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A NEMATODE PARASITE FOR
BIOLOGICAL CONTROL OF SLUGS

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ABSTRACT

A Nematode Parasite for Biological Slug Control

A nematode, identified as a species of Phasmorhabditis (Phasmorhabditis hermaphrodita), previously known to be associated with slugs but not previously thought to be a parasite, was shown to be a parasite capable of killing several species of pest slugs. The parasite infects slugs in their shell cavities reproducing and causing a disease with characteristic symptoms including mantle swelling and feeding inhibition which leads to the death of the slug, usually between seven and twenty-one days after infection. The nematode then spreads and multiplies in the cadaver.

The nematode which is a bacterial feeder, was grown in vitro on a mixed bacterial flora on foam chips impregnated with kidney medium, and in deep liquid cultures of kidney medium; two techniques which have been used to produce insect parasitic nematodes commercially.

Nematodes grown on the mixed bacterial flora on foam chips were used in two outdoor mini-plot trials to test the capacity of the nematode to protect Chinese cabbage seedlings and wheat seeds from slug damage. In the first trial slug damage was significantly less in plots treated with a single high dose of nematodes (2 x 10^6/m², 2 x 10^10/ha) than in untreated plots and plots treated with methiocarb bait pellets, a standard product for slug control. In the second trial a range of different nematode doses from 10^8 to 10^10/ha was used, plant protection equivalent to that given by methiocarb was given by nematode doses equivalent to 8 x 10^8/ha and above.

The relationships between Phasmorhabditis sp. and several species of bacteria associated with it were investigated. Phasmorhabditis sp. was found to be a bacterial feeding nematode capable of growth in monoxenic culture with many different bacteria but the reproductive capacity of the nematode was influenced by the bacterial species. Nematodes grown in monoxenic culture with five different bacteria were bioassayed against the slug Deroceras reticulatum. Only two of the nematode/bacterium combinations were found to be consistently pathogenic; one other species gave inconsistent results and the remaining two species were consistently non-pathogenic. The reasons for the differences in pathogenicity between nematodes grown on different bacteria were not a result of differences in the pathogenicity of the bacteria alone. No evidence was found that Phasmorhabditis sp. vectors a specific pathogenic bacterium into its host, as do certain insect parasitic rhabditid nematodes.

It was concluded that Phasmorhabditis sp. has much potential as a commercial biological molluscicide. A priority patent application has been filed by the project sponsors, the Agricultural Genetics Company.
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I would like to thank my supervisor Dr. David Glen for his constant support and guidance throughout the course of this study. Particular mention must be made of his help with analysis of data from the two mini-plot trials.

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Grahame Stroud also deserves thanks for the work he did in developing the soil based bioassay system which was used extensively in this study and for taking some of the photos.

I should like to thank Chris Wiltshire for extracting slugs and other soil invertebrates from the soil samples taken in the first mini-plot trial. I should also like to thank Clive Lyons who identified the earthworms and Julie Ellis who helped set up the first mini-plot trial. Chris Wiltshire also took some of the photographs.

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My thanks must also go to Bob Hughes and the glasshouse and controlled environment staff for growing the Chinese cabbage used to feed slugs, for providing controlled environment rooms for raising and storing both slugs and nematodes, for preparing the mini-plots and for irrigating the slug collection sites.

I am grateful to both David Hooper of Rothamsted for identifying the nematode and for teaching me the basics of practical nematology and to many of the nematologist at Littlehampton (Both AGC and HRI staff) for their helpful suggestions.
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Finally I should like to thank the Agricultural Genetics Company who provided financial support for this work.
DECLARATION

The work reported in this thesis is based upon the author’s independent studies under the supervision of Dr. D. M. Glen. The experimental work was carried out by the author with the assistance of Susan George and others as acknowledged, and the author was responsible for the interpretation of the results and all conclusions described herein. Assistance and advice received from colleagues has been acknowledged.

The views presented within this thesis are those of the author and may not represent those of the University of Bristol.

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1. INTRODUCTION

1.1. Slugs as Pests
Slugs (Gastropoda: Pulmonata) have been reported as pests of agriculture and horticulture throughout the world. In India slugs damage potatoes and a wide variety of garden produce (Raut & Mandal 1984). In central America slugs are major pests of dry beans and also act as an intermediate host for the causal agent of human abdominal angiostrongiliasis (Andrews 1983; Andrews 1989). In North America slugs are considered to be pests of maize, alfalfa and turnips in conservation tillage cropping (Barratt, Byers, & Bierlein 1989) and in New Zealand slugs are pests of pasture establishment (Barker 1989). Slugs also damage a wide range of crops in Northern Europe and are especially troublesome in the mild, moist climate of the United Kingdom (Godan 1983). This thesis will concentrate on the pest status and control of slugs in this country.

1.1.1. Pest Species

There are 28 species of slugs resident in the United Kingdom, twenty native species, and eight introduced species. (Kerney & Stubbs 1980). These species represent four gastropod families. Three of these families have species considered to be pest species: the Arionidae, Milacidae and Limacidae families. The fourth family, the Testacellidae are mainly carnivorous. Deroceras reticulatum (Muller), from the Limacidae, Arion hortensis agg., Arion circumscriptus (Johnston), Arion silvaticus (Lohmander), and Arion intermedius, (Normand) from the Arionidae and Tandonia budapestensis (Norris) and Milax sowerbyi (Norris) from the Milacidae are all considered to be pests (Anon. 1984). Runham and Hunter (1970) give a general description of the biology, ecology and taxonomy of these animals. D. reticulatum is the most widely distributed pest species in the U.K. and is generally regarded as the most important pest species. However, Glen (1989) states that in cereals D. reticulatum typically occurs together with one or more Arion species. Furthermore, Tandonia budapestensis and Arion hortensis agg. are known to be common pests of potatoes. Thus to be effective any molluscicide must be capable of controlling a wide range of slug species.
1.1.2. Crops Damaged

Slugs damage a wide range of crops. In pastoral agriculture slugs are pests of pasture establishment. They eat grass seeds at, or shortly after germination (Savage and Thomas 1985), and may also be pests of established swards by grazing on clover. (Clements & Bentley 1983).

A wide range of vegetable crops are damaged by slug grazing, e.g. Brussels sprouts, celery and carrots (Godan 1983). Slugs may also cause problems by contaminating harvested vegetable products e.g. peas (Wharton & Enser 1969), and lettuce (Anon. 1984). It has also been suggested that slugs may act as vectors for certain plant pathogens and parasites e.g. *Erwinia caratovora* in Potatoes (Dawkins et al. 1986), white clover mosaic virus, and the stem nematode *Ditylenchus dipsicai* (Cook, Thomas and Mizen 1989). While these phenomena can be demonstrated in the laboratory, it is not known whether the effect of slugs on the spread of these plant diseases is significant in economic terms.

Oilseed rape seedlings are attacked shortly after germination. While slugs are considered a serious pest of this crop in Belgium (Moens 1989) and France (Hommay and Briard 1989), in the U.K. they are regarded only as minor pests (Port & Port 1986; Winfield 1986). However, the incidence of slug damage in this crop is likely to increase as the glucosinolate content of new double-low varieties decreases, since varieties low in glucosinolates are known to be more palatable to slugs (Moens 1989; Glen, Jones & Fieldsend 1989; 1990).

In the United Kingdom the most severe slug damage in economic terms is done to maincrop potatoes and autumn sown wheat. In the case of potatoes slugs damage the maturing tubers in late summer and early autumn. The tuber yield by weight is rarely reduced substantially but the quality, and hence the value of the crop may be considerably reduced (Port & Port 1986). Harvested crops with more than 5% of tubers showing slug damage are not suitable for the top value pre-pack potato market, and crops with more than 10% showing slug damage are only suitable for processing (Beer 1989). Beer (1989) states that at 1988 prices, growers whose crop did not meet the top quality markets (i.e. > 5% damage) were not likely to cover their costs.
In winter wheat the most severe damage is done soon after sowing. Slugs may hollow the seeds by eating the embryo, or they may eat through the base of the seedling stem shortly after germination, thus killing the whole plant at an early stage of development. Slugs also graze on the leaves of mature plants. This damage is not usually considered to be important, but it has been suggested that slug grazing on the flag leaf may lead to a reduction in yield (Kemp & Newell 1987).

Recent changes in agricultural practices have increased the incidence of slug problems in autumn sown cereals (see section 1.2.2.), to the extent that in 1986-87 slugs were the pests causing greatest concern to wheat growers in the U.K. (Glen 1989).

1.2. Control Methods

1.2.1. Cultural control and factors affecting slug damage.

Recent changes in agricultural practices have led to a considerable increase in slug damage to cereals. Martin & Kelly (1986) and Port & Port (1986) considered that the increased use of direct drilling for cereals, straw incorporation as opposed to burning, and the increase in the area of oilseed rape to be important factors leading to the higher incidence of slug damage. Fields with directly-drilled crops are likely to harbour more slugs than ploughed fields. Ploughing is thought to reduce slug numbers by a combination of direct mechanical damage and exposure to desiccation and predation by birds and other vertebrates (Martin & Kelly 1986). Straw burning usually results in lower slug populations than where straw is incorporated, (Glen, Wiltshire & Milsom 1984; 1988) but the impending ban on straw burning will prevent this form of slug control.

Early sown cereals are generally thought to be at less risk of slug damage than late sown crops because the seed beds tend to be less favourable for slug activity but Martin & Kelly (1986) suggest that a dry seed-beds in early autumn may inhibit seed germination more than slug activity.

Wheat following crops with dense foliage or crops which leave much debris after harvest is at high risk because such crops provide excellent conditions for slug populations to
build up by providing abundant food and maintaining ground moisture at high levels. Wheat following dry harvested peas or oilseed rape is especially at risk.

Differences in soil types and soil conditions are known to affect slug activity. Slugs are favoured by heavy clay or silt soils and they are more able to move through soil to damage seed if it is sown into a cloddy seed-bed. Consolidation of seed-beds is generally considered to reduce the likelihood of slug damage by breaking down soil clods and destroying the natural soil spaces through which slugs move (Stephenson 1975; Gair, Jenkins & Lester 1987) but as noted by Stephenson (1975) and Glen, Milsom and Wiltshire (1989), this may not always happen. Glen, Milsom and Wiltshire (1989) investigated the differences in slug numbers and slug damage to wheat resulting from different seed-bed preparations following ploughing. They found that more slugs were present on consolidated rather than loose seed beds and also that seeds in seed-beds consolidated before sowing were sown at shallower depth and suffered more slug damage. They attributed the lower numbers of slugs in loose seed beds compared with consolidated, to be a result of the extra cultivation being done to loosen them which killed slugs or pushed them down to greater depth where they could not damage seeds. In consolidated seed beds in this experiment, consolidation did not result in the clods being broken down, but did reduce the depth to which the seed drill could penetrate. The authors concluded, contrary to popular belief, that shallow sown seed was more susceptible to slug damage than deep sown seed. Glen, Milsom and Wiltshire (1990) tested this hypothesis and confirmed that deep sowing of wheat seed is beneficial in reducing slug damage. They also found that deep sowing increased yield, but they could not directly attribute this yield increase to the reduction in slug damage (Glen et al. unpublished data).

