslug and Cedarwood	0.3 ± 0.3	7.3 ± 7.3
2	3	Water	4.7 ± 0.3	0 ± 0
		Tween	5 ± 0	3.3 ± 3.3
		Nemaslug	4.7 ± 0.3	1.7 ± 1.7
		Cedarwood	1.3 ± 0.9	0 ± 0
		Nemaslug and Cedarwood	0.7 ± 0.3	5.3 ± 5.3
3	3	Water	5 ± 0	23.7 ± 19.3
		Tween	4.3 ± 0.7	0 ± 0
		Nemaslug	5 ± 0	0 ± 0
		Cedarwood	4 ± 0.6	0 ± 0
		Nemaslug and Cedarwood	2.3 ± 0.3	0 ± 0

Table 3: The mean number of live *A. vulgaris* and mean number of slug eggs found in propagators exposed to five treatments on day 14. N=5, n=9 for each treatment.

5.4 Discussion

A single application of *P. hermaphrodita* reduced slug damage in two out of three of the propagator experiments with *D. invadens*, but , this was only towards the latter stages of the experiment on days 7 and 14. Although protection against *D. invadens* was demonstrated this was never comparable to the cedarwood treatment or the combination of cedarwood and nematodes – these two treatments were highly efficient and killed the slugs and subsequently reduced damage quickly. There was no significant difference between the effect of cedarwood versus cedarwood and nematodes; this means the nematodes had no effect and the slug killing and reduction of slug damage was solely due to cedarwood oil. *P. hermaphrodita* survives when exposed to cedarwood oil (Barua *et al*., 2020) (unlike other essential oils such

as garlic etc.), so the oils were not inhibiting the effect of the nematodes, it is more likely the oil is so toxic to slugs, they were killed quickly before the nematodes had a chance to infect the slugs. These results show cedarwood could be a promising alternative control method for slugs. There is not much information about the effect cedarwood has on invertebrates or pests but Flor-Weiler *et al*. (2022) found cedarwood was toxic and repellent to four species of tick, and had potential to be an eco-friendly acaricide. Also, cedarwood was found to be the most efficient essential oil (out of 26 tested) affecting activity and urease activity against the bacterium *Helicobacter pylori* (Korona-Glowniak *et al*., 2020).

P. hermaphrodita can only kill juvenile stages of *A. vulgaris* and not adults (Grimm *et al*., 2002; Speiser *et al*., 2001), therefore it was perhaps not surprising nematodes performed poorly in all three propagator experiments. All lettuce was eaten and the nematodes were unable to cause any mortality or induce feeding inhibition. The inability of *P. hermaphrodita* to cause death to non-susceptible slugs has been problematic in field trials and the nematodes have failed to reduce slug damage (e.g. Rae *et al*., 2009). Interestingly, cedarwood oil also failed to reduce slug damage. Previous to the experiment, it was predicted the oil would be as efficient as in the experiment with *D. invadens* (as based on previous research by Klein *et al*. (2020) and Mc Donnell *et al*. (2016) the oils killed slugs and large snails – *C. aspersum*), but this was not the case. It should therefore be emphasized that although it has been stated that essential oils could be used to control pestiferous gastropods, and have shown promise by Klein *et al*. (2020) there are some slug species e.g. *A. vulgaris* that are still particularly problematic to kill. This is unfortunate as *A. vulgaris* is a difficult to kill due to its size, reproductive rate and is currently spreading round northern Europe rapidly (Zemanova *et al*. 2017).

There are only a handful of field trials that have looked at increasing the effectiveness of *P. hermaphrodita* by combing with other methods of control, such as chemical control. Hass *et al*. (1999a) applied methiocarb and *P. hermaphrodita* as a furrow treatment and found the combination worked well to decreased slug damage in sugar beet. The rationale of the study was that pellets could kill the slugs on the soil surface, while nematodes would hunt out the ones in the subterranean soil layers. Interestingly, chemical bait pellets such as methiocarb can affect the survival of *P. hermaphrodita* but not infectivity (metaldehyde has no effect on nematode survival) (Wilson *et al*. 2000b). Thus, there is potential to combine both treatments (apart from the fact metaldehyde is going to be banned in the U.K.).

There is evidence to show the combination of other nematodes (EPNs) and essential oils can also be effective at killing pests. For example, Monteiro *et al*. (2021) showed the combination of *Heterorhabditis indica* and essential oil from *Lippa triplinervis* was 90% efficient at killing cattle tick (compared to 73% using just the essential oil). Also, EPNs (*S. carpocapsae*) and diallyl disulfide (an essential oil component of garlic) has a nematocidal effect and reduces numbers of the plant parasitic nematode *Meloidogyne javanica* (Anastasiadis *et al*., 2011), compared to nematodes applied on their own.

In summary, application of *P. hermaphrodita* was effective at reducing slug damage by *D. reticulatum* in two out of three trials, but only towards the later stages. Cedarwood worked well in all trials, though only for *D. reticulatum* and not *A. vulgaris*. The combination of nematodes and cedarwood was never more efficient than individual applications of each biocontrol agent. Finally, cedarwood oil shows promise as a biological control agent for slugs and slug eggs (see previous chapter).

Chapter 6: Field trials to investigate the efficacy of *P. hermaphrodita* combined with cedar oil to control slugs

6.1 Introduction

Although the application of *P. hermaphrodita* has been shown to reduce slug damage in agricultural crops such as oil seed rape (Speiser and Andermatt, 1996), and winter wheat (Wilson *et al*., 1994a), and in high value crops such as strawberries (Glen *et al*., 2000) and asparagus (Ester *et al*., 2003a), also in orchids (Ester *et al*., 2003b) and Hostas (Grewal *et al*., 2001), there are some reports of *P. hermaphrodita* not providing protection against slug damage or reducing slug numbers (Glen *et al*., 2000, Iglesias *et al*., 2001, 2003, Wilson *et al*., 1995). It is therefore important to investigate other application methods that are more targeted and could offer superior protection. Similarly, as the standard application rate of *P. hermaphrodita is* 3×10^9 dauer juveniles per hectare (Rae *et al*., 2007), which is standard for most nematode based applications, can be prohibitively expensive for growers compared to chemical treatments (Campos-Herrera *et al*., 2015), such as metaldehyde, hence new more economical, and just-as-effective methods for nematode application are needed. These have been investigated for use of *P. hermaphrodita* but have shown mixed results. For example, Rae *et al*. (2009b) sprayed *P. hermaphrodita* round the base of plants, at three lower doses of nematodes, and dipped lettuce roots in a mixture of carboxymethylcelluose and *P. hermaphrodita* and compared these treatments to a broadcast application. They reported variable results a reduction in slug damage but there was no reduction in slug numbers (and those collected were non-susceptible species such as *A. subfuscus*).

Clearly, there is ample room for investigation of new application methods (including the combination of methods e.g. cedarwood oil which has shown promise in Chapters 4 and 5) to try to reduce the numbers of nematodes applied to soil and reduce the cost of *P. hermaphrodita* as well as maintain or increase efficacy. Furthermore, as demonstrated in this thesis, the ability of *P. hermaphrodita* to kill slugs, as well as the survival in soil can be severely affected by soil type (chapters 2 and 3). This has rarely been investigated for *P. hermaphrodita* in field studies and could account for the sub-optimal results sometimes obtained in the field.

Therefore, the aims of this chapter were to:

1. Investigate if under field conditions cedar oil, *P. hermaphrodita* and a combination of *P. hermaphrodita* and cedar oil, could provide superior slug

control in lettuce. Cedar oil was used as it has no effect on the survival of *P. hermaphrodita* (Barua *et al.* 2020; Appendix 1) and showed promising results in propagator trails in Chapter 5.

2. Whether soil type (compost and loam soil) would affect the efficacy of these slug control measures

6.2 Methods

6.2.1 Field trial methodology

The field trials were performed at Parkhaven, Maghull (SD 36957 02122) on land previously used for horticultural uses. Approximately 70 x 2 m of land was prepared by digging up weeds and using weed killer. Seventy-two plots, each consisting of 60 x 40 cm were marked out and thirty-six had top soil (sourced from a local supplier) or a 50/50 mix of garden bed soil with Sylva peat free compost added. Between each plot there were buffers of 60 x 40 cm of normal untreated soil. The plots were left for 14 days to encourage slugs and then plastic lawn edging was added (60 x 40 cm) fitted with copper slug tape and pushed into the soil to a depth of 5 cm. The lawn edging was used to prevent emerging or immigrating slugs from crossing over into other plots. At the end of the field trial all soils were removed and new soil was added to the plots before the second field trial. The entire area of land containing the plots was fenced off using mesh and covered with netting to prevent birds and other pests affecting the results.

Chinese cabbage (*Brassica rapa var. pekinensis*) was grown from seed in a glasshouse on the property. After 4 weeks of growth 6 seedlings were planted in 2 rows of 3 seedlings prior to treatment application.

The treatment for each plot was randomly allocated using a random number generator (using random.org). Treatments used were the same as in Chapter 5 and consisted of: 1. Water (2 litres) 2. 1% Tween (2 litres of water and 20 ml of Tween), 3. The recommended rate of iron phosphate pellets (1.2 g of Sluggo®) and 2 litres of water 4. The recommended rate of *P. hermaphrodita* (72,000 nematodes per plot using Nemaslug® from BASF Agricultural Specialities in 2 litres of water) 5. 0.5% Cedarwood oil (from www.pipingrock.com) mixed with 10 ml Tween in 2 litres of water) 6. 0.5% Cedarwood oil and the recommended rate of *P. hermaphrodita* (72,000 nematodes per plot), applied in 2

litres of water. Water, cedarwood oil and nematodes were applied using a watering can fitted with a rose.

In replicate 1 each treatment was applied to the two different soil types but in replicate 2, the treatments were only applied to compost. Replicate 1 was carried out on the 26th of April 2021 until the 7th of June 2021 (42 days) and replicate 2 began on the 29th of July until the 9th of September (49 days).