While it can be seen how farmers are able to modify their husbandry techniques to reduce the likelihood of slug damage, often economic or agronomic factors or weather will prevent the farmer from choosing or achieving conditions or rotations which deter slug activity. In such cases the farmer may use his knowledge of slug behaviour to assess whether treatment with molluscicide in necessary. Forecasting slug damage accurately is difficult because any forecasting system needs to take into account the numbers of slugs present. The most accurate methods of assessing slug numbers within a field rely on digging soil samples and flooding them over time, thus forcing the slugs to the surface
where they are counted (Glen & Wiltshire 1986) or by using defined area trapping (DAT) in which galvanised iron rings of 357 mm diameter and 150 mm deep are hammered into the ground, covered and slugs collected every two - three days (Ferguson, Barratt & Jones 1989). Soil flooding methods are extremely labour intensive and while DATs are less labour intensive, they are still not practical for farm use. Farmers currently rely on traps baited with methiocarb pellets, which provide an estimate of surface activity but are known to select for larger individuals. Thus they are not reliable in indicating the number of slug species present if one species has recently bred and is only present as juveniles (Glen & Wiltshire 1986). New traps, devised by Hommay and Briard (1988) consisting of 0.5 m² water absorbent mats covered with silver coloured reflective material are being tested (Clements & Murray 1991). The silver coloured surface reflects heat and keeps the traps cool while the absorbent underside keeps the traps moist providing a suitable habitat for slugs. It is possible that traps may become more useful if they are used in conjunction with information on population dynamics and life-cycles of slugs, but more research is needed in these areas before this approach can be used.

1.2.2. Chemical Control

In the U.K. chemical slug control relies largely on two active ingredients, methiocarb or metaldehyde, which are formulated into baited pellets. In cereals the pellets are either broadcast before, or shortly after sowing, or mixed with the seed, which may give good results with directly drilled crops (Hogan 1985). In maincrop potatoes pellets are broadcast between July and September while the tubers are maturing. Rayner (1975) and Rayner et al.,(1978) found repeated applications over this period gave better control than a single application.

Methiocarb is a stomach and nerve poison, which also has some contact toxicity but this is thought to be unimportant. In metaldehyde the contact action is thought to be important. Metaldehyde poisoning immobilises slugs, causes excessive slime production and eventual death by dehydration (Cragg & Vincent 1952). Consequently slugs may recover from metaldehyde poisoning in wet weather. Furthermore, metaldehyde is not thought to be as effective as methiocarb at killing Arion species of slugs. Thus methiocarb is considered to be more reliable than metaldehyde and is more widely used, despite being more expensive. However, the control given by either chemical is
frequently ineffective (Henderson & Parker 1986). Cereals may still need to be re-drilled as a result of slug damage even after an application of slug pellets. Beer (1989) states that some potato growers use up to six applications of slug pellets and still experience economic levels of slug damage. It has been estimated that an application of slug pellets to cereals results in only $\leq 50\%$ reduction in slug numbers (Wiltshire & Glen 1989).

Both chemicals are known to be toxic to both vertebrate and invertebrate non-target organisms. Methiocarb is known to be toxic to earthworms (Martin & Forrest 1969; Symonds 1975; Bieri, Schweizer & Czarnecki 1989), carabid beetles (Martin, Davis & Morris 1969; Buchs, Heimbach & Czarnecki 1989), and reductions in populations of carabid beetles, many of which are considered beneficial, have been detected in the field following application of methiocarb pellets (Kennedy 1988; 1990). Metaldehyde is considered to be less of a threat to non-target invertebrates than methiocarb, but more cases of vertebrate poisoning are attributed to metaldehyde than to methiocarb (Fletcher & Stanley 1981; Stanley & Fletcher 1982; Fletcher & Hardy 1983, 1984).

1.2.3. Future Prospects in Slug Control

1.2.3.1. Chemical control.

New formulations of existing molluscicides are being developed with the aim of making the pellets more persistent, more attractive and more palatable to slugs. Methiocarb and metaldehyde are known to reduce the amount of pelleted bait eaten to the extent that a sub-lethal dose may be ingested (Wedgewood & Bailey 1986). Bourne, Jones & Bowen (1990) investigated the potential of mixing the two active ingredients in pellets in different proportions and found it was possible in combination to reduce the overall concentration of active ingredients. They found the ratio of 2:1 metaldehyde:methiocarb (wt:wt) to be particularly effective. Henderson et al. (1989) described the molluscicidal properties of various chelates of aluminium or iron. The chelates can be formulated into baited pellets. Henderson et al. (1989) showed that in field trials these baits killed slugs as effectively as methiocarb or metaldehyde and were more efficient in preventing
recovery of poisoned slugs under very wet conditions. Furthermore, preliminary data suggests that these compounds may be non-toxic to non-target organisms e.g. beneficial carabid beetles. A rice and vegetable insecticide, bensultap, has been developed as a molluscicide by I.C.I. in a formulation said to be repellent to hares and birds. A product has been launched in France under the trade name Malice. However, while these newer active ingredients may offer certain advantages over metaldehyde and methiocarb, they are not likely to be wholly effective at controlling slugs because of the problems associated with using baited pellets as molluscicides (Henderson & Parker 1986). These problems include possible repellent effects of active ingredients in baits; the fact that only surface active slugs, which are not necessarily the slugs causing crop damage come into contact with the pellets, and the fact that baited pellets have to be formulated with fungicides (which may repel slugs) to stop the growth of moulds.

Airey et al. (1989) tried to identify new and specifically molluscicidal compounds by investigating materials considered likely to interfere with physiological processes of molluscs. They also investigated the possibility of using semiochemicals in slug control systems. These authors did not find any specific molluscicides with potential for slug control and concluded that more research on molluscan physiology will be needed before this approach is likely to succeed. They identified a number of attractant and phagostimulant compounds but they found no clear evidence for the use of sex pheromones, aggregation pheromones or alarm pheromones by slugs and concluded that the use of semiochemicals to control molluscs is likely to be confined to compounds which interfere with mollusc feeding.

1.2.3.2. Plant resistance.

The possibility of breeding plants resistant to slug damage has not yet been explored in any detail. Certain cereal species are known to be more palatable to slugs than others e.g. slugs prefer wheat to barley (Port & Port 1986), and differences between varieties of winter wheat in palatability to slugs have been demonstrated in laboratory tests (Spaul & Eldon 1990). As yet there is no evidence of differences between wheat varieties in susceptibility to slug damage in the field. Certain varieties of potatoes (e.g. Maris Piper) are known to be much more susceptible to slug damage than others, and the National Institute of Agricultural Botany give a slug susceptibility rating from 1 (most susceptible)
to 9 (least susceptible) on their list of approved potato varieties (Anon. 1990). Johnston, Kershaw & Pearce (1989) found low molecular weight compounds were essential for the expression of slug resistance in potatoes. Phenolics and glycoalkaloids are two such compounds which have been implicated in slug resistance (Storey, 1985). Johnston, Kershaw & Pearce (1989) demonstrated that phenolics are of primary importance, but they considered that the important factor is the rate at which phenolics are oxidised when the potato is damaged, rather than the concentration of phenolics. They concluded that there is potential for enhancing the rate of phenolic oxidation by genetically manipulating the genes for the enzymes involved. It is worth noting that while Maris Piper potatoes are at great risk of slug damage they are resistant to *Globodera rostochiensis* (yellow potato cyst nematode).

1.2.3.3. Biological control

The possibility of controlling slugs biologically has received scant attention in the past and little is known about the natural enemies of slugs. More interest has been shown in recent years, but none of the natural enemies studied has much potential for commercial use in U.K. agriculture. (see section 1.3.)

1.3. Natural Enemies of Slugs and Snails.

1.3.1. Viruses

There are as yet no confirmed reports of viruses infecting slugs. David *et al.* (1977) reported virus-like particles in *D. reticulatum*, but these were later found to be particles of galactogen and glycogen (Kassanis, Woods and Macfarlane 1984).

1.3.2. Bacteria

Like all animals, slugs have an associated bacterial flora. Mead (1961) reviewed reports of bacteria associated with molluscs and mentioned the genera *Escherichia*, *Alcaligenes* and *Bacillus* but gave no indication of whether or not any of these species were pathogenic. Mead (1961) also reported an infection by *Aerobacter* in a snail farm where *Helix aspersa* (Muller) was being reared. Elliot (1969) and Shrewsbury & Barson (1947)
investigated bacteria associated with *Limax maximus* and *Arion ater* respectively, but the emphasis of their studies was to isolate human pathogens which might be transmitted by these slugs. Yamada, Yonemoto & Matsumoto (1960) investigated human pathogens associated with slugs in Japan. In all three of these studies the effects of the bacteria on the host slugs were not investigated.

The aquatic snail *Biomphalaria glabrata* has been investigated as a possible target for biological control because it is a vector of schistosomiasis in man. Pan (1956) reported an acid-fast pathogen in this snail but stated that it had low virulence and had little potential as a control agent. Another bacterium, *Bacillus pinottii* Cruz and Dias has been successfully used in field trials against this snail in Egypt (Dias & Dawood 1955), but Trip (1961) and Wright (1968) did not achieve control of the snail using the same bacterium. *Vibrio parahaemolyticus* has been shown to be pathogenic to this snail (Ducklow, Tarraza & Mitchell 1980) and Singer (1989) states that some isolates of *Bacillus brevis* are also pathogenic against this species. However, none of these bacteria have been assayed against slugs.

It has been reported that certain commercially available insecticidal strains of *Bacillus thuringiensis* are highly pathogenic to *D. reticulatum* (Terytze & Hofmann 1986), but Hommay (unpublished data), using the same commercially available strains found no activity and suggested that the high mortality of slugs recorded by Terytze & Hofmann (1986) might be a result of the high temperature at which they kept their slugs (22°C).

The symptoms of eight disease types have been described for pest slugs and snails in India (Raut & Panigrahi 1989), seven of these diseases being found in slugs. The aetiology of these diseases has not yet been investigated. One of the diseases, a leucodermia type disease in the pest slug *Laevicaulis alte* Ferussac had been previously described (Raut & Mandal 1986). This disease has symptoms identical to a disease in the giant African snail *Achatina fulica* Bowdich, thought to be caused by infection with the bacterium *Aeromonas liquefaciens* (= *A. hydrophila*) (Dean, Mead & Northey 1970). Field observations suggest that this disease is capable of reducing populations of both slugs (Raut & Mandal 1986) and snails (Raut & Ghose 1977). The mode of transmission of this disease is not understood and the potential of *Aeromonas hydrophila* as a biological control agent for these slugs and snails has not been investigated.
1.3.3. Fungi

There are no recorded cases of fungi parasitising slugs. There are, however, reports of fungi attacking the eggs of slugs. Tervet and Esslemont (1938) described a strain of *Verticillium chlamydosporium* which caused 97% mortality of *D. reticulatum* eggs. Arias and Crowell (1963) found *Arthrobotrys* on the eggs of *Arion circumscriptus*. There have not been any studies on the feasibility of using either of these fungi as biological control agents, possibly because Tervet and Esslemont (1938) considered that they would be unsuitable.

1.3.4. Protozoa

Stephenson and Knutson (1966) reviewed the many reported associations between protozoa and slugs. In most cases these studies concentrated on the parasites rather than on their effects on the host slugs. It is not possible to assess from these studies which, if any of the protozoa would be of use as biological control agents. Since then the biology and parasitic action of two species of holotrichous ciliates, *Tetrahymena rostrata* (Khal) and *Tetrahymena limacis* (Warren) have been studied in detail (Brooks 1968). *T. rostrata* was found to be highly pathogenic, reducing the longevity and fecundity of infected *D. reticulatum*. The parasite can be grown in artificial medium, is capable of surviving in the soil as a free living organism and can be transmitted trans-ovum. This ciliate has been found in nine different slug species, and Brooks (1968) considered it to have considerable potential as a biological control agent but no further work has been published. Brooks (1968) did most of his bioassays at room temperature, and there is some evidence that the pathogenicity of this ciliate is reduced at lower temperatures (Brooks 1968, Coyne 1989, Selman pers comm.). *T. limacis* was found to have low pathogenicity, poor survival in the field and was more difficult than *T. rostrata* to cultivate in vitro.

Jones (1985) and Jones and Selman (1984) studied the parasitic action and biological control potential of a microsporidian parasite, *Microsporidium novocastriensis* (Jones and Selman 1985). This parasite caused chronic infection inhibiting growth, longevity, fecundity and feeding activity. Its potential as a biological control agent may be limited.
because it only infects *D. reticulatum* and like all microsporidia, cannot be grown in vitro.

1.3.5. Nematodes

As with protozoa there are a great many records of nematodes being associated with slugs, but there is little information on the effects of these worms on the slugs. These records are summarised by Stephenson & Knutson (1966) and Godan (1983). Mengert (1953) examined many slugs and snails for the presence of nematodes in order to study the full range of relationships between nematodes and terrestrial slugs and snails, and concluded that nematodes are more likely to be associated with slugs than snails because slugs live in the soil and are more likely than snails which often live on plants above soil level, to come into contact with nematodes.

Mengert (1953) found 29 species of nematodes associated with slugs and divided these species into four groups based on the degree of adaptation shown by the nematodes to life within slugs. The first group contained 23 species which Mengert considered to be non-parasitic. These species were either transiently associated with the slug slime or were able to survive ingestion by the slug and passage through the gut.

The second group contained two species, *Rhabditis papillosa*, and *R. neo*papillosa, which form resistant dauerlarvae which can find their way into the body of slugs and survive there until the slug dies, after which the nematodes feed on the dead slug. Mengert considered that although these nematodes did show a degree of adaptation to life within slugs, they did not live as parasites. (See also Section 1.4.). The third group contained only one species, *Allionema appendiculata* which is a facultative larval parasite of the foot of slugs. The larvae can enter the foot and develop, feeding on the slug, but are later expelled as a result of increased slime production by the slug. The nematodes develop into adults in the soil.

The fourth group of nematodes contained three species: *Limaconema limacis* (=*Angiostoma limacis*) *L. stammeri*, and *L. dentifera*. These species are obligate parasites of the slugs. Eggs of the nematodes are ingested by healthy slugs. The eggs then hatch and develop into adults within the slug’s gut. More eggs are produced and passed out in the slug’s faeces and are later ingested by other slugs as eggs or juvenile larvae.
Arias & Crowell (1963) recorded *Rhabditis* cf. *lambdiensis* (Maupus), *Panagrolaimus* sp., and *Rhabditis* sp. parasitising laboratory cultures of *D. reticulatum*. They considered that these nematodes were parasitic, but gave no details.

There have been no published investigations on the potential of any nematodes as biological control agents for slugs. Slugs often act as the intermediate host for nematode parasites of vertebrates including domestic animals and man e.g. *D. reticulatum* is considered to be the most important vector of the sheep lungworm, *Cystocaulus ocreatus* (Railliet et Henry), (Rose, 1960). This obviously precludes the use of certain nematodes as biological control agents.

1.3.6. Platyhelminthes

Again there are many records of platyhelminthes being associated with slugs and these have been summarised by Stephenson and Knutson (1966). Much research has concentrated on species which are parasites of Man and Domestic animals and use molluscs as intermediate hosts. While some of these mammalian parasites are known to be lethal to the mollusc intermediate host (e.g. *Brachyleaema* sp. are known to kill *D. reticulatum*,) and may thus be important in natural control of slug species, they have no potential as biological control agents.

1.3.7. Insects

Certain insects are known to be parasitoids and predators of slugs. Several glow-worms (Coleoptera: Lampryidae) are known to eat slugs (Schwalb 1960). The larvae of *Schiomyzid* flies (Diptera) feed exclusively on molluscs and eight species are known to be more or less exclusively predators of slugs (Reidenbach, Vala & Ghamizi 1989). Reidenbach, Vala & Ghamizi (1989) studied the predation capacity of *Euthycera cribrata* larvae on *D. reticulatum* in the laboratory and concluded that this, and probably other species of *Schiomyzids* may well have potential for biological slug control. Many beetles are known to feed on slugs. Both carabid and staphylinid beetles are known to feed on slugs (Tod 1973), and they are considered to exert a degree of natural control in the field. The possible use of beetles as biological control agents has attracted much attention.
in recent years. Altieri et al. (1982) demonstrated a significant reduction in slug and snail populations following the release of ground beetles, *Scaphinotus striatopunctatus* (Chandoir). In plot trials, Symondson (1989) demonstrated that *Abax parallelepipedus* was capable of reducing numbers of *D. reticulatum* and damage caused by this slug to lettuce grown in polythene tunnels. While use of these beetles for slug control may have some potential in gardening and niche markets, e.g. organic horticulture, dispersal of the animals following release (*A. parallelepipedus* is predominantly a woodland species) and the cost of mass rearing would make them unsuitable as control agents for general use in U.K. agriculture.

1.3.8. Vertebrates

Badgers, foxes, birds, hedgehogs, slow-worms and weasels are just some of the many vertebrates known to feed on slugs. These animals are not amenable to manipulation for biological control, and their impact on slug numbers is likely to be small (Port & Port, 1986).

1.4. Phasmorhabditis neopapillosa/hermaphrodita

In the most recent taxonomic review of the sub-order Rhabditina (Nematoda: Secernentia) Andrassy, (1983) lists two species of *Phasmorhabditis* in the family Rhabditida which are identical morphologically, but differ in the proportion of males present in the populations. In *P. neopapillosa* males and females are equally abundant, whereas in *P. hermaphrodita* males are extremely rare. It is not yet known if *P. hermaphrodita* is a separate species or a hermaphrodite form of *P. neopapillosa* (Andrassy, 1983).

*P. hermaphrodita* was first described from slugs by Maupas (1900), (who named the nematode *Rhabditis caussaneli*). He found resistant larval forms in the intestine of *Arion ater* which he collected in Normandy. He maintained cultures of the nematode on rotten flesh for two years. He found that the adult worms were predominantly protandrous autogamous hermaphrodites. Males were present in very small numbers (1 male for 1300 females) and the number of males in cultures was not affected by nutritional conditions.
Maupas never witnessed males mating with females, which showed no change in their fecundity, or the sex ratio of their offspring, in the presence of males.

*Phasmorhabditis neopapillosa* was described by Mengert (1953), (who named the nematode *Rhabditis neopapillosa*) in his studies on the relationships between nematodes and terrestrial molluscs. He found the nematodes as resistant larval stages ('dauerlarvae') in the hind gut of the slug *Limax cinereoniger*. Mengert (1953) considered *P. neopapillosa* to be a saprophyte which thrives on decaying material for many generations, but when conditions become unfavourable the juveniles form resistant non-feeding dauerlarvae, which find their way, when the opportunity arises into the hindgut of slugs. He considered that the dauerlarvae of this species wander, when the opportunity arises, into the body cavity of slugs where they remain as dauerlarvae until the slug dies, after which they develop and reproduce feeding on the corpse. The stay in the slug is not a necessary part of the nematode life cycle but Mengert (1953) considered that the dauerlarvae of this species did show a degree of adaptation to life within slugs. Mengert (1953) considered the lifestyle of *P. neopapillosa* to be identical to two other species, *Phasmorhabditis papillosa* and *R. caussanelli* (=*P. hermaphrodita*). However, he did not find *P. hermaphrodita* in his studies.

Neither Mengert (1953) nor Maupas (1900) considered either *P. neopapillosa* or *P. hermaphrodita* to be parasites of slugs.

1.5. Nematodes as biological control agents of insect pests.

Many species of nematodes from diverse nematode groups are known to be parasites of insects and some have been used successfully to control various insect pests either by introducing an exotic nematode parasite into a pest population or by using an inundative approach. The use of nematodes as biological control agents has been reviewed in detail by Poinar (1979). Some of the more successful examples are described briefly here.
1.5.1. Romanomermis culicivorax

Romanomermis culicivorax is a mermithid nematode which parasitises a broad range of mosquito species, many of which are important as vectors of human disease. Infective (pre-parasitic) juveniles of this nematode hatch from eggs laid in sand in the bed of the water containing mosquitoes. The larvae swim and actively seek out mosquito larvae. The nematode larvae penetrate the cuticle of the mosquito larvae and develop as parasitic juveniles, then leave the mosquito larvae (killing the host in the process) as post-parasitic juveniles. These develop in the water to adults which then lay eggs in the sand. The entire life cycle takes about four weeks. Peterson and Willis (1972) developed a method for mass rearing the nematodes in vivo and for storing viable eggs ready for use. This nematode has been extensively used in field trials, often with considerable success. In El Salvador a 0.11 km² area of lake was treated to control Anopheles albimanus resulting in a 94% reduction in numbers of this mosquito. This nematode was produced commercially for a short time but did not achieve commercial success because of shipping problems and lack of technical backup (Poinar 1979).

1.5.2. Deladenus siricidicola

Deladenus siricidicola, a neotylenchid nematode, has been used to control siricid woodwasps, which are introduced pests of pine forests in Australia. These woodwasps lay eggs carrying a symbiotic fungus into healthy trees. The eggs hatch and the larvae tunnel through the wood feeding on the fungus, eventually killing the tree. The nematode D. siricidicola has two distinct life cycles, one involving insect parasitism and the other involving fungal feeding. The insect parasitic stages develop into adults in female insects and produce larvae which can enter the eggs in the insect ovary. The parasitised eggs which are usually killed by the nematodes are oviposited into new trees, still carrying the symbiotic fungus. The nematode larvae then enter the mycetophagous phase and feed on the parasitic fungus. If siricid larvae are present in the wood, the mycetophagous females produce insect parasitic larvae, which enter the woodwasp larvae. The woodwasp larvae
mature and carry the nematodes to new trees, where again the nematodes are introduced within the wasp’s eggs.

When used in biological control, nematodes are introduced either by injecting the nematodes into infected trees through punched holes, or by releasing parasitized females into forests infested with the siricid pest. This nematode has been released at many sites in Australia and has become well established. The use of this nematode has been summarised by Bedding (1984a).

1.5.3. *Tripius sciarae*

*Tripius sciarae*, a sphaerulariid nematode has been used to control sciarid and mycetophilid flies in glasshouses. Fertilised females of the nematode penetrate the cuticle of the fly larvae. The mature female nematodes swell up and evert their uterine cell out through the vulval opening to absorb nutrients directly from the host’s haemolymph. Eggs are deposited into the insect and develop into pre-adult larvae which leave the host through the mouth, anus, or reproductive system of the female fly during oviposition. When the nematodes leave a female fly through the reproductive tract they usually destroy all the eggs within the insect, effectively sterilising the female. The nematode can be mass reared in cultures of sciarid flies.

1.5.4. *Pristionchus uniformis*

*Pristionchus uniformis* is a diplogasterid nematode known to be a parasite of *Leptinotarsa decemlineata*, the Colorado potato beetle. This bacterial feeding nematode forms resistant third stage dauerlarvae which enter the haemocoel of the beetle larvae via the gut and mature and replicate resulting in death of the insect. This nematode has been reared in *vitro* under xenic conditions and has reduced populations of overwintering Colorado beetle larvae in field trials in Poland (Fedorko & Stanuszek, 1971).

1.5.5. *Heterotylenchus autumnalis*

*Heterotylenchus autumnalis* is an allantonematid nematode parasite of the face fly *Musca autumnalis*. Fertile adult female nematodes living in dung penetrate the cuticle of face fly
larvae, also in the dung, and produce eggs which all develop into parthenogenic females. These in turn produce offspring which develop into pre-adult larvae of both sexes. These pre-adult nematodes enter the reproductive system of the adult fly and pass out into dung along with oviposited fly eggs, where they mature feeding on the dung. This nematode can be mass reared in vivo (Stoffolano 1973) and has been released in California to control an introduced pest face fly (Poinar 1979).

1.5.6. Steinernema and Heterorhabditis

While all of the above mentioned nematodes have proven successful in field trials, by far the most promising nematodes in terms of commercial potential are members of the families Heterorhabditidae and Steinernematidae within the sub-order Rhabditina, which also includes Phasmorhabditis spp. These two families both contain a single genus, Heterorhabditis, and Steinernema respectively. Much research has been done in many countries on this group of nematodes and the taxonomy, biology, ecology, behaviour, and genetics of these nematodes has been reviewed in detail by Gaugler & Kaya (1990). Members of these two families are already available for use as biological insecticides in several countries including the U.K.