6.2.2 Data collection

In order to assess the effect of the treatments on slug feeding the number of leaves on each of the 6 Chinese cabbages in each plot was counted. In replicate 1 this happened on days 0, 7, 14, 21, 28, 36 and 42. In replicate 2 the number of leaves on each Chinese cabbage was quantified on days 0, 7, 14, 21, 28 and 49.

As well as quantifying the number of leaves, a plant score was assigned to each Chinese cabbage on the last day of the experiment (based on information form the Royal Horticultural Society) which consisted of estimating the damage to each plant between 0 and 5 with 0 = no sign of gastropod damage, 1 = minimal – few nibbles, and only observed when looking closely between leaves, 2 = mild – enough damage to prevent product being saleable but signs of damage are often unnoticeable from a distance 3 = medium – 50% of the leaves show damage, 4 = severe – damage to the plant that will render the plant very weak and likely to not recover 5 = complete – plant entirely (or very nearly) gone; the plant stem may remain but all leaves eaten.

At the end of the experiment each plant was cut at base, removed and weighed to record the wet and dry weight (by placing in a paper bag and then drying at 80°C for 48 hrs).

Slug populations were assessed using refuge traps set and assessed every 7 days for 6 weeks. Refuge traps consisted on using an upside down 15 cm plant saucer pot placed in the centre of each plot containing a small amount of bran underneath it. At the end of the field trials soil cores were removed from each plot and flooded to extract slugs from the soil; however, none were found on each occasion.

6.2.3 Data analysis

The effect of water, Tween, *P. hermaphrodita*, iron phosphate, cedar oil or *P. hermaphrodita* and cedar oil on the number of leaves on the lettuces grown in compost or top soil was analysed using a Two Way ANOVA. Differences in wet and dry weight (g) of the plants and plant score at the end of the trial were compared using a One Way ANOVA with Tukey's post hoc test. A chi squared test was used to understand if there was a significant difference between the total number of *D. reticulatum* and *D. invadens* found in the treatment plots in replicate 2 with compost used. This was not carried out in replicate 1 or with any other slug or snail species as n was frequently <5, and it is discouraged to use chi squared analysis when frequencies are so low.

6.3 Results

6.3.1 Investigating the efficacy of *P. hermaphrodita*, iron phosphate, cedarwood oil (with and without *P. hermaphrodita*) to reduce slug damage in Chinese cabbage grown in top soil- replicate 1

In the first field trial using loam soil there was a significant effect of time ($P<0.001$), treatment ($P<0.001$) and both factors interacted significantly ($P<0.05$) (Fig 21). However, there was no significant difference between the numbers leaves in the cabbages treated with water (control) and the other slug treatments (Tween, iron phosphate, cedar, cedar and *P. hermaphrodita*) ($P>0.05$) over 42 days apart from the single treatment of *P. hermaphrodita*, which had significantly more leaves than cabbages exposed to water ($P=0.001$), iron phosphate ($P<0.05$), cedar oil ($P<0.001$) and cedar and *P. hermaphrodita* ($P<0.001$).

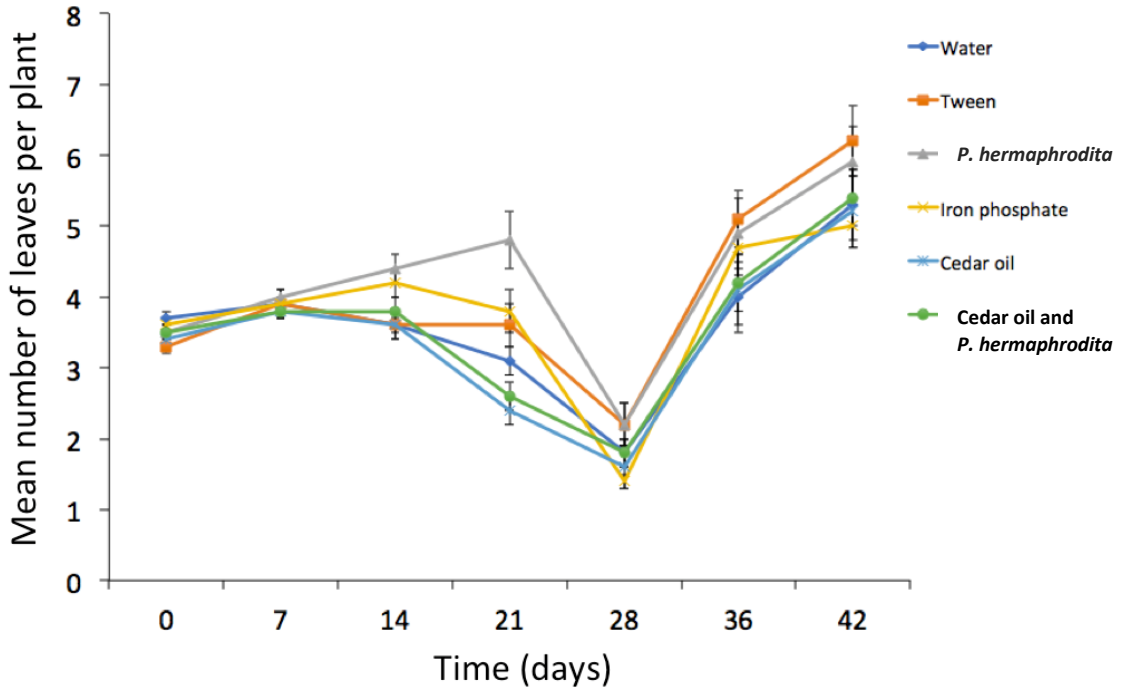

Figure 6.1: Replicate 1: Mean number of leaves per cabbage grown in loam soil over 42 days exposed to water, Tween, *P. hermaphrodita*, iron phosphate, cedar oil or *P. hermaphrodita* and cedar oil. N6, n=6 for each treatment. Bars represent ± one standard error.

At the end of the field trial cabbages grown in top soil or compost were weighed and their wet weight (g), dry weight (g) and plant score was calculated. There was no significant difference between mean wet weight, dry weight or plant score of cabbages exposed to water and Tween, *P. hermaphrodita*, iron phosphate, cedar oil or *P. hermaphrodita* and cedar oil ($P>0.05$) (Fig 22-24) grown in top soil after 42 days.

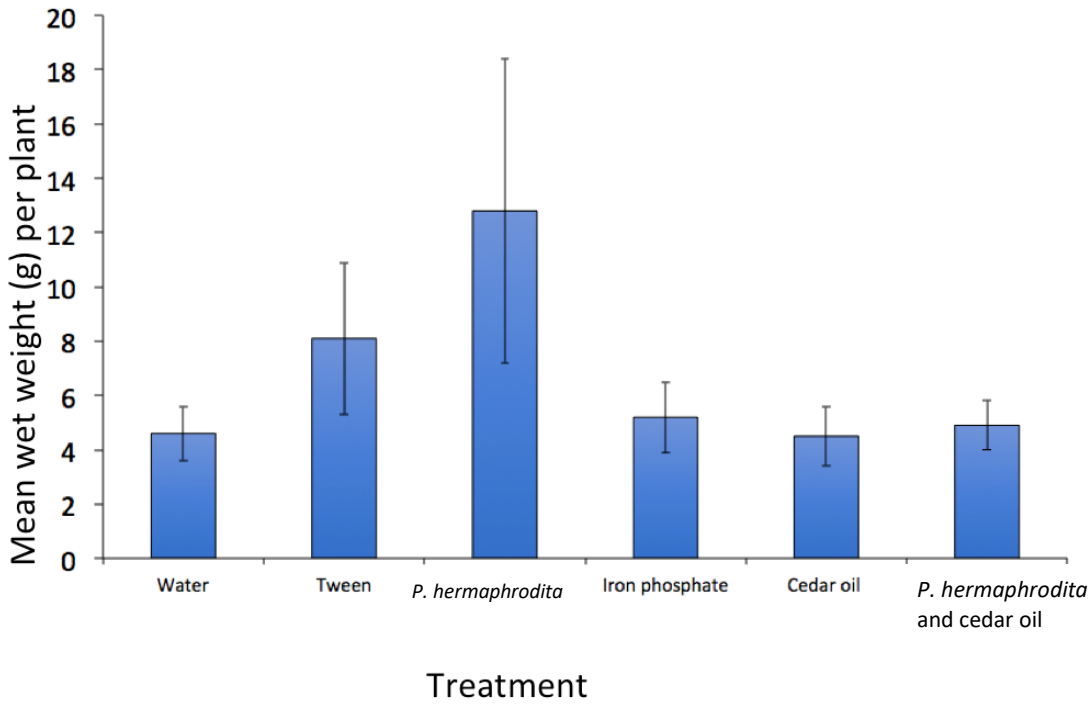

Figure 6.2: Replicate 1: Mean wet weight (g) of all cabbages grown in loam soil exposed to water, Tween, *P. hermaphrodita*, iron phosphate, cedar oil or *P. hermaphrodita* and cedar oil. N6, n=6 for each treatment. Bars represent ± one standard error.