Nematodes of these two families are mutualistically associated with bacteria from the genus Xenorhabdus. The nematodes form resistant non-feeding third instar juveniles (dauer larvae) which carry the Xenorhabdus bacteria in their intestine. The dauerlarvae enter an insect host usually through the spiracles or other body openings and release the bacteria into the insect’s haemolymph. The bacteria multiply rapidly in the haemolymph and are in part responsible for the death of the insect. The nematodes develop and reproduce, feeding on the bacteria and the insect corpse which is broken down by enzymes released by the bacteria. As nutrients are depleted and conditions become unfavourable for nematode reproduction the juveniles develop into dauerlarvae and leave the insect corpse to search for a new insect host (Poinar 1966; Poinar & Thomas 1966; 1967).

The nematodes produce proteins which inhibit the insect’s immune system and allow the bacteria to multiply rapidly in the host (Gotz, Boman & Boman 1981; Burman 1982), and the bacteria produce antibiotics which inhibit the growth of other bacteria which may not
be suitable for nematode growth (Poinar et al. 1980; Akhurst 1980; Paul et al. 1981). The nematodes' association with the bacteria is very close but is not thought to be an absolute necessity; axenic nematodes are capable of invading and killing insects, the nematodes are capable of growth in combination with bacteria other than *Xenorhabdus* (Poinar & Thomas 1966) and not all infective larvae carry the bacteria (Poinar 1966). Species of *Xenorhabdus* associated with nematodes have never been isolated from soil or water but *X. luminescens* has been isolated from a human wound (Colepicolo et al. 1989). Members of the genus *Steinernema* are associated with the bacterium *X. nematophilus* whereas *Heterorhabditis* are associated with *X. luminescens*. The two genera are also distinguished by morphological characteristics and by differences in their life-cycles; Infective *Steinernema* larvae develop into either males or females, so at least two larvae must enter the insect in order to reproduce, whereas infective *Heterorhabditis* larvae develop into autogamous hermaphrodites, which then produce offspring of both sexes, thus only one infective larvae need enter the insect to initiate reproduction.

Nematodes of these two genera have an extremely wide host range; *S. carpocapsae* alone is known to infect over 250 different species of insects from over 75 families in 11 orders (Poinar 1986) and so have potential to control a wide range of pest insects. The nematodes are also easy to produce in mass monoxenic culture with their associated bacteria. Bedding (1981; 1984b) devised a method of growing the nematodes on polyether polyurethane sponge chips, soaked in chicken offal. Three Kg of the foam chip medium is put into 1.0 x 1.5 metre autoclavable plastic bags. Once the medium is sterilised, it is first inoculated with *Xenorhabdus* bacteria, then 24 hours later the nematodes are added. This method on average produces $13 \times 10^8$ infective *S. feltiae* nematode dauer larvae per bag. While this method of production does not require a large initial investment, it is sensitive to contamination and is labour intensive. In scale-up models economy of scale is obtained only up to a production level of approximately $10 \times 10^{12}$ nematodes per month; at levels above this labour costs remain constant and significant (Friedman 1990). This method of production is still used in developing countries where labour cost are lower, e.g. China, and in some small scale industrial concerns. However, for mass production in countries where labour costs are high, liquid cultivation systems seem to offer more hope of producing large numbers of nematodes at acceptable prices. Friedman (1990) states that in scale up models, nematode production at levels of $1 \times 10^{12}$ per month and above is most economical in liquid systems, and that economy of scale is maintained
up to $50 \times 10^{12}$ nematodes per month. Monoxenic liquid culture of *S. feltiae* was first described by Beucher and Popiel (1989). Liquid media based on kidney and yeast extract, or soy flour, yeast extract, corn oil and egg yolk have been used and yields of up to 100,000 infective juveniles per ml have been obtained (Georgis 1990a). Biosys (Palo Alto, California), currently produce steinernematid nematodes in 15,000 litre fermenters, and plan to scale up production to use 60,000 litre fermenters. Application costs of their products range from 40 - 400 U.S. dollars/hectare, depending on the application dose, and the market segment (Georgis, 1990a).

The dauer larvae of *Steinernema* spp. and *Heterorhabditis* spp. are capable of surviving for long periods away from insect hosts, and are thus more amenable to storage and formulation than other nematode stages. *Steinernema* larvae are capable of survival in aerated tap water under refrigeration for over 2 years (Bedding 1976). Early nematode products were sold as bags of peat containing infective juvenile nematodes, the peat being mixed in with potting compost to protect ornamental plants from subterranean pests, e.g. black vine weevil larvae (Georgis 1990a). In recent years much progress has been made in formulating nematodes by partly immobilising the larvae in carriers such as clay or reversible gels formed with polyacrylamide or alginate. These carriers are thought to reduce nematode metabolism and thus improve their tolerance to temperature extremes (Georgis 1990a). *Steinernema* nematodes are sold by Biosys on alginate sheets 12 x 60 cm containing approximately 10 million nematodes stored in 2.5 litre containers. These products remain viable for three months at ambient temperature, or six months if refrigerated.

Future formulation research will include the possibility of using anhydrobiotic nematodes. Many species of nematodes can remain viable under anhydrobiotic conditions, and already it is possible to desiccate and revive *Steinernema* nematodes but currently the shelf life of these desiccated nematodes is less than the industry standard of one year (Georgis, 1990a).

The wide host range, ease of production and relative ease of formulation and the fact that nematodes are exempt from pesticide registration schemes has made the use of these nematodes in biological control widespread. They have been used to control a wide range of insect pests in many habitats. The nematodes, being soil organisms are generally most
effective when applied to soil to control soil pests and usually supplied for controlling pests in this habitat (Klein 1990). However, the nematodes have also been used successfully to control insects in cryptic habitats, e.g. the carpenter worm Prionoxystus robiniae in evergreen oak trees (Begley 1990). Foliar applications tend to be less successful than applications to soil or other cryptic habitats because the nematodes are exposed to greater environmental extremes (Begley 1990).

It should be noted that while the above overview treats the genera Steinernema and Heterorhabditis together, the two genera, and indeed different species within both genera, pose their own unique production and formulation problems, and are thought to behave differently in the field. In general Heterorhabditis sp. are more difficult to produce than Steinernema sp., particularly in liquid culture, but are thought to be more effective in the field.

1.6. Summary and Aims of this Study

Several species of slugs are important pests of many U.K crops. Current chemical control measures rely largely on two active ingredients, methiocarb and metaldehyde, formulated into baited pellets. These products often fail to provide adequate protection for crops, particularly when the part of the crop attacked by slugs is underground, e.g. winter wheat seeds and tubers of maincrop potatoes. Due to recent changes in agricultural practices slugs have become increasingly important as pests of winter wheat and while certain cultural techniques are known to reduce the risk of slug damage, in many cases other agronomic or economic or weather factors preclude their use.

While certain crop cultivars of potatoes are known to be more resistant than others to slug attack, susceptible varieties are often grown because of market preferences and plant breeders do not consider breeding for slug resistance to be a high priority. Furthermore there is little information available on natural enemies of slugs, and none of the pathogens, parasites, or predators investigated in previous studies shows much promise as a biological control agent for general use in U.K. agriculture. Thus slugs are likely to remain a problem in the near future and there is a need for a novel approach to slug control.
Nematodes, particularly nematodes within the suborder Rhabditina of the genera Steinernema and Heterorhabditis which vector pathogenic bacteria (entomopathogenic nematodes) show considerable promise as biological control agents for many insect pests, and are particularly effective at controlling soil pests.

This study aims to assess the potential of a nematode from the suborder Rhabditina, previously found associated with slugs but not previously believed to be parasitic, as a biological control agent for slugs. Specific aims were:

1. To show whether the nematode is a parasite which is capable of killing or inhibiting feeding of pest slugs and to investigate the host range of this nematode.

2. To determine whether it is possible to rear this nematode in vitro, with particular emphasis on cultivation methods amenable to scale up for industrial production, and also to make preliminary investigations on storage of these nematodes.

3. To determine whether these nematodes are capable of protecting plants from slug damage under field conditions, and if so at what concentration, and to compare the protection to plants given by nematodes to that given by methiocarb pellets.

4. To gain some insight into the parasitic mode of action of the nematode and the way it interacts with bacteria and slugs, with particular emphasis on discovering whether this nematode is mutualistically associated with bacteria as in the case of the entomopathogenic nematodes Steinernema spp. and Heterorhabditis spp..

1.7. Layout and Presentation of Experimental Results in this Thesis.

The aims of this study necessitated the undertaking of a broad range of experimental work covering many topics. In order to simplify the presentation of results, after a short section detailing general methods relevant throughout the thesis, the experimental methods and results are described in eight separate sections with the results being discussed at the end of each section.
The first three sections (Sections 3 - 5) describe initial work on the potential of the nematode parasite as a biological control agent. These are:

**Section 3. The taxonomy and parasitic action of the nematode on slugs.**
In this section the taxonomy of the nematode is discussed and a brief description of the disease caused by the nematode in the slug *D. reticulatum* is given. This is followed by details of experiments to study the effect of nematode dose on this species of slug and experiments investigating the host range of the nematode parasite.

**Section 4. Xenic cultivation and storage of nematodes.**
This section describes experiments on in-vitro growth of the nematode on a mixed bacterial flora: methods include simple petri dish cultures, mass production techniques using foam chip support medium, and deep liquid cultures suitable for scale up for industrial production. Preliminary experiment investigating the survival of the nematode in aerated tap water are included in this section.

**Section 5. Mini-plot field experiments using xenically cultivated nematodes.**
This section describes two experiments using outdoor mini-plots to investigate the ability of the nematode to protect plants from slug damage. The first experiment used a single high dose of nematodes, whereas the second experiment investigated the effect of different doses of nematodes.

The next four sections investigate the relationships between slugs, bacteria and the nematode, with particular reference to the importance of bacteria associated with the nematodes in the pathogenicity of the nematode to slugs:

**Section 6. Isolation, Selection and Identification of Bacteria Associated with Phasmodrhabditis sp. for Further Studies.**
This section describes how bacteria associated with the nematodes were isolated, identified and selected for use in later experiments.
Section 7. Growth with Known Bacteria.
This section describes experiments investigating the ability of different bacteria to support growth of the nematode parasite in monoxenic culture.

Section 8. Effects on the host slug D. reticulatum of bacteria which support growth of Phasmorhabditis.
This section describes experiments in which growth supporting bacteria were fed to slugs or injected into slugs to see if they are pathogenic to slugs without the nematode.

This section describes dose response experiments in which nematodes grown in monoxenic culture with five different bacteria were bioassayed against D. reticulatum. Bacteria used to grow the nematodes included bacteria known to be pathogenic and bacteria known to be non-pathogenic to this slug.

After these seven sections dealing with experimental work Section 10 is a general discussion which summarises the findings of the thesis and briefly outlines areas where further research is needed.
2. GENERAL METHODS

2.1. Collection and Maintenance of Experimental Organisms.

2.1.1. Slugs.

As stated in section 1.1.1. D. reticulatum is generally considered to be the most important pest species of slug. For this reason it was decided that most experiments would be done on this slug, with the exception of host range studies. This species of slug takes from five to six months to reach maturity (Arias & Crowell 1963) and is prone to epizootic outbreaks when kept in crowded culture. Thus, the most economical way to obtain stocks for experimental use is by field collection rather than by breeding (Henderson & Parker 1986). Slugs were collected from under up-turned flower-pot saucers, baited with bran and left in an area of rough grassland which was irrigated by an overhead pipeline in the summer. D. reticulatum is capable of breeding throughout the year if conditions are suitable, so adult slugs were available throughout the year except during periods of freezing weather. Other species have defined seasonal life cycles and were collected as and when available for host range studies.

Once collected the slugs were kept in non-airtight clear plastic boxes lined with moistened absorbent cotton wool. They were fed Chinese cabbage leaves and carrots, and kept at 10°C with a twelve hour night and day cycle. The slugs bred profusely in these boxes and when juveniles were required (e.g. for mini-plot trials) the eggs were removed from the culture boxes and washed in tap water to remove any parasites present on the egg surface. They were then transferred to small plastic boxes lined with moist absorbent cotton wool and kept at 15°C with a twelve hour night and day cycle. Under these conditions eggs hatched within about four weeks. Young slugs were fed young Chinese cabbage leaves and moistened dog biscuits and maintained at 15°C until required.
2.1.2. Nematodes

The nematode was isolated from mature individuals of *D. reticulatum* collected from the field and kept in culture boxes as described above. The nematode was found to be associated with a disease in slugs with characteristic symptoms (see 3.3.1), most noticeably a swelling of the slug's mantle.

The nematode was maintained over several generations and the nematodes and the disease were spread from one slug culture box to another by transferring diseased or dead slugs or even food from one box to another. However, after preliminary experiments showed that the nematode could be grown on artificial medium (see 4.2.1.) it was decided to maintain the nematode *in vitro*. Throughout this thesis different types of nematode cultures will be described using terms proposed, or endorsed by Dougherty (1953, 1959) and further endorsed by Hooper (1986). These are:

- Xenic - with an unknown number of associated organisms (in this thesis meaning a mixed microbial flora).
- Gnotobiotic - with known associated organisms (known in number but not necessarily to species).
- Monoxenic - with one known associated organism.
- Axenic - with no associated organisms.

In this thesis the processes by which nematodes are rendered free from all contaminating bacteria shall be referred to as axenisation, and such nematodes will be referred to as axenic nematodes. Introducing axenic nematodes into culture with a single specific bacteria shall be referred to as monoxenisation, and nematodes grown in such cultures will be referred to as monoxenic nematodes.

Nematodes were generally maintained in xenic culture using a modification of Bedding's (1984b) technique in which the nematodes were grown on foam chips impregnated with medium and inoculated with bacteria and nematodes. (See Section 4.2.2)
2.1.3. Bacteria.

Bacteria associated with nematodes were isolated from within nematodes, from thriving xenic nematode cultures, from within the mantle of living slugs infected with nematodes and from the bodies of slugs supporting large nematode populations (See Section 6 for Methods). In all cases, once bacterial colonies had been selected the bacteria were subcultured on nutrient agar plates for examination and given a reference number. Cultures were stored on slopes of nutrient agar refrigerated at 4°C following 24 hours initial growth at 25°C. These cultures were subcultured at least once every six months. Two duplicate tubes of each culture were kept, one as a working culture for use in experiments and one as a library culture which was not used between subcultures unless the working culture died or became contaminated.

2.2. Counting nematodes in suspension.

For preparation of bioassays, the micro-plot trials and investigations into liquid cultivation it was necessary to count nematodes in liquid suspensions. Procedures used were similar to those recommended by Shepherd (1986). Counts were done in one of two types of nematode counting chamber. The commercially available Peter’s 1 ml counting chamber (Hawkesely, London)(Plate 1) was used. This consists of a single glass cell with a counting grid 3 x 2 cm divided into twenty-four 5 mm squares. The volume over the grid is 1 ml. These single chambers are expensive and only one count can be done at a time with each chamber between washing. For these reasons perspex multiple chambers were made by the LARS workshop (plate 1). These were a modification of the multiple counting chamber described by Doncaster et al. (1967). They were made out of three layers of perspex, the bottom layer being marked with a grid similar to that in the Peter’s chamber, the middle spacer layer having holes cut out to form the actual chambers, and the top layer having inlet and outlet holes drilled. The middle spacer in these slides was 2 mm thick so that the individual chambers had a volume of 1.2 ml over the grid. Each slide contained five chambers.

Usually at least five individual counts were done on each sample. Suspensions were diluted in known volumes of tap water so that ideally there were between two and three hundred nematodes over each grid and the numbers of nematodes in the original
suspension could be calculated. Nematodes showing any movement, however slight, were counted as living. All nematode suspensions were shaken vigorously to achieve an even distribution of nematodes. When aliquots of suspensions were transferred to counting chambers this was done as quickly as possible to avoid settling out in the pipette.

Plate 1. Peter's 1 ml nematode counting chamber (below) and multiple nematode counting chamber made in LARS workshops.

2.3. Analysis of Data.

Data from experiments in which one or more factors with discrete levels were considered to affect the results (e.g. micro-plot experiments, nematode growth experiments) were analysed using analysis of variance (ANOVA). This technique estimates the variation in the results between all factors and their sub-classes in the experiment separately, and then compares these variances with the natural variance measured throughout the experiment (the residual variance). If the variance attributed to an experimental factor is much greater than the residual variance it shows that this particular factor is having a large effect on the results. The significance of this effect can be determined by dividing the treatment variance by the residual variance to obtain an F value. The probability of obtaining this F value in an experiment with
same design can be checked in statistical tables. ANOVA was done using GENSTAT 5. (Payne et al., 1987).

ANOVA assumes that the recorded experimental results for all experimental factors are normally distributed and that the variability of the results, measured by the variance, is the same for all the individual experimental factors. ANOVA also assumes that the effect on the results of all the individual experimental factors are additive. These three assumptions were checked by plotting the residual values (the difference between the observed results and those predicted by the ANOVA model) against the fitted values (those predicted by the model). If the assumptions were correct, then the spread of residual values would be independent of the fitted values (Lane, Galwey, and Alvey 1987). If these assumptions were not correct the data were transformed to better fit these assumptions.

In experiments in which the measured variable was dependent on one or more continuous variables (e.g. bioassays, second micro-plot trial) the results were analysed using regression analysis. This technique produces an equation to describe the dependence of the measured variable on the variables set in the experimental design, which represents the line of best fit on a graph of dependent verses non-dependent variables. The line of best fit is the line in which the sum of the squared vertical deviations of the observed values from the line is least. The relationship may be linear, or non linear. Both types of regression analysis were done using GENSTAT 5.

Contingency tables of the results of certain experiments (e.g. slug injection experiments, host range studies) were analysed using chi² test in the case of two way contingency tables or by using MINITAB (Ryan, Joiner & Ryan 1980) for larger contingency tables. This technique assumes the null hypothesis that the experimental treatments do not effect the numbers in different treatment populations, and compares the predicted numbers in the populations assuming this hypothesis with the observed values. A value of chi² can be calculated for the ratio of observed to predicted values and its significance checked in statistical tables. Both GENSTAT and MINITAB were run on a MICROVAX 3800 running under a VMS operating system.
3. THE PARASITIC ACTION OF PHASMORHABDITIS SP. ON SLUGS.

3.1. Introduction.

This section discusses the identity and the taxonomic problems associated with parasitic nematode Phasmorhabditis sp. and describes observations made on the disease in D. reticulatum caused by infection with this nematode. Also described are preliminary dose response experiments attempting to quantify the relationship between dose of xenically cultured Phasmorhabditis sp. and its effects on D. reticulatum together with studies of the ability of the nematode to kill a range of slug species. Initial dose response experiments were done by keeping individual D. reticulatum in plastic tubes containing different numbers of nematodes. However, following problems with this system a method was devised in which larger numbers of slugs were kept in plastic boxes containing soil aggregates that had been sprayed with different numbers of nematodes. After an initial infection period the slugs were removed from the boxes of soil and transferred individually to petri dishes where their feeding and general appearance could be monitored. This system was used by Stroud (1990) in a LARS MSc. student project to investigate the relationship between the nematode dose and effect on D. reticulatum and was also used to investigate the ability of the nematode to kill a range of slug species.

All the experiments used nematodes grown in foam chip xenic cultures.

3.2. Materials and Methods.

3.2.1. Identification of the Nematode.

Nematodes were taken from dead slugs on several occasions, heat killed by adding Ringer’s solution at 60°C, and then fixed in TAF, a triethanolamine and formaldehyde mixture (Poinar & Thomas 1984). Fixed nematodes and diseased slugs carrying live nematodes were sent to David Hooper, Rothamsted Experimental Station (RES) for identification.
3.2.2. Effects of Phasmorhabditis sp. on D. reticulatum.

Field collected D. reticulatum infected with Phasmorhabditis sp. and slugs infected in the laboratory were examined and the symptoms recorded. Slugs were infected in the laboratory using either universal-tube infection chambers (See sections 3.2.3.) or in soil bioassay boxes (Section 3.2.4.). Observations of nematodes within slugs were made by dissecting slugs under water using a binocular microscope after killing slugs by immersion in boiling water for approximately 2 seconds.

3.2.3. Bioassays in which Slugs were Kept in Universal Tubes

3.2.3.1. First tube bioassay.

Individual adult D. reticulatum were kept in 30 ml plastic 'Sterilin' universal tubes. Each tube contained a plug of absorbent cotton wool at the bottom (plate 2). Seven doses of nematodes ranging from 10 to 1,000 infective larvae per tube were used with 50 replicate tubes being used for each dose. The correct number of nematodes suspended in tap water were placed on the cotton wool plug using a pipette and the total volume of tap water on each bung was made up to 4 ml. A further 50 tubes containing 4 ml of tap water without nematodes were used as controls. The slugs were fed strips of Chinese cabbage, 5 x 1.5 cm which were replaced every three days. The slugs were then kept at 10°C for three weeks and the number of dead slugs in each treatment was recorded every two days.

3.2.3.2. Second tube bioassay.

This experiment was also an investigation of the effect of dose of Phasmorhabditis sp. on D. reticulatum and was similar to the first tube bioassay in design and methods, but used the improved assay tubes. The bottoms of the tubes were blacked out using insulating tape and the opaque plastic screw caps were replaced by nylon netting held in place with rubber bands (Plate 2). This made the bottom of the tube, where the nematodes were placed, the darkest part of the tube and encouraged the slugs to rest there, in contact with the nematodes. The slugs fed on bran sprinkled at the bottom of the tube. Five nematode doses at half logarithmic intervals were used ranging from
30 to 1000 nematodes per slug. Fifty slugs were used for each nematode dose, and a further fifty slugs were left untreated. The number of dead individuals in each treatment group was recorded at 3, 5, 7 and 9 days after which the experiment was ended.

Plate 2. Universal tubes used to house slugs and nematodes in the first (left) and second (right) tube bio-assays.

3.2.4. Soil based bioassays.

3.2.4.1. First Soil Bioassay.

Soil is the natural habitat of both nematodes and slugs, so a soil based bioassay system was devised to measure the activity of Phasmodrhabditis sp. against slugs. Air dried clay soil from LARS was sieved using a shaking machine for 10 seconds. Soil was first passed through a sieve of hole size 24 mm and then through another sieve of hole size 13 mm separating the soil aggregates into three sizes. The middle size aggregates which were retained on the second sieve (approx 12 - 24 mm in diameter) were used for bioassays. These aggregates were used to fill two clear non-airtight plastic boxes measuring 135 mm x 75 mm x 60 mm (Plate 3). The amount of soil needed to fill the boxes was weighed then the aggregates were taken out of the
box and replaced back in layer by layer. 50,000 infective-juvenile nematodes were suspended in sufficient water so that the final water content of the aggregates was 30% w/v, and this was sprayed using a wash bottle over each layer of aggregates as they were replaced in the box. Twenty *D. reticulatum* were placed between the middle layers of the soil aggregates. The second box also received twenty *D. reticulatum* but was moistened with 30% w/w of tap water without added nematodes. The boxes were left at 10°C for 14 days after which time they were dismantled and the slugs examined.