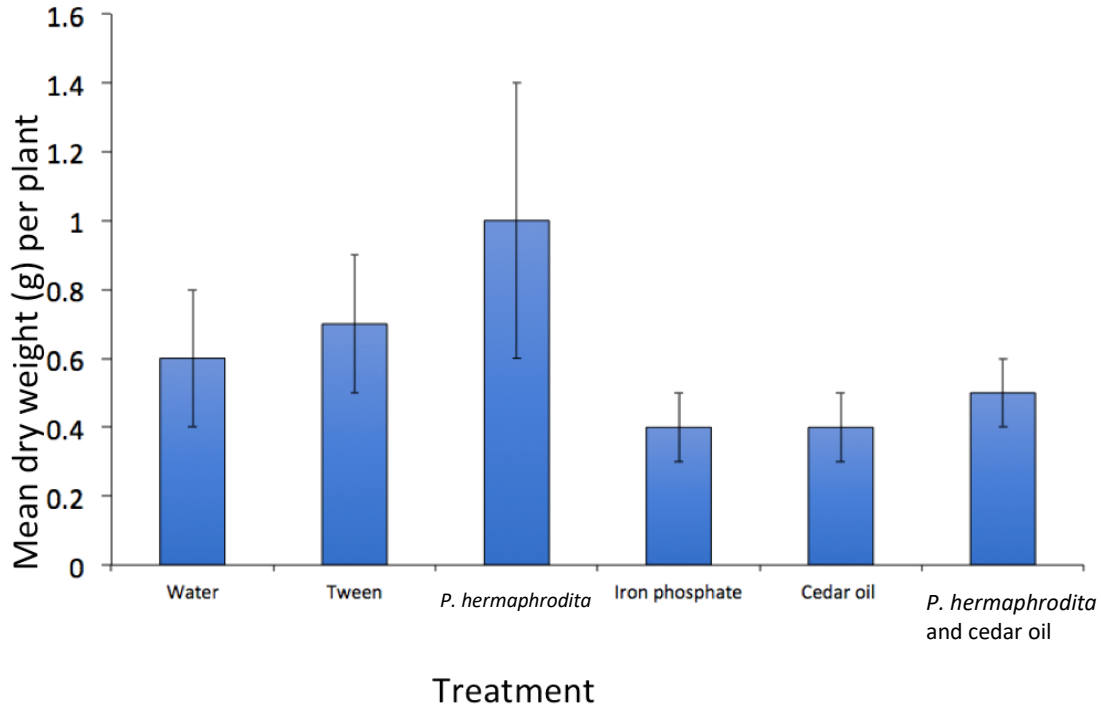

Figure 6.3: Replicate 1: Mean dry weight (g) of all cabbages grown in loam soil exposed to water, Tween, *P. hermaphrodita*, iron phosphate, cedar oil or *P. hermaphrodita* and cedar oil. N6, n=6 for each treatment. Bars represent ± one standard error.

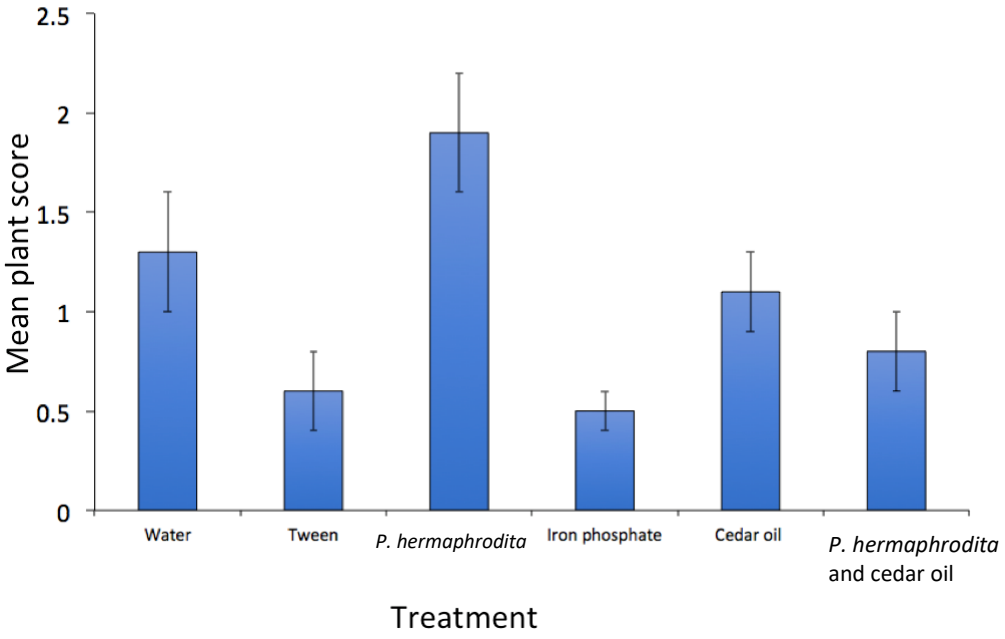

Figure 6.4: Replicate 1: Mean plant score of all cabbages grown in loam soil exposed to water, Tween, *P. hermaphrodita*, iron phosphate, cedar oil or *P. hermaphrodita* and cedar oil. N6, n=6 for each treatment. Bars represent ± one standard error.

Every 7 days for 7 weeks slugs were collected from the plots and counted. There were very few slugs found over the 7 weeks, the most common was *D. reticulatum*, followed by *D. invadens* and *A. vulgaris* (Table 5).

Table 4: Total number of slugs collected every week over 7 weeks from plots with loam soil in replicate 1

	D. reticulatum	*D. invadens*	*A. hortensis*	*A. vulgaris*	*T. budapestensis*	*Limax spp.*	*A. valentianus*	*Cepaea spp.*
Water	3	0	0	1	0	0	1	1
Tween	2	1	0	0	0	0	0	0
Iron phosphate	3	2	0	0	0	0	1	0
P. hermaphrodita	2	1	0	1	0	0	0	0
Cedar oil	0	0	0	1	0	0	0	0
Cedar oil and P. hermaphrodita	0	0	0	1	0	0	0	0

6.3.2 Investigating the efficacy of *P. hermaphrodita*, iron phosphate, cedarwood oil (with and without *P. hermaphrodita*) to reduce slug damage in cabbages grown in compost - replicate 1

In the first field trial using compost there was a significant effect of time ($P<0.001$), treatment ($P<0.001$) and both factors interacted significantly ($P<0.001$) (Fig 25). There was no significant difference between the number of leaves on the cabbages exposed to water and tween, *P. hermaphrodita*, iron phosphate ($P>0.05$) over 42 days, but there were significantly fewer leaves on cabbages treated with cedar oil ($P<0.01$) and cedar oil and *P. hermaphrodita* ($P<0.001$) compared to the water control. There was no significant difference between the numbers of leaves on the cabbages treated with cedar oil and cedar oil and *P. hermaphrodita* ($P>0.05$).

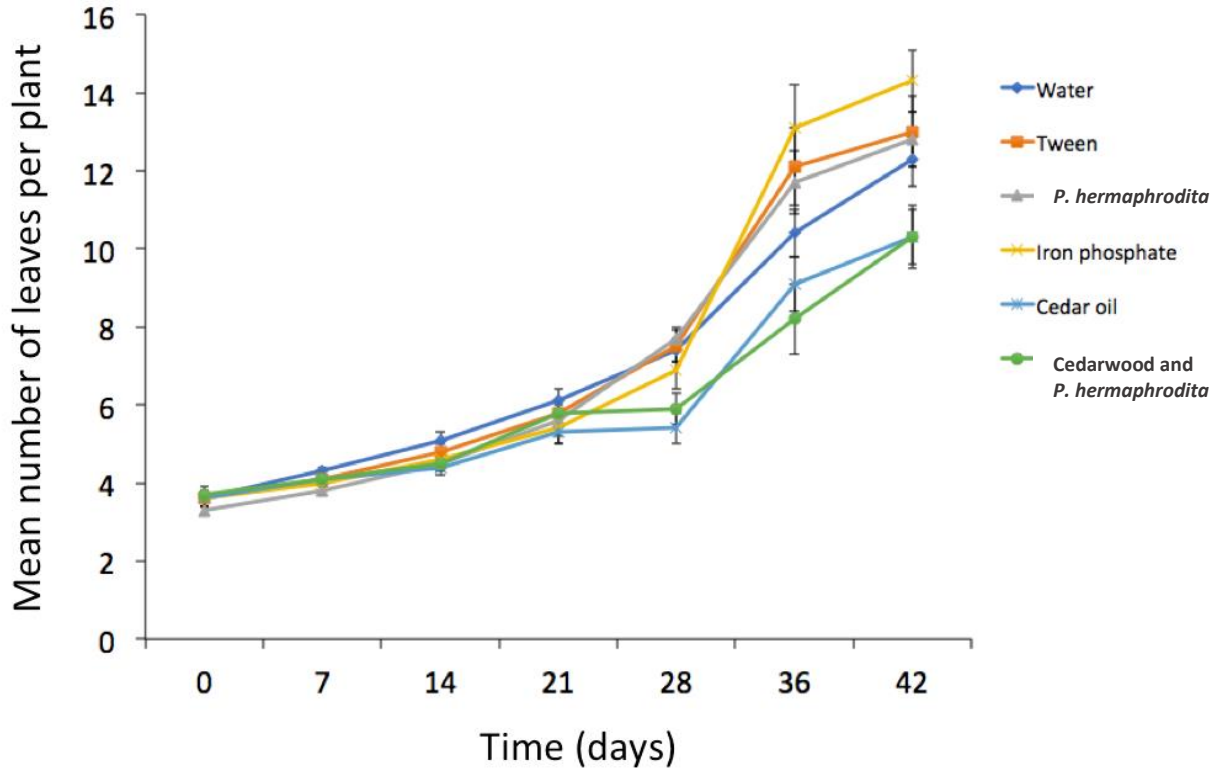

Figure 6.5: Replicate 1: Mean number of leaves per cabbages grown in compost over 42 days exposed to water, Tween, *P. hermaphrodita*, iron phosphate, cedar oil or *P. hermaphrodita* and cedar oil. N6, n=6 for each treatment. Bars represent ± one standard error.

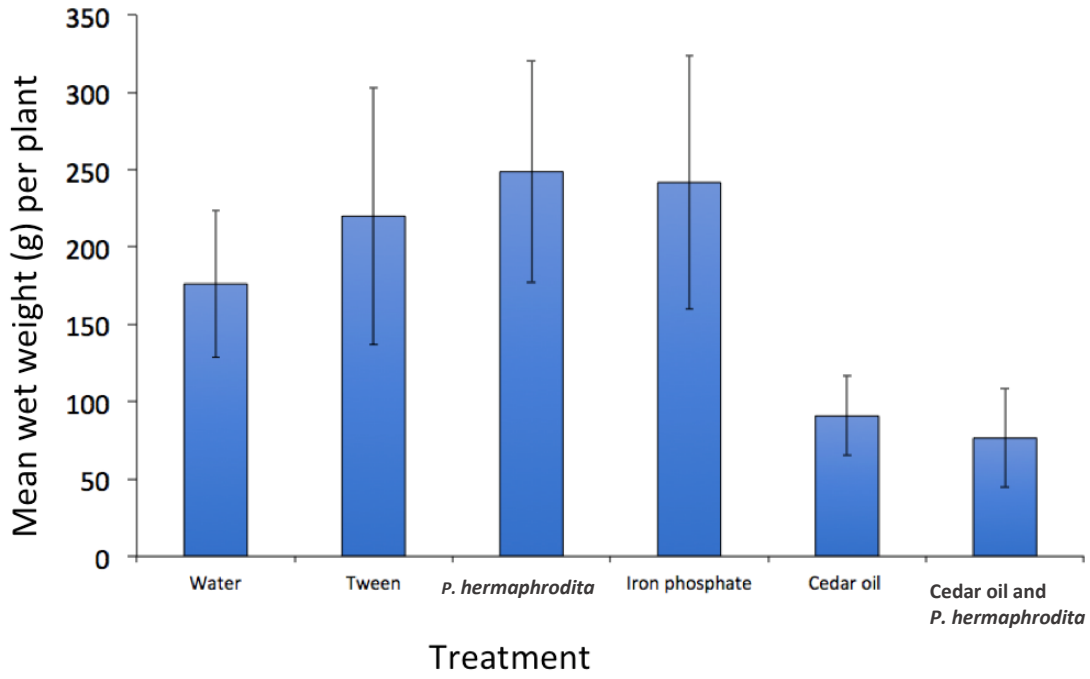

Figure 6.6: Replicate 1: Mean wet weight (g) of all cabbages grown in compost exposed to water, Tween, *P. hermaphrodita*, iron phosphate, cedar oil or *P. hermaphrodita* and cedar oil. N6, n=6 for each treatment. Bars represent ± one standard error.

There was no significant difference between mean wet weight, dry weight or plant score of cabbages exposed to water and Tween, *P. hermaphrodita*, iron phosphate, cedar oil or *P. hermaphrodita* and cedar oil ($P>0.05$) grown in compost after 42 days (Fig 25-28).

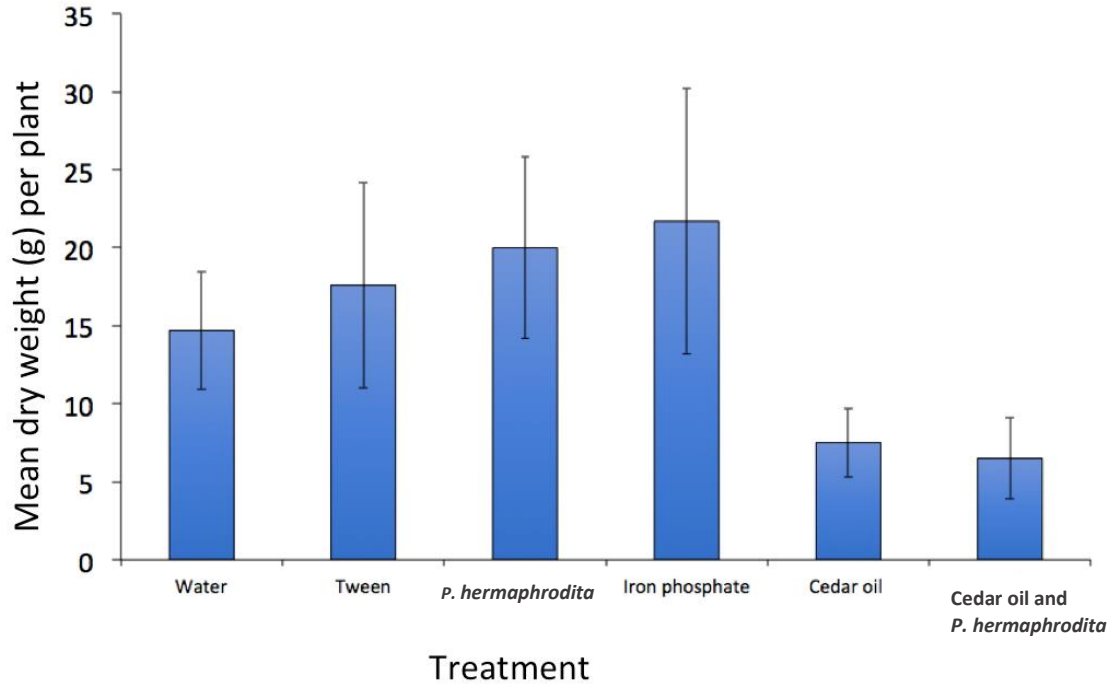

Figure 6.7: Replicate 1: Mean dry weight (g) of all cabbages grown in compost exposed to water, Tween, *P. hermaphrodita*, iron phosphate, cedar oil or *P. hermaphrodita* and cedar oil. N6, n=6 for each treatment. Bars represent ± one standard error.

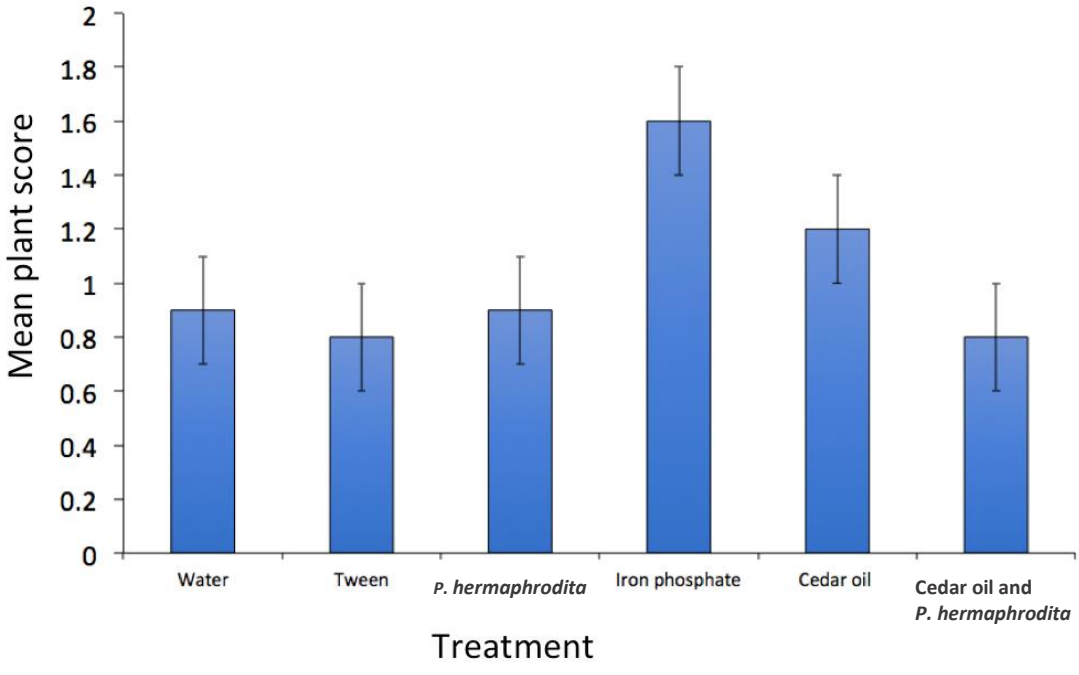

Figure 6.8: Replicate 1: Mean plant score of all lettuces grown in compost exposed to water, Tween, *P. hermaphrodita*, iron phosphate, cedar oil or *P. hermaphrodita* and cedar oil. N6, n=6 for each treatment. Bars represent ± one standard error.

As with the plots with loam soil, the plots with compost had very few slugs recorded over the 7 weeks, the most abundant were *D. reticulatum* and *D. invadens* (Table 6).

Table 5: Total number of slugs collected every week for 7 weeks in plots with compost in replicate 1

	D. reticulatum	*D. invadens*	*A. hortensis*	*A. vulgaris*	*T. budapestensis*	*Limax spp.*	*A. valentianus*	*Cepaea spp.*
Water	6	1	0	0	0	0	0	1
Tween	7	0	0	1	0	0	0	0
Iron phosphate	4	2	0	0	1	1	1	1
P. hermaphrodita	1	1	0	0	0	0	0	1
Cedar oil	3	0	0	0	0	0	2	0
Cedar oil and *P. hermaphrodita*	4	0	0	1	0	0	0	0

6.3.3 Investigating the efficacy of *P. hermaphrodita*, iron phosphate, cedarwood oil (with and without *P. hermaphrodita*) to reduce slug damage in cabbage grown in compost - replicate 2

In the second field trial using only compost there was a significant effect of time ($P<0.001$), treatment ($P<0.001$) and both factors interacted significantly ($P<0.001$) (Fig 29). There was no significant difference between the number of leaves on cabbages exposed to water or iron phosphate, *P. hermaphrodita* ($P>0.05$) over 49 days, but there were significantly fewer leaves on cabbages exposed to cedar oil ($P <0.001$) and *P. hermaphrodita* and cedar oil ($P<0.001$) (Fig 29). There was no difference between the numbers of leaves on cabbages exposed to cedar oil ($P >0.05$) and *P. hermaphrodita* and cedar oil ($P>0.05$) (Fig 29).

There was no significant difference between mean wet weight, dry weight or plant score of cabbages exposed to water and Tween, *P. hermaphrodita,* iron phosphate, cedar oil or *P. hermaphrodita* and cedar oil ($P>0.05$) (Fig 30-32) grown in compost after 49 days.