3.2.4.2. Development of Soil Based Bioassay System.

Dry soil aggregates as used in 3.2.4.1. were divided into 440 g batches, placed in clear non-airtight plastic boxes measuring 135 mm x 75 mm x 60 mm and soaked for 24 hours with 80 ml (18% w/v) of tap water during which time the aggregates absorbed all the water. The following day the desired number of infective nematode larvae per box was suspended in 50 ml tap water in a wash bottle. The moistened soil aggregates were removed from the boxes and replaced layer by layer in the non-airtight plastic boxes. As the layers were being built up, the 50 ml of nematode suspension was evenly distributed over the surface of the soil. The final moisture content of the soil was thus 30% w/w, approximately the field capacity of soil. Ten slugs (fewer in host range studies on larger slugs, see Section 3.2.5.1) were placed between the middle layers. Slugs were left in the soil for an initial infection period after which slugs were transferred individually to 9 cm petri-dishes lined with moistened "Whatman No. 1" filter paper and containing a 3 cm disc of Chinese cabbage leaf. The ten petri dishes containing the slugs from each infection box were then kept at 10°C in another non-airtight box lined with moistened absorbent tissue paper to maintain 100% humidity (plate 4). After three days the amount of leaf disc consumed by each slug was estimated by eye to the nearest 5%. The old leaf discs were removed and replaced with new leaf discs and the consumption of the new discs was estimated after a further three days. New discs were added and read again once more after a further three days. The experiments were ended fourteen days after the initial exposure of slugs to the nematodes.
Plate 3. Plastic non-airtight box containing moistened soil aggregates used for bioassays.

Plate 4. Ten slugs kept in individual petri-dishes lined with moistened filter paper and containing a 3 cm disc of Chinese cabbage.
This system was used by Grahame Stroud (Stroud 1990) who used five doses of nematodes (15000, 23000, 35000, 55000, and 75000 nematodes per box of ten slugs) and included boxes of ten slugs without nematodes as untreated controls.

3.2.5. Host Range Studies.

Two experiments were done to investigate the host range of Phasmorhabditis sp. These experiments compared mortality in slugs treated with a single high dose of nematode with untreated control slugs. Experiments were done using the soil-based bioassay system (Section 3.2.4.).

3.2.5.1. First Host Range Experiment

Phasmorhabditis sp. nematodes were tested for their ability to kill six pest species of slugs. These were Deroceras reticulatum, D. caruanae, Arion ater, A. intermedius, A. distinctus and Tadonia sowerbyi. The slugs were collected from bran-baited traps at Long Ashton Research Station during November 1990. All slugs were adults except for A. ater which were juveniles (mean weight 770 mg). The nematodes were reared in xenic foam-chip bag cultures. Slugs were kept in plastic assay boxes of coarse soil aggregates as described in (Section 3.2.4). Ten slugs were placed in each box except for the larger slug species (T. sowerbyi and A. ater), for which five slugs were kept in each box. Approximately $1.9 \times 10^5$ infective larvae of Phasmorhabditis sp. were added to each of the nematode-treated boxes. Twenty slugs of each species were treated and a further twenty were left as untreated controls except for A. distinctus, seventeen of which were treated with nematodes and eighteen were kept as untreated controls. The slugs were left in the soil for a five-day infection period after which the soil boxes were dismantled and the numbers of dead slugs recorded. Surviving slugs were transferred to 9 cm petri dishes lined with moist filter paper where they were kept individually and fed leaf discs of Chinese cabbage. Numbers of dead slugs were recorded twice more at three day intervals.
3.2.5.2. Second Host Range Experiment

A second experiment was done to test the nematode for pathogenicity against *Arion silvaticus* and *Tandonia budapestensis*, with *D. reticulatum* also included as a positive control. Thirty slugs of each species were treated with nematodes and a further thirty were kept as untreated controls. Each nematode-treated box received $1.9 \times 10^5$ nematodes. Numbers of dead slugs were recorded at three day intervals.

### 3.3. Results

#### 3.3.1. Identification of the nematode.

David Hooper of RES initially identified the nematode as belonging to the Sub-Order Rhabditina. The main taxonomic characteristics of this group are the mouthparts and the male reproductive structures. No males were present in the samples of nematodes sent to RES, making identification difficult. The females had a distinctive, short stoma with an isomorphic metastom, characteristics shared by two genera, *Phasmorhabditis* and *Oschieus*, these two genera being distinguished by the morphology of the bursa in males.

After much searching of diseased slugs, a male was found on a dead slug, which was sent to David Hooper. The population of nematodes from this slug contained many males, which had peloderan bursas, fitting the species *Phasmorhabditis neopapillosa*. The females in this culture were morphologically indistinguishable from the females in the original samples. Thus it would seem that, according to Andrassy (1983), the original nematodes were *Phasmorhabditis hermaphroditica* whereas the second sample were *P. neopapillosa* (See 1.4.). The two species both caused disease in slugs with no apparent differences in symptoms.

In subsequent work males have been very rarely seen in the laboratory maintained nematode culture, or on dead slugs infected with this culture, indicating that the species used in all experiments is *P. hermaphroditica*. However, since it has yet to be
decided if *P. hermaphroditid* is a separate species or just a biological variety of *P. neopapillosa* (Andrassy 1983), throughout this thesis the nematode will be referred to as *Phasmorhabditis* sp.

### 3.3.2. Effects of *Phasmorhabditis* sp. on *Deroceras reticulatum*

*Phasmorhabditis* sp. nematodes infect slugs under the mantle and in diseased slugs this region becomes swollen, particularly the rear half of the mantle (Plates 5, 6 & 7). In severe infections the shell pierces the mantle and is often shed (plate 9). The mantle cavity below the mantle contains the slug’s kidney, lungs and heart, but dissection of infected slugs showed that the nematodes do not infect and multiply in this cavity, but in the region above this cavity surrounding the animal’s vestigial shell. This shell cavity is separated from the organs of the mantle cavity by membranous tissue. Nematodes undergo 4 cuticle moults during their life cycle and the five stages are designated L1, L2, L3, L4 and L5. The dauerlarvae (infective stage) of *Phasmorhabditis* sp appears to be an L3 larva in which the mouth and anus are closed, surrounded by a retained L2 cuticle (plate 11 and plate 12). Observations suggest that the infective stage enters the slug host through the dorsal integumental pouch immediately posterior to the mantle, formed where the back of the slug abuts with the posterior edge of the mantle. This pouch is connected to the shell cavity area by a short canal and it is through this canal via the pouch that the nematodes enter the mantle region. (See Fig 1.). The pouch and canal were first described by Brooks (1968) in his studies on ciliate parasites of slugs. He did not know their function but he considered the pouch to be the route of infection for the ciliates. Evidence for the canal via the pouch being the route of entry for the *Phasmorhabditis* sp. is:

a) Infective juveniles placed on the slug aggregate around the opening of this pouch and appear to be striving to gain entry.

b) Shortly after exposure to nematodes a characteristic lesion develops in the region of the central opening of the canal (plates 7 & 8). As the disease progresses this lesion becomes larger, and nematodes can be found within the pouch as well as round the shell.
Figure. 1. Sagittal section through the centre of *D. reticulatum* at the region where the body (A) joins the mantle (B), showing the integumental pouch (C), and the canal (D) linking the pouch with the cavity containing the shell (E). (After Brooks 1968).

Once the dauerlarvae have gained entry to the shell region they develop into adults (plate 12) and produce young. During the course of infection fluid accumulates in the shell cavity which leads to the characteristic swelling associated with nematode infection. At least two generations of nematodes (adults and juveniles) can be found in the swelling. Numbers of nematodes increase, and following the death of the slug they spread from the mantle region and feed and reproduce on the rest of the body of the slug (plate 10) which can support many thousands of nematodes. Eventually juvenile nematodes fail to develop into adults and form new dauerlarvae, presumably in response to lack of food or build up of toxic waste products.
**Plate 5.** Extended profile of healthy *D. reticulatum*.

**Plate 6.** Extended profile of *D. reticulatum* infected with *Phasmorhhabditis* sp.. The rear half of the mantle is swollen.
Plate 7. Healthy (left) and nematode infected (right) *D. reticulatum* viewed from above showing the mantle swelling and the lesion where the mantle joins the body on the infected animal.

Plate 8. Lesion seen at an early stage of infection with *Phasmorhabditis* sp. at the of the opening of the integumental pouch where the mantle meets the body.
Plate 9. Shell protruding from the mantle of *D. reticulatum* showing nematodes. Extreme pressure of fluid within the swelling has forced the shell to tear the mantle. Often the shell is shed.

Plate 10. *D. reticulatum* that has died following nematode infection. The nematodes have spread from the mantle region and are feeding on the dead slug.
Plate 11. Infective juvenile dauerlarvae (above) and non-infective juvenile Phasmorhabditis sp. (below). The mouth of the infective larvae is closed and the retained L2 cuticle can be seen. (x 100)

Plate 12. Hermaphroditic adult Phasmorhabditis sp. (above) and infective juvenile dauerlarvae (below). (x 35)
The disease has been studied in detail only for *D. reticulatum* but other species of slugs show similar symptoms, although the swelling of the mantle is usually less pronounced.

### 3.3.3. Bioassay in which Slugs were Kept in Universal Tubes.

#### 3.3.3.1. First Tube Based Bioassay.

Mortality of slugs over time is show in Table 1 and the results for day 21 are plotted against log nematode dose in Figure 2. It can be seen from Table 1 and Figure 2 that there was no increase in slug mortality with increasing dose of nematodes. Very few slugs in any of the nematode treated tubes showed any signs of nematode infection suggesting that either the nematodes were not pathogenic or that they were not reaching the slugs.

**Table 1.** Percent slug mortality during 21 days confinement with different numbers of nematodes in the first tube bioassay.

<table>
<thead>
<tr>
<th>Time in days</th>
<th>0</th>
<th>10</th>
<th>40</th>
<th>60</th>
<th>120</th>
<th>180</th>
<th>330</th>
<th>1000</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
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<td>7</td>
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<td>12</td>
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<td>4</td>
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</tr>
<tr>
<td>21</td>
<td>40</td>
<td>25</td>
<td>20</td>
<td>28</td>
<td>24</td>
<td>28</td>
<td>32</td>
<td>48</td>
</tr>
</tbody>
</table>
3.3.3.2. Second Tube Based Bioassay.

Mortality of slugs treated with different numbers of nematodes at time intervals throughout the experiment is shown in Table 2. It can be seen that by day 9 there was 32% mortality in the untreated slugs although slug mortality was higher in all slugs except those treated with the lowest dose of nematodes. To remove the effect of the background mortality from the results, slug mortality at days 9 and 12 was expressed as percent control using Abbott's (1925) formula:

\[
\text{corrected } \% \text{ mortality} = \frac{\left(\text{mortality of treated} - \text{mortality of control}\right)}{100 - \text{mortality of control}}
\]

These data were plotted against log nematode dose (Fig. 3.), showing that there was higher mortality in slugs treated with the lowest dose of nematodes than in the untreated slugs, which were thus calculated to have negative values for slug mortality thus making these results unsuitable for regression analysis. To test the significance of the trend for increasing slug mortality with increasing nematode dose, Kendall's coefficient of rank correlation was calculated (Campbell 1989) for the data at days 9 and 12. This showed that there was a significant correlation between slug mortality and nematode dose on both days 9 (P < 0.01), and 12 (P < 0.05).
Table 2. Percent mortality over 14 days confinement with different numbers of nematodes in the second tube bioassay.

<table>
<thead>
<tr>
<th>Numbers of nematode per tube</th>
<th>0</th>
<th>30</th>
<th>110</th>
<th>230</th>
<th>350</th>
<th>925</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time in days</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>0</td>
<td>2</td>
<td>6</td>
<td>4</td>
<td>0</td>
<td>8</td>
</tr>
<tr>
<td>5</td>
<td>0</td>
<td>2</td>
<td>12</td>
<td>4</td>
<td>0</td>
<td>12</td>
</tr>
<tr>
<td>7</td>
<td>16</td>
<td>12</td>
<td>30</td>
<td>14</td>
<td>10</td>
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<td>46</td>
<td>32</td>
<td>56</td>
<td>56</td>
<td>80</td>
<td>92</td>
</tr>
</tbody>
</table>

Figure 3. Dose response relationship for Phasmorhabditis sp. against D. reticulatum in the second tube bioassay.
3.3.4. Soil Bioassays.