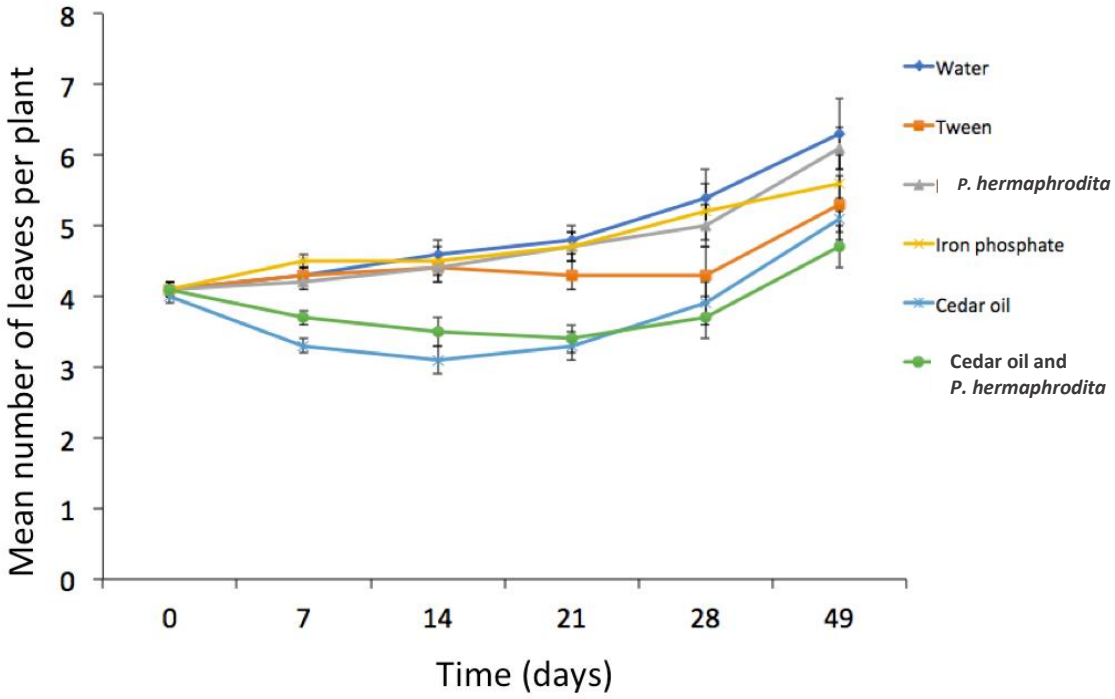

Figure 6.9: Replicate 2: Mean number of leaves per cabbage grown in compost over 49 days exposed to water, Tween, *P. hermaphrodita*, iron phosphate, cedar oil or *P. hermaphrodita* and cedar oil. N=6, n=12 for each treatment. Bars represent ± one standard error.

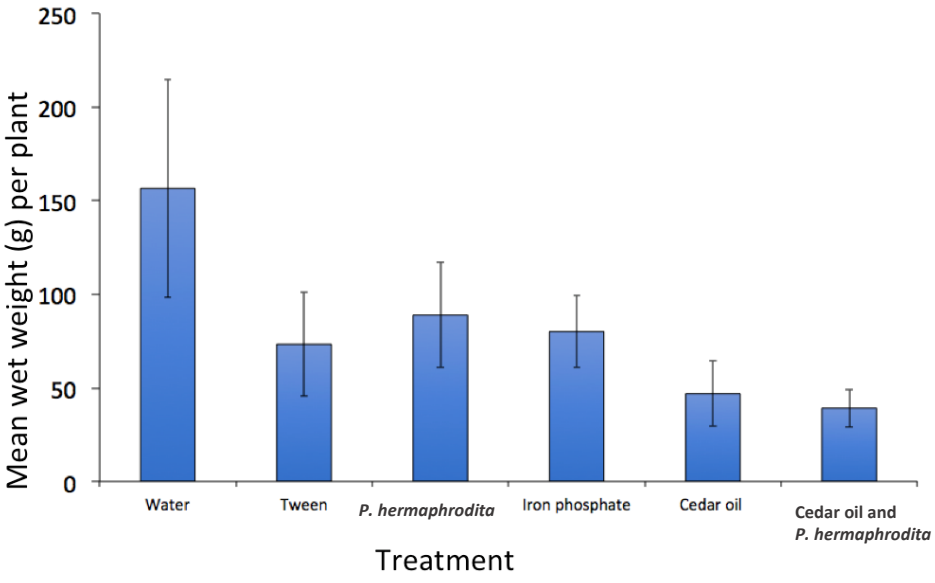

Figure 6.10: Replicate 2: Mean wet weight (g) of all cabbages grown in compost exposed to water, Tween, *P. hermaphrodita*, iron phosphate, cedar oil or *P. hermaphrodita* and cedar oil. N=6, n=12 for each treatment. Bars represent ± one standard error.

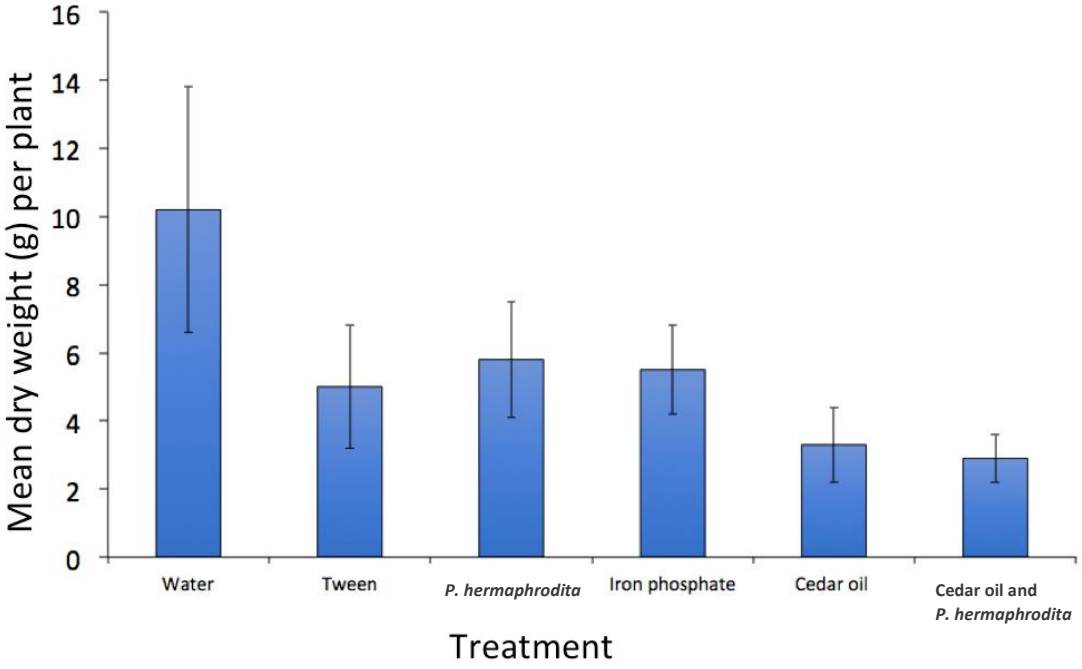

Figure 6.11: Replicate 2: Mean dry weight (g) of all cabbages grown in compost exposed to water, Tween, *P. hermaphrodita*, iron phosphate, cedar oil or *P. hermaphrodita* and cedar oil. N=6, n=12 for each treatment. Bars represent ± one standard error.

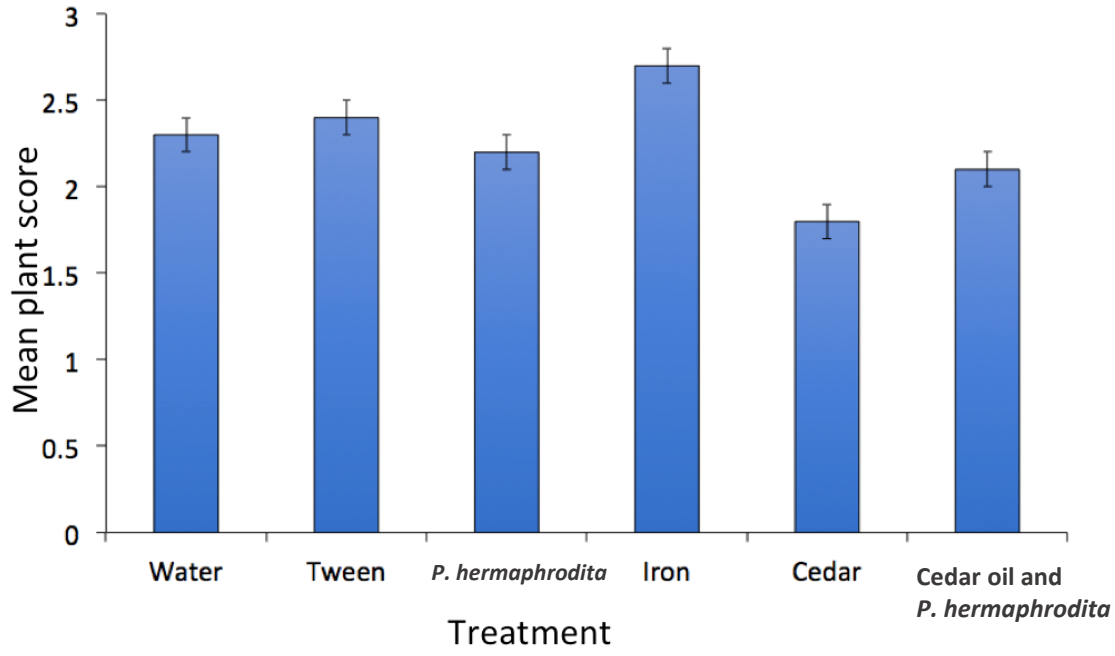

Figure 6.12: Replicate 2: Mean plant score of all cabbages grown in compost exposed to water, Tween, *P. hermaphrodita*, iron phosphate, cedar oil or *P. hermaphrodita* and cedar oil. N=6, n=12 for each treatment. Bars represent ± one standard error.

A greater numbers of slugs were recorded in replicate 2 using compost. By far the most common was *D. reticulatum*, followed by *D. invadens* and *A. hortensis* (Table 7). There was no significant difference between the numbers of *D. reticulatum* and *D. invadens* in the different treatments ($X_2 = 6.22$, $P = 0.28$).

Table 6: Total number of slugs collected every week for 7 weeks from plots with compost in replicate 2

	Deroceras reticulatum	*Deroceras invadens*	*Arion hortensis*	*Arion vulgaris*	*Tandonia budapestensis*	*Limax spp.*	*Ambigolimax valentianus*	*Cepaea spp.*
Water	42	10	3	3	0	0	2	0
Tween	22	7	1	0	0	0	2	0
Iron phosphate	35	8	6	0	0	1	2	2
P. hermaphrodita	15	11	4	0	1	1	4	3
Cedar oil	20	6	1	0	2	0	3	0
Cedar oil and *P. hermaphrodita*	13	5	2	1	2	1	5	2

6.4 Discussion

In the first replicate of the field trial using loam soil, the only treatment that had an effect on numbers of cabbage leaves, was a single application of *P. hermaphrodita*, which had significantly more leaves that the water based control and iron phosphate, cedar oil and cedar and *P. hermaphrodita*, although there was no difference in the wet or dry weight of the cabbages or in the mean plant score. The nematodes performed poorly as a control in the first replicate with compost. There was no difference in the treatments apart from the use of cedar oil (alone or with *P. hermaphrodita*), which resulted in a significant reduction in number of leaves. It may be under these conditions the cedar oil had a phytotoxic effect, but further research is needed. The wet and dry weights were unaffected, as well as the mean plant score. The loam soil leaf counts in replicate 1, on day 28 shows a dip in the number of leaves present. We were at that time have dry conditions with the loam soil showing desiccation at a faster rate than compared to composts. Rainfall that came after this time enable the regrowth of the plants still surviving in the loam after day 28. In the second replicate with compost, similar results were reported, with none of the treatments having any significant effect on the number of leaves on the cabbages apart from cedar oil applied in one application on its own or with nematodes, where there were a significantly lower number of leaves recorded (although there was no difference in wet or dry weight, or mean plant score).

The lack of demonstrable effect of the slug control treatments (apart from nematodes) in the first replicate must be due to the lack of slugs in the area. Though preliminary slug

survey data of Parkhaven recorded an abundance of different slug species such as *D. reticulatum*, when the trial was carried out the numbers dwindled. In contrast this was not the case in the second replicate carried out with just compost. High numbers of *D. reticulatum* and *D. invadens* were collected and quantified over the 7 weeks of the trial; however, there was little effect of the slug control treatments. Presumably the variability in slug numbers was due to the weather. The weather during the first repetition (April to June 2021) was noted by the Met Office as being 'very mixed'. For example, April was the fourth driest on record which was then followed by May that had heavy downpours. The cold, stormy conditions, unseasonably strong winds and low pressure of May 2021 would also have impacted the number of slugs present as they tend to retreat underground. This was in contrast to the second trial in August, which was warmer and wetter, perfect for slug movement and proliferation.

Interestingly, cedar oil had some sort of phytotoxic effect on the plants and reduced plant health. A major drawback in the use of essential oil is many are phytotoxic e.g. *Eucalyptus* essential oils kill lettuce (Aragão *et al*., 2015) and oregano negatively affects seed germination and seedling growth of cucumber and tomato (Ibáñez and Blázquez, 2020). There are many more examples, so much so some have been suggested as herbicides to kill problematic weeds (see Abd-ElGawad *et al*., 2021 for a review).

In conclusion, it is encouraging to see application of *P. hermaphrodita* can prevent slug damage in replicate 1 with loam soil, however, the nematodes performed poorly in replicate one and two with compost. This difference in the ability of *P. hermaphrodita* to control slug damage in two different soil types in the field has never been reported. From data collected in Chapter 2, it is apparent the nematodes exit the dauer stage when added to compost. This may be the reason *P. hermaphrodita* performed so poorly under field conditions. It is therefore imperative farmers and gardeners record the soil type before application of *P. hermaphrodita* as soil type does not just affect the ability of the nematode to exit the dauer stage, it also affects dispersal ability and behaviour (Macmillan *et al*., 2009). However, other factors played a role in poor performance of the slug treatments, for example, in the first replicate there was a severe lack of slugs, which may be due to the weather. Finally, though other treatments, such as cedar oil should have a lethal effect on slug eggs and juveniles in lab based studies, we did not replicate this in the field. Cedar oil failed to offer any significant slug control and it negatively affected plant health, which rules out its use in commercial farming.

Chapter 7: Profiling the grey field slug microbiome and investigating whether it changes upon *P. hermaphrodita* infection

7.1 Introduction

A poorly understood factor in the interaction between slugs and *P. hermaphrodita* is the role of microbiome in both the host and parasite. From the nematode point of view it was thought, upon infection, *P. hermaphrodita* vectors *Moraxella osloensis* into the haemocoel of the slug and was responsible for death (Tan and Grewal, 2001a,b; 2002). However, recent research failed to find this bacterium in the next generation of infective juveniles of *P. hermaphrodita* (and two other *Phasmarhabditis* species, *P. californica* and *P. neopapillosa*) after killing a slug using 16S rRNA metagenomic sequencing (Sheehy *et al*., 2022). Furthermore, these authors showed, using 16S rRNA amplicon sequencing, that *M. osloensis* is actually a *P. faecalis*. Therefore, the role bacteria play in causing death to slugs is currently unknown. This warrants further research as *Phasmarhabditis* nematodes are the only genus of Nematoda that have evolved to infect and kill slugs and snails out of the entire Nematoda phylum (consisting of an estimated 1 million species). From the slug point of view, the role the microbiome plays in the health of these animals is unknown. There are only few microbiomes that have been studied in terrestrial gastropods. For example, Cardoso *et al*. (2012) examined the microbiome of the giant land snail *Lissachatina fulica* and found an abundance of Proteobacteria and when it ate a diet rich in sugarcane there was a greater abundance of *Bacteroidetes* and *Firmicutes*. Joynson *et al*. (2017) investigated the microbiome of the common black slug, *A. ater* and found the most abundant genera were *Enterobacter, Cirobacter, Pseudomonas, Escherichia, Acinetobacter* and *Sphingobacteriaceae*. Reich *et al*. (2018) showed the most abundant phyla in the protected Kerry slug *Geomaculus maculosus* were Proteobacteria, Bacteroidete, Planctomycetes, Acdiobacteria, Verrricomicrobita, Firmicutes and Actinobacteria.

The microbiome plays an important role in the health of animals (Fan and Pederen, 2021; Peixoto *et al*., 2021; Wu and Wu, 2012) but how the slug microbiome changes in response to *P. hermaphrodita* infection is unknown. Hence the aim of this chapter was to profile the microbiome of the pestiferous slug *D. reticulatum* and to understand if it changes upon infection of *P. hermaphrodita.*

7.2 Materials and methods

7.2.1 Source of invertebrates

Slugs (*D. reticulatum*) were collected from a garden in Oakworth, Keighley, Liverpool (OS grid reference SE045389) and kept in non-airtight plastic boxes (35 x 23 x 22 cm) lined with moist tissue paper at 15°C for 7 days and checked daily for any signs of infection by *Phasmarhabditis* nematodes e.g. swollen mantle, lesions. The slugs were immediately fed a diet of iceberg lettuce and carrots using protocols by McDonald-Howard *et al*. (2022).

P. hermaphrodita (strain DMG0001) was purchased from BASF Agricultural Specialities and stored at 15°C for one week before the experiment.

7.2.2 Infection of *D. reticulatum* with *P. hermaphrodita*

To infect slugs with *P. hermaphrodita* we used a modified soil based bioassay (Cutler *et al*., 2020; Sheehy *et al*., 2022). Briefly, to 50 ml Falcon tubes a cotton wool bung was added to the bottom and 1,000 infective stage *P. hermaphrodita* were added in 0.5 ml of tap water. Control received water and no nematodes. Two *D. reticulatum* were added to each tube, a cotton wool bung was loosely placed on top and the lid was added and stored at 10°C. The survival of the slugs was monitored daily and after 5 days the slugs were placed in new 50 ml Falcon tubes with fresh carrot and lettuce discs.

7.2.3 Harvesting of gut microbiota in faeces of *D. reticulatum*

Sixteen individual faecal samples were collected from each tube and weighed (mean weight = 0.036 ± 0.005 g; n = 16).

Group 1: Faeces collected from wild *D. reticulatum* (without infection with *P. hermaphrodita*) collected on day 0.

Group 2: Faeces collected from wild *D. reticulatum* (without infection with *P. hermaphrodita*) after days being fed a diet of lettuce and carrot for 7 days.

Group 3: Faeces collected from wild *D. reticulatum* (infected with *P. hermaphrodita*) after days being fed a diet of lettuce and carrot for 7 days.

We checked bacteria DNA was present after extractions by using PCR amplification of the hypervariable regions of the 16S rRNA gene was carried out using the primers 27f (5’-

AGAGTTTGATCMTGGCTCAG-3') and 1492r (5'-TACGGYTACCTTGTTACGACTT-3') and the following conditions: 3 min at 95°C followed by 35 cycles of 15 sec at 95°C, 30 sec at 55°C, 1.5 min at 72°C and a final step of 8 mins at 72°C. Amplicons were visualised using agarose gel electrophoresis to ensure that the PCRs had worked correctly; in all cases bands of the correct size were present and no amplification of bacterial DNA could be seen in the negative controls.