3.3.4.1. First Soil Bioassay.

After 14 days in the soil boxes, 14 of the twenty slugs treated with nematodes had died compared with only 2 of the untreated slugs. A $X^2$ test shows that this difference is highly significant ($P < 0.001$) showing that the nematodes are capable of infecting and killing slugs in this bioassay system.

3.3.4.2. Development of Soil Based Bioassay System.

Stroud (1990) found that it was best to use five nematode doses (15,000, 23,000, 35,000, 55,000 and 75,0000 nematodes per box) and keep the slugs in the soil with the nematodes for five days. Using this system he estimated the nematode to have an $ED_{50}$ (The number of nematodes needed per box of ten $D. reticulatum$ to kill 50% of the population) of approximately 35,000. This system was used to bioassay nematodes which had been grown in monoxenic culture with different bacteria (Section 9).

3.3.5. Host range studies

3.3.5.1. First Host Range Experiment.

Table 3 shows mortalities of individual slug species in treated and untreated cells at 5, 8 and 11 days after introduction to the bioassay boxes. Mortalities of treated and untreated individuals of each species were compared using $X^2$ tests. After the five day infection period the differences in mortality between nematode-treated and untreated slugs were highly significant ($P < 0.001$) for $D. reticulatum$, $D. caruanae$, and $A. intermedius$. For the other three species differences in mortalities between treated and untreated slugs were not significant at this stage, but after eight days the differences in mortality between treated and untreated slugs were significant for all species tested ($P < 0.001$ for $D. reticulatum$, $D. caruanae$, $T. sowerbyi$ and $A. distinctus$, and $P < 0.01$ for $A. ater$, and $A. intermedius$). By day 11 all slugs treated with nematodes had died and the differences in mortality between treated and untreated slugs were
significant for all species \((P<0.01\) for \(A.\) intermedius and \(P<0.001\) for all other species). The difference was not as great for \(A.\) intermedius because many of the untreated slugs had died.

Table 3. Percent mortality at 5, 8 and 11 days in different species of slugs exposed to xenically cultured \(Phasmo\) rhabditis sp. nematodes or left untreated (First host range experiment).

<table>
<thead>
<tr>
<th>Species</th>
<th>Day 5</th>
<th>Day 8</th>
<th>Day 11</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>un-treated</td>
<td>nematodes</td>
<td>un-treated</td>
</tr>
<tr>
<td>Deroceras reticulatum</td>
<td>10</td>
<td>100</td>
<td>25</td>
</tr>
<tr>
<td>Deroceras caruanae</td>
<td>10</td>
<td>70</td>
<td>15</td>
</tr>
<tr>
<td>Arion ater</td>
<td>0</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>Arion distinctus</td>
<td>6</td>
<td>6</td>
<td>11</td>
</tr>
<tr>
<td>Tandonia Sowerbyi</td>
<td>15</td>
<td>20</td>
<td>15</td>
</tr>
</tbody>
</table>

3.3.5.2. Second Host Range Experiment.

The results are shown in Table 4. After the five day infection period in this experiment there were no significant differences in mortalities between treated and untreated slugs of any species. However, eight days after exposure, significantly \((P<0.001)\) more \(T.\) budapestensis had died in the nematode-treated group than in the untreated group, but there were still no significant differences in mortalities for other species. By day 11 mortality was significantly greater in nematode-treated \(A.\) silvaticus and \(T.\) budapestensis \((P<0.05\) and \(P<0.01\) respectively) than in the
untreated slugs. By day 14 mortality was significantly higher for nematode-treated
than untreated slugs of all species. (A. silvaticus, P<0.05, T. budapestensis P<0.01,
D. reticulatum P<0.001).

Table 4. Percent mortality at 5, 8, 11 and 14 days in different species of slugs
exposed to xenically cultured Phasmorhabditis sp. nematodes or left untreated (Second
host range experiment).

|--------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| D. reticula
tum       | 7               | 7               | 10              | 17              | 23              | 27              | 23              | 63              |
| A. silva
ticus       | 10              | 13              | 13              | 17              | 17              | 30              | 20              | 43              |
| T. budape
stensis      | 7               | 23              | 10              | 63              | 20              | 83              | 26              | 87              |

3.4. Discussion.

Preliminary investigations of the pathogenicity of Phasmorhabditis sp. showed that
this nematode is a parasite capable of killing a range of species of slugs: in the host
range studies, mortality was significantly greater in slugs treated with nematodes than
in untreated slugs, and there was a significant increase in mortality of D. reticulatum
with increasing nematode dose in the second tube assay. In both host range
experiments and the second dose response experiment, infected slugs showed
symptoms typical of nematode infection and nematodes could be re-isolated from the
dead slugs.

These findings are different from those of Maupas (1900) and Mengert (1953) who
considered that Phasmorhabditis hermaphrodita and P. neopapillosa respectively,
while being associated with slugs, did not live as parasites. One possible explanation
for the differences in these author's findings and those of the present investigation

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could be differences in the size of the slugs used in the studies. Maupas (1900) studied *P. hermaphrodita* in adult *Arion ater* and Mengert (1953) found *P. neopapillosa* in *Limax cinereoniger*. These two slugs are extremely large species, much larger even than *Tandonia sowerbyi*, the largest species used in the host range studies, and thus they may possibly only be killed by extremely high numbers of nematodes. Juvenile *Arion ater* were found to be killed by the nematode but adults have not been assayed. Furthermore Maupas (1900) and Mengert (1953) found the nematodes by dissecting apparently healthy field-collected slugs which had probably been exposed to fewer infective juveniles of *Phasmorhabditis* sp. than the experimental slugs in the present study. As the nematode disease is dose responsive (Fig 3.), it is possible that the slugs studied by Mengert (1953) and Maupas (1900) were capable of inhibiting the development of the small numbers of nematodes that they carried, but were not capable of killing them.

It is not clear from the present studies precisely how *Phasmorhabditis* sp. kills the host slug. Examination of fluid from shell cavities infected with nematodes reveals many bacteria, which the nematodes are presumably feeding on. It is possible that the slug's death arises from either a general septicemia following spread of the bacteria throughout the body, or as a result of toxins produced by the nematodes or the bacteria.

The lack of a dose response in the first tube based bioassay was probably a result of slugs in these universal tubes sitting on the lid at the top of the tube, where they were sheltered from the light. The nematodes in the cotton wool bung at the bottom of the tube may have came into little contact with the slugs. In the second bioassay the opaque plastic top of the tube was replaced by nylon netting and the bottom of the tube was blacked out using insulation tape making the bottom of the tube the darkest part. Thus the slugs tended to rest in contact with the nematodes and hence became infected. The high mortality in untreated slugs kept in these tubes meant, however, that even the improved tubes of the second bioassay were not particularly suitable for use in dose response experiments.

The soil bioassay gave better results, and as well as being used in these host range studies was used for detailed dose response studies using xenically cultivated
nematodes (Stroud 1990) and with nematodes grown in monoxenic culture with different bacteria (Section 9).

In the host range studies, *Phasmorhabditis* sp. was found to be capable of killing all the pest species tested. This is encouraging because as previously discussed (Section 1.1.1.) a population of mixed species of slugs is often present in arable fields and thus a biological control agent for slugs would need a wide host range to be reliably effective. *Phasmorhabditis* sp. has not been bioassayed against the largest species of slugs and the observations of Maupas (1900) and Mengert (1953) suggest that these species may possibly be resistant. However, in the bioassays *Arion ater* juveniles were shown to be susceptible, and *Limax cinereoniger*, the largest U.K. slug, feeds on decaying plant material and thus is not a pest. The wide host range of *Phasmorhabditis* sp. is a characteristic shared with entomopathogenic nematodes.
4. XENIC CULTIVATION AND STORAGE OF NEMATODES.

4.1. Introduction

Having established that *Phasmorhabditis* sp. was pathogenic to slugs and thus showed some potential as a biological control agent for slugs (Section 3), it was decided to produce sufficient numbers of the nematodes *in vitro* to test the ability of the nematodes to control slugs under field conditions in micro-plot trials (Section 5.).

Initial attempts were made to establish xenic cultures of the nematode in petri-dishes using simple methods recommended for free living rhabditid nematodes (4.3.1.). Having shown that *Phasmorhabditis* sp. could be grown in xenic culture, the nematodes were grown using modifications of the foam chip culture techniques (Bedding 1981, 1984b), developed to mass produce insect-parasitic nematodes. These techniques were used to produce sufficient numbers for storage experiments, bioassays and mini-plot trials. However, no experiments were done to optimise growth in xenic foam chip cultures because this method of cultivation is labour intensive and not economical for mass production in developed countries with high labour costs. In such countries liquid fermentation systems are more economically viable (Friedman 1990). Thus, two experiments were done to investigate the feasibility of growing *Phasmorhabditis* sp. in xenic liquid cultures. In a commercial production system it would be preferable to grow the nematodes in monoxenic culture, but it was felt necessary at an early stage in the project to show that *Phasmorhabditis* sp. was capable of *in vitro* growth in liquid, before nematode growth in monoxenic culture had been fully investigated.

Experiments were done to investigate the survival of nematode dauer larvae in aerated water, the most simple method for storage of entomopathogenic nematodes. The first experiment investigated survival of nematodes stored at temperatures equivalent to, and above room temperature. A second, more detailed experiment was done to study the survival of nematodes stored in aerated tap water at four lower temperatures.
4.2. Materials and Methods.

4.2.1. Xenic Growth of Nematodes in Petri-Dish Cultures.

According to Hooper (1986), many bacterial feeding nematodes can be grown with ease in xenic culture. An experiment was done to compare the growth of *Phasmarhabditis* sp. in xenic petri-dish cultures on three different media. Two of these media, Nigon's agar (Nigon 1949), and quarter strength bacteriological nutrient agar were standard media for rearing rhabditid nematodes as recommended by Hooper (1986). The third medium was one based on slugs and was made as follows. 500 g of slugs, previously killed by freezing, were added to 1000 ml of distilled water. This mixture was homogenised in a domestic blender and refrigerated overnight. The following day, the mixture was passed through muslin to remove solids. The medium was then supplemented with 10 g of Oxoid brain-heart infusion broth, 15 g of peptone, 5 g of NaCl and 20 g Oxoid Agar Technical No. 3. This medium was then autoclaved and poured into petri-dishes.

Gravid adult nematodes were taken from slugs which had died following nematode infection and were supporting large numbers of nematodes. Nematodes were washed in three successive changes of sterile distilled water then placed onto 9 cm petri-dishes containing growth medium. Five gravid nematodes were placed on each plate and ten such plates were prepared for each test medium. The plates were incubated at 20°C for three weeks prior to counting.

Because many nematodes had burrowed down into the agar after three weeks, it was necessary to melt the agar in order to count the nematodes. Two one-eighth pie sections of each plate were cut out at random and put into 30 ml universal bottles. The bottles were put in a water bath of boiling water where the agar melted. The agar was poured in a thin layer into a 6 cm petri dish which was then put on a graph paper counting grid. The nematodes were counted using a binocular microscope and hand tally.