7.2.4 16S rRNA bioinformatic analysis

DNA samples were sent for 16S rRNA Metagenomic sequencing (Novogene). The V4 hypervariable region of the 16S rRNA gene was amplified using the primers 515F (5'-GTGCCAGCMGCCGCGGTAA-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3'), all PCR reactions were carried out with Phusion® High-Fidelity PCR Master Mix (New England Biolabs). The libraries were generated with NEBNext® UltraTM DNA Library Prep Kit for Illumina and quantified via Qubit and Q-PCR. These libraries were sequenced on an Illumina NovaSeq 6000 platform to generate 2 x 250 bp paired-end reads.

Novogene carried out all the bioinformatic analysis including filtering, alpha and beta diversity analysis to obtain richness and evenness information, Principal Component Analysis (PCA) and Unweighted Pair Group Method with Arithmetic Mean (UPGMA). Further details on bioinformatic analysis can be found in Sheehy *et al*. (2022).

A One-way Analysis of Variance (ANOVA) with Tukey's post hoc test was used to compare the abundance of Proteobacteria and Bacteroidota in groups 1, 2 and 3.

7.3 Results

The microbiome of wild *D. reticulatum* (without infection with *P. hermaphrodita*) collected on day 0 (group 1) consisted mainly of Proteobacteria and Bacteriodota, as well as smaller amounts of Actinobacterota, Verrucomicrobiota, Firimicutes, Desulfobacterota, Campilbacterota and other phyla (Fig 33). Faeces was harvested from slugs 7 days later and were fed a diet of lettuce and carrot (group 2). There was no significant difference between the abundance of phyla between Group 1 and 2 (Fig 33; $P = 0.1$), therefore the lab diet of lettuce and carrot did not significantly affect the microbiome of the slugs. However, there was a significant difference in the abundance of phyla found in the microbiome from *D. reticulatum* infected with *P. hermaphrodita* (group 3) and the abundance of phyla found in the microbiome from *D. reticulatum* (group 1) collected on day 0 (Fig 33; $P = 0.007$).

Specifically, there were fewer Proteobacteria and more Actinobacteriota. Similarly, there was also a significant difference between the abundance of phyla found in the microbiome from *D. reticulatum* fed lab diet (group 2) and the microbiome from *D. reticulatum* infected with *P. hermaphrodita* (group 3) (Fig 33; P <0.001).

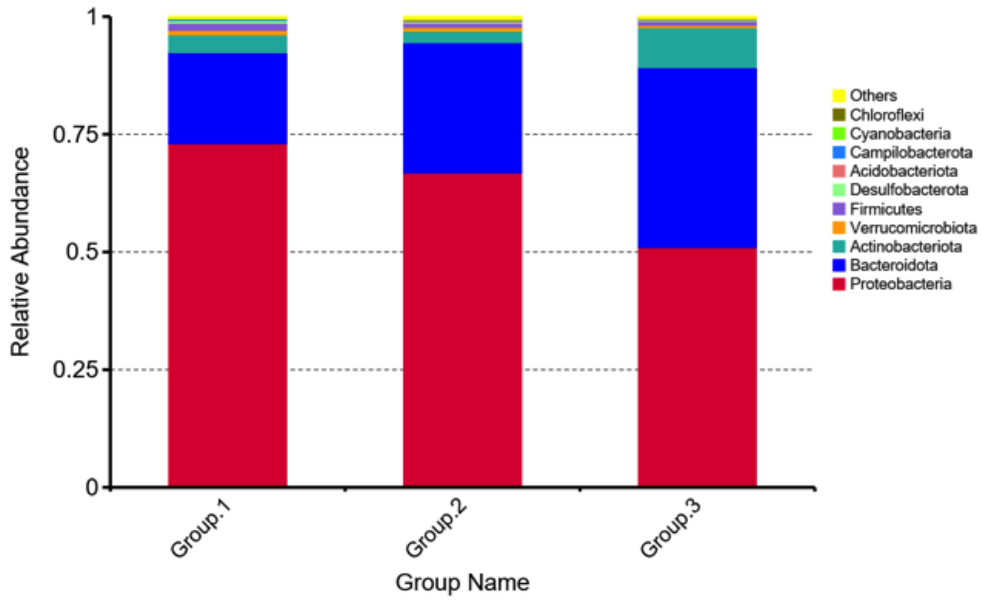

Fig 7.1: The abundance of the most prevalent phyla in the three treatments. Group 1: Microbiome from *D. reticulatum* collected on day 0; Group 2: Microbiome from *D. reticulatum* fed lab diet; Group 3: Microbiome from *D. reticulatum* infected with *P. hermaphrodita*. n=16 for each treatment.

Each group has its own unique microbiota (Fig 34). For example, faeces from *D. reticulatum* collected on day 0 from the wild had 168 unique Operational Taxonomic Units (OTUs), *D. reticulatum* fed on a lab diet for 7 days had 450 and *D. reticulatum* infected with *P. hermaphrodita* had 321 OTUs (Fig 34). However, there was a core microbiome found between all 3 groups which consisted of 774 OTUs (Fig 34).

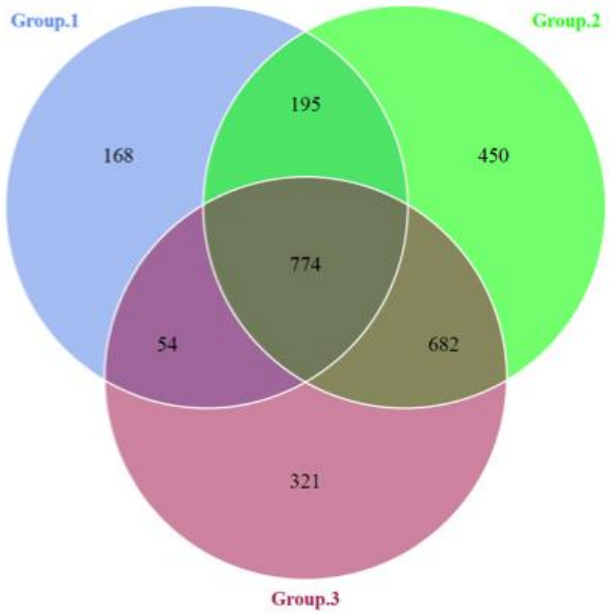

Fig 7.2: Venn diagram showing the number of OTUs (operational taxanomic units) in the three treatments. Group 1: *D. reticulatum* collected on day 0; Group 2: *D. reticulatum* fed lab diet; Group 3: *D. reticulatum* infected with *P. hermaphrodita* n=16 for each treatment.

In terms of understanding which groups were more closely related to each other UPGM cluster analysis was used (Fig 35), which showed phyla found in faeces from *D. reticulatum* collected on day 0 and *D. reticulatum* fed a lab based diet for 7 days were more similar in community structure than group 3 – *D. reticulatum* infected with *P. hermaphrodita* (Fig 35). This strongly suggests the microbiome of *D. reticulatum* is affected by infection by the nematode. Further evidence of this can been seen from Fig 36 which shows Principal Component Analysis (PCA) of beta diversity of each group 3 which clearly clusters in its own group away from groups 1 and 2.

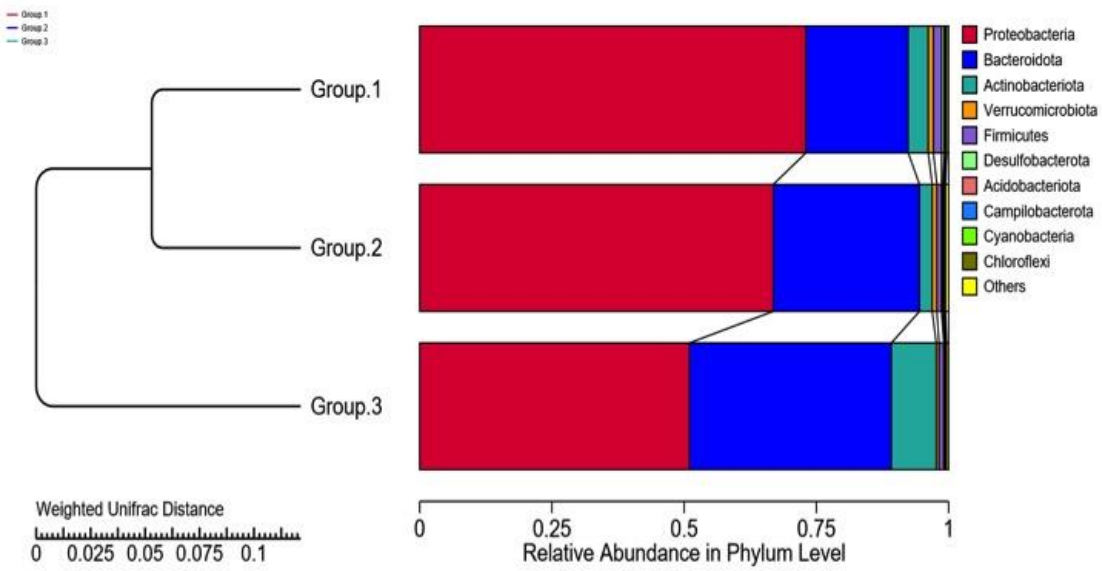

Fig 7.3: UPGM cluster tree based on Weighted UniFrac distances at the phylum level of the three treatments. Group 1: *D. reticulatum* collected on day 0; Group 2: *D. reticulatum* fed lab diet; Group 3: *D. reticulatum* infected with *P. hermaphrodita*. n=16 for each treatment.

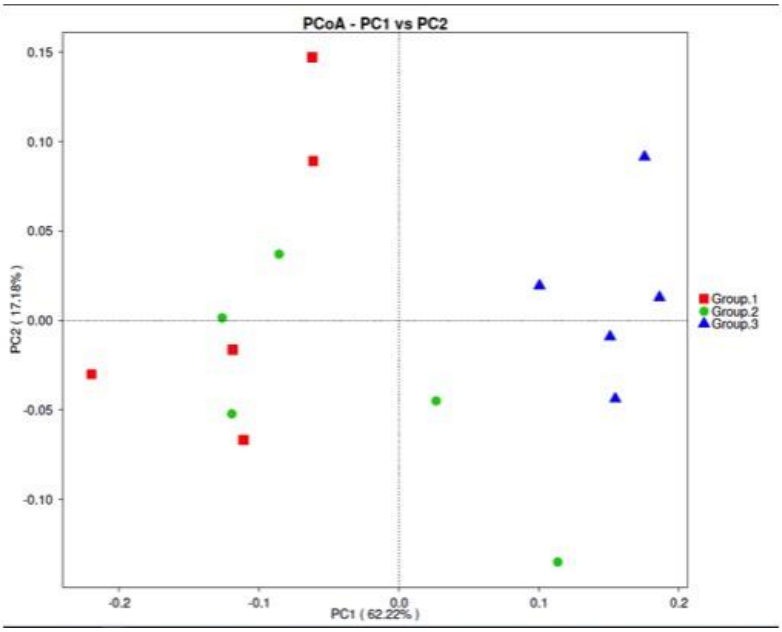

Fig 7.4: Beta diversity comparison for each sample was completed via a Principal Component Analysis (PCA) showing the relatedness of the three replicates from the three treatments. Group 1: *D. reticulatum* collected on day 0; Group 2: *D. reticulatum* fed lab diet; Group 3: *D. reticulatum* infected with *P. hermaphrodita*. n=16 for each treatment.

Of all phyla that differed in abundance between the groups, there were significantly more Proteobacteria found in wild *D. reticulatum* collected on day 0 (and *D. reticulatum* fed a lab based diet for 7 days) compared to *D. reticulatum* infected with *P. hermaphrodita* (Fig 37A) (P *reticulatum* 0.05). In contrast there were significantly more Bacteroidota found in *D. reticulatum* collected in day 0 compared to *D. reticulatum* infected with *P. hermaphrodita* (Fig 37B) ($P < 0.05$). Therefore, infection with *P. hermaphrodita* seems to change the microflora of *D. reticulatum* and specifically the abundance of Proteobacteria and Bacteroidota.

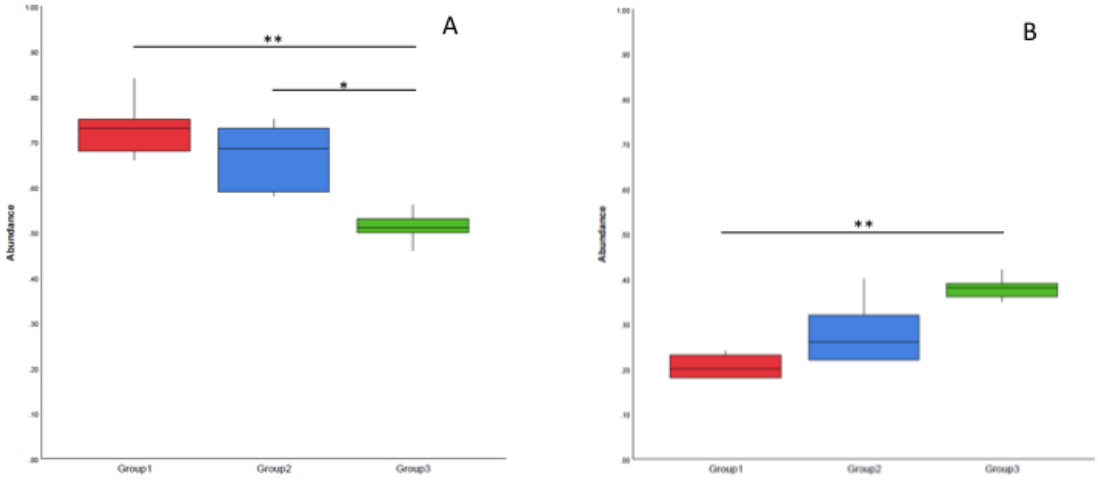

Fig 7.5: Abundance of Proteobacteria (A) and Bacteroidota (B) in the three replicates from the three treatments. Group 1: *D. reticulatum* collected on day 0; Group 2: *D. reticulatum* fed lab diet; Group 3: *D. reticulatum* infected with *P. hermaphrodita*. n=16 for each treatment.

7.4 Discussion

The native microbiome of *D. reticulatum* collected from the wild consists of mainly of Proteobacteria and Bacteriodota, as well as smaller amounts of Actinobacterota, Verrucomicrobiota, Firimicutes, Desulfobacterota and Campilbacterota. This is similar to results by Cardoso *et al*. (2012) who found an abundance of Proteobacteria in *L. fulica* and Reich *et al*. (2018) who found the most abundant phyla in *G. maculosus* were Proteobacteria, Bacteroidete, Planctomycetes, Acdiobacteria, Verrricomicrobita, Firmicutes and Actinobacteria, but differs from research reported by Joynson *et al*. (2017) who found the

most abundant genera in the microbiome of *A. ater* were Enterobacter, Cirobacter, Pseudomonas, Escherichia, Acinetobacter and Sphingobacteriaceae.

The microbiome did not change significantly when the slugs were kept under lab conditions and fed lettuce and carrots (based on methods developed in Chapter 4); however, there was a significant difference in the microbiome when *D. reticulatum* was infected with *P. hermaphrodita*. Specifically, the abundance of Proteobacteria decreased in *D. reticulatum* infected with *P. hermaphrodita* (compared to *D. reticulatum* collected on day 0) and the abundance of Bacteroidota increased when comparing the same treatments. There are two reasons for this: either *P. hermaphrodita* is able to change the microbiome of the *D. reticulatum* or, due to the infection process and *D. reticulatums* immunodeficiency state is impacted which subsequently alters the microbiome. There is evidence in other host/parasite systems to show parasites can directly affect the microbiota. For example, infection with whipworm (*Trichuris muris*) reduced microbiome alpha-diversity in mice (Houlden *et al*., 2015) but promoted growth of *Lactobacillus* (Holm *et al*., 2015). Also, Hahn *et al*. (2022) they showed the microbiome of sticklebacks changes with not just infection of the cestodes parasite *Schistocephalus solidus* but this is also dependant on the genotype of the parasite. Similar changes to the microbiome have been found in other host/parasite systems such as *Daphnia* waterfleas infected with pathogenic *Pasteuria* (Preiswerk *et al*., 2018).

Chapter 8: General discussion and conclusions

In Chapter 2 and 3 I studied the effect of six different soils on the survival, movement and ability to kill slugs. *P. hermaphrodita* survived best in compost without peat, and unusually it was observed exiting the dauer stage and reproducing prolifically. This is concerning. All other stages of the nematode life cycle do not infect slugs therefore, poor slug control may occur. In the movement experiment the nematodes stayed at the point of application and there was no different in the ability to move through the soils. Presumably, the species of *Phasmarhabditis* species tested are 'ambushers' and wait for slugs to pass before latching on and infecting the slugs. The acidity of the soils and well as the soil structures and composition may be influencing the outcomes, as the compost with peat (a known more acidic substrate) which triggered a high number of dauer to progress into their next life stage, however the yield will still significantly lower that the compost with no peat. *P. hermaphrodita* added to compost with peat caused earlier morbific states of the slugs. (for reasons unknown). Therefore it can be surmised that compost with peat was a less habitable environment than that without peat. As soil affected survival of the nematodes and they exited the dauer stage it is imperative farmers and gardeners pay attention to soil type before application of these nematodes. It remains unknown what the exact properties of the soils are which affect the ability of the nematode to kill slugs and cause nematodes to exit the dauer stage. Further studies into the preference of nematode feeding sources would certainly help direct us to the answers to the questions generated by this study.

In Chapter 4 I developed a slug rearing protocol, which allowed me to grow enough slugs to expose them to numerous essential oils and test their ability to kill slug eggs and juveniles. All the essential oils killed slug eggs and juveniles (apart from birch tar). Cedarwood was a clear candidate for future mesocosm (Chapter 5) and field studies (Chapter 6), especially as it was shown to not affect the survival of *P. hermaphrodita*. A combination of nematodes and cedarwood oil may act synergistically and could be beneficial for slug control with slug eggs being killed by cedarwood oil and the nematodes killing the juvenile slugs. In propagator experiments cedarwood oil worked very well, but nematodes were variable. There was no clear benefit of the combination of nematodes and cedarwood oil. However when extracting the data and looking at it through individual periods of time (climatic periods) the controls efficiency varied during the temperatures. The nematodes were most successful during the cooler periods and cedarwood essential oil out competing during the higher temps, at medium temperatures the oils and nematodes combination demonstrated

their best results. Therefore, a possible combination of a synergistic and independent controls that could be administered with seasonal adjustment. In the field trial (Chapter 6), using the same treatments (but with two different soils), there was no difference in any of the treatments, but this was due to a lack of slugs and potentially a problem in the experimental design. Future research and repetition of this field trial should concentrate on using plastic barriers and adding a specific number of slugs to the plots rather than relying on natural populations to invade the plots. In order to avoid phytotoxic effect, the oils could be applied directly to soils, and as the morbidity rates of the oils at 0.25% on *D. reticulatum* was very efficient, there is potentially room to lower dose. There is also a strong viability of using the synergic control to eliminate mollusc pests within greenhouses (protected from UV rays) which are deemed often perfect humid environment for slug pests.

Finally, in Chapter 7 I investigated the microbiome of slugs and whether it was affected by infection by *P. hermaphrodita*. I found the microbiome was significantly altered when infected with *P. hermaphrodita*. This is due to either 1. the nematodes having a direct effect on the microbiome, perhaps by introducing its own concoction of bacteria or 2. the slugs are dying causing a selection of microbes to proliferate. Only further studies attempting to culture the microbiome for further experiments with slugs can answer this.

In summary, this study has shown how important temperature and soil type is to the survival and efficacy of *P. hermaprhrodita*. It has suggested cedarwood oil could be used as a new slug control method and interestingly, the nematode can severely affect the gut microbiome of the slugs. There is much more to explore in the unfolding world of nematology and *P. hermaphrodita.*

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