4.2.2. Large Scale Nematode Production in Foam Chip Cultures.

Nematodes were mass cultivated for experimental work in foam chip cultures similar to those used for mass cultivation of entomopathogenic nematodes (Bedding 1984b). The medium consisted by weight of 65% pigs kidney, 15% beef dripping and 20% water. The
kidney was chopped into small pieces, the water was added and the mixture was 'liquidized' in a Waring blender. The beef dripping was melted in a large pan over a gas ring then the kidney homogenate was added and mixed thoroughly with the fat and cooked until brown. The mixture was then returned to the Waring blender and ground once again. This mixture was then mixed with polyether-polyurethane foam chips (without fire retardant), with 12 parts by weight of medium being added to 1 part foam chips (Plate 13). 'Sterilin' autoclave bags were prepared for use as culture bags as follows: A small slit (approx 3 cm) was cut in the middle of the bag and two lengths of autoclavable polythene tubing (each approximately 25 cm long) were inserted through the slit facing in opposite directions, one to act as an air inlet and one as an air outlet. The entry site of the two tubes was sealed by wrapping non-absorbent cotton wool round the two tubes in a figure-of-eight pattern and then sealing with strips of 5 cm width insulating tape. A 2 µm cellulose acetate filter was connected to the inlet pipe to prevent fungal spores and unwanted bacteria from being introduced into the culture bag (plate 14). After problems with blow-flies crawling into the bags, the outlet tube was covered with muslin netting. One Kg of medium was added to each bag then the bags were autoclaved at 121°C for thirty minutes. The sterile bags were kept deep frozen until needed. Bags were inoculated with nematodes and bacteria simultaneously. Each bag was slit open at the top and 75 ml of overnight culture of bacterium or bacterial mix was added. For xenic cultures this mix was obtained by removing one foam chip from a thriving xenic nematode culture and using it to inoculate nutrient broth. The nematode inoculum consisted of foam chips, carrying nematodes and bacteria, taken from mature foam chip cultures which were ready to harvest. The chips were poured from one bag directly into the new bag through the inoculation slit without being handled. Approximately 100 g of mature culture was used to inoculate each 1 kg bag. After inoculation with nematodes and bacteria the bags were sealed with tape and vigorously shaken to distribute the inoculum evenly. The bags were aerated by an aquarium pump, the air being bubbled through water to maintain high moisture levels in the bag. Culture bags were incubated at 15°C for three weeks. After this time many infective juveniles could be seen on the inside of the bags having left the spent medium.

Nematodes were harvested from the foam chips by a modified funnel extraction technique, similar to that used for collecting nematodes from soil samples. A 17.5 cm diameter milk filter (Clares Ltd, Midsomer Norton) was placed on top of a 17.5 cm diameter coarse mesh copper soil sieve which was then placed in a 50 cm diameter plant
pot saucer. The foam chips from the bags were placed in the sieve to a depth of approximately 2 cm and the plant pot base was filled with water until the water level just reached the bottom of the foam chip layer (Plate 16). Six such sieves were needed to harvest each bag. The sieves were then left overnight during which time live nematodes swam through the milk filters and collected in the water below. The water containing the nematodes was poured from the plant pot bases into plastic buckets which were left for at least six hours at 10°C while the nematodes settled to the bottom of the bucket. The water containing bacteria and spent medium was syphoned off to a level about 5 cm above the nematode sediment, and the buckets were filled with fresh tap water. This process was repeated at least six times, until the water in the buckets was clear and the nematode sediment at the bottom could easily be seen. The nematode suspension was then aerated using aquarium pumps and kept at 10°C until nematodes were required.

Non-infective nematode stages tended to die quickly in the water and could be removed by passing the suspension through milk filters or facial tissue in soil sieves, as used in harvesting. When smaller numbers of nematodes were required, approximately 90 g of the foam/kidney mixture was placed in 250 ml wide-neck conical flasks which were inoculated with either foam chips from mature cultures or slices of agar from mature petri dish cultures. These could be incubated without aeration and harvested in the same way as foam bag cultures.

Plate 13. Homogenised kidney medium being mixed with foam chips for use in foam bag cultures.
Plate 14. Completed foam chip bag with 2 µm cellulose acetate filter on inlet pipe.

Plate 15. Nematodes being harvested from foam chips using a modified Baerman funnel technique.
4.2.3. Liquid Cultivation.

Liquid cultures were incubated at 15°C and rotated at 200 rpm (a speed slightly higher than that used for entomopathogenic nematodes (J. Pearce AGC, pers comm.)) in a "New Brunswick Scientific" gyrotatory incubator/flask shaker. A liquid kidney medium, similar to the solid medium used in foam chip cultures was prepared as follows:

An aqueous suspension of 3.5% homogenised pigs kidney, 2.5% yeast extract and 3% corn oil (% w/v) was prepared and autoclaved at 121°C. During the heating, proteins congealed and the precipitated lumps were then filtered out through muslin. The desired volume of medium was then dispensed into flasks which were then plugged with bungs of non-absorbent cotton wool and re-autoclaved.

This medium has been used successfully to grow Steinernema sp. insect parasitic nematodes (J. Pearce, AGC, pers comm.).

4.2.3.1. The effect of different volumes of liquid in 250 ml baffled and unbaffled flasks on nematode yield.

A factorial experiment was done to investigate the effect of different volumes of liquid medium on nematode growth in standard 250 ml and baffled 250 ml conical flasks. The experiment consisted of six treatments; baffled or unbaffled flasks containing either 25 ml, 50 ml or 100 ml of liquid kidney medium with five replicate flasks of each treatment. The flasks were inoculated with infective juveniles grown in xenic foam chip bags. The nematodes were washed three times in sterile distilled water, before the final suspension of nematodes in distilled water was concentrated and counted. All flasks were inoculated with 175 nematodes per ml of medium. The flasks were simultaneously inoculated with 0.4μl/ml overnight nutrient broth cultures of Providencia rettgeri, a bacterium which had been shown to support good nematode growth in experiments on monoxenic culture on solid medium (See Section 7). Although Pr. rettgeri was added as a starter culture, the cultures were xenic because after the infective larvae had exsheathed and started feeding, their gut flora would have been released, so that mature cultures contained an unknown number of unidentified bacteria in addition to Pr. rettgeri.

After one week, two weeks, three weeks and four weeks, one 0.5 ml sample was taken from each flask and the numbers of nematodes counted, at least three counts being done
on each sample. After three and four weeks the proportion of infective juveniles was recorded in each sample, and the numbers of infective juveniles per ml estimated. Nematode numbers were transformed to square roots prior to analysis.

4.2.3.2. The effect of different inoculation levels on final nematode yield.

This was an experiment to investigate the effect of different initial numbers of nematodes on final nematode yield in xenic culture. Five different inoculum levels (50, 110, 170, 250 and 330 nematodes per ml) were added to 50 ml of liquid kidney medium in 250 ml baffled flasks, with four replicate flasks per treatment. The nematodes used to inoculate the flasks had been grown in xenic foam chip bag cultures and then stored in aerated tap water. The nematodes were washed three times in sterile distilled water prior to inoculation. In all cases the number of nematodes used to inoculate the flasks were suspended in 2 ml of sterile distilled water. Flasks were simultaneously inoculated with 50 µl of overnight broth culture of a xenic mix of bacteria taken from a thriving foam chip xenic culture. Samples of 1 ml were then taken from each flask after one week and then twice weekly for the next three weeks and the total numbers of nematodes per ml counted. In all but the first sample the number of infective juveniles per ml was also counted.

4.2.4. Storage Experiments

4.2.4.1. Survival of nematodes at temperatures above room temperature.

An initial experiment was done to investigate the effects of high temperature on nematode survival. One 100 ml conical flask containing 80 ml of tap water was placed in each of three incubators running at temperatures just above those required and the flask was aerated with an aquarium pump connected to an air-stone. The water temperatures in the test flasks were lower than the air temperatures in the incubators because of evaporation caused by the aeration. The temperature of the incubators was adjusted until the water in the flasks was at 25, 27, or 29°C. 10 ml samples of previously counted nematode suspension which had been stored at 10°C were placed in boiling tubes with thermometers and heated in a 50°C water-bath for approximately 10 seconds until the nematode suspension had reached the test temperature. The nematode suspensions were then added
to the conical flasks in the incubators resulting in a nematode density of approximately 100/ml. Three 1 ml samples were then taken from each flask at intervals throughout the day. The numbers of living and dead nematodes in each sample were counted and the percentage of nematodes alive calculated.

4.2.4.2. Survival of nematodes at temperatures below room temperature.

A second experiment was done to investigate the effect of temperature on long term nematode survival in aerated water at lower temperatures than those used in experiment 1. Infective nematode larvae were raised and harvested from xenic foam-chip bag cultures using methods described in Section 3.2.2. Before use the nematodes were washed three times in artificial tap water (Greenaway, 1970). Nematodes were then suspended in artificial tap water at a density of 5,000 nematodes per ml, and 100 ml of this nematode suspension was placed into each of twelve, 250 ml conical flasks. Three flasks were kept at each of four temperatures: 5°C, 10°C, 15°C and 22°C. The flasks were sealed with bungs of non-absorbent cotton wool to reduce water loss by evaporation and aerated using aquarium pumps placed inside the incubators, thus blowing air of the same temperature through the suspension. Numbers of live nematodes were estimated at frequent intervals by taking one 0.5 ml sample from the flasks during the following two months.

4.3. Results.

4.3.1. Xenic Growth of Nematodes in Petri-Dish Cultures.

The numbers of nematodes after three weeks on the three different culture media are shown in Table 5. Nematode numbers were transformed to logarithms for statistical analysis. Most nematodes were found on the slug based agar, but numbers on this medium were not significantly different from numbers on either nutrient agar or Nigon's agar.
Table 5. Numbers of nematodes after three weeks growth in xenic culture on different media.

<table>
<thead>
<tr>
<th></th>
<th>Nutrient agar</th>
<th>Nigon's agar</th>
<th>Slug agar</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean number of nematodes/plate</td>
<td>1695</td>
<td>2846</td>
<td>4279</td>
</tr>
<tr>
<td>Mean log nematode numbers/plate</td>
<td>3.085</td>
<td>3.291</td>
<td>3.456</td>
</tr>
</tbody>
</table>

S.E.D. for comparing log nematode numbers = 0.1964 (27 D.F.)

The numbers of nematodes present on different plates of the same medium varied considerably, possibly as a result of slight differences in the bacterial flora on each plate.

4.3.2. Xenic Foam Chip Cultures.

No detailed experimental work was done to investigate nematode yields from xenic foam chip cultures. These were merely used to produce large numbers of nematodes for experimental work. The bags generally yielded from 5 to 10 x 10^7 nematodes with generally over 90 % being infective juveniles. Occasionally these bags yielded very low numbers and many nematodes died within the bags. The reasons for the poor yields were not known.

4.3.3. Liquid Cultivation

4.3.3.1. The effect of different volumes of liquid in 250 ml baffled and unbaffled flasks on nematode yield.

The total numbers of nematodes recorded from different volumes of liquid in baffled and unbaffled flasks in this experiment are shown in Figure 4, and the proportion and numbers of infective juveniles at weeks 3 and 4 are shown in Table 6. Good growth was observed in all baffled flasks which supported better growth than unbaffled flasks (Fig 4). Nematode numbers in baffled flasks increased over the first two weeks with sharp increases being observed during the second week. During the third week numbers levelled off or fell slightly.
Figure 4. Nematode numbers during four weeks of growth in 250 ml baffled or unbaffled flasks containing either 25, 50 or 100 ml of liquid kidney medium.

In unbaffled flasks containing 50 ml and 100 ml of medium, numbers of nematodes remained low for the four weeks duration of the experiment. Numbers in these flasks were significantly (P < 0.05) lower in weeks two and three than in baffled flasks containing the corresponding volume of medium. Good growth of nematodes was observed in unbaffled flasks containing 25 ml of medium and growth was not significantly different from that seen in 25 ml baffled flasks except at week 2, when the baffled 25 ml baffled flasks had significantly (P < 0.001) more nematodes than the unbaffled 25 ml flasks. Growth in the 25 ml unbaffled flasks followed a similar pattern to that seen in all baffled flasks.
Table 6. Numbers of infective juveniles per ml and infectives juveniles as a percentage of nematodes populations grown in baffled and unbaffled flasks containing 25, 50 or 100 ml of liquid kidney medium.

<table>
<thead>
<tr>
<th></th>
<th>Unbaffled Flasks</th>
<th>Baffled Flasks</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Week 3</td>
<td>Week 4</td>
</tr>
<tr>
<td></td>
<td>%Infec-tives</td>
<td>Infectives/ml</td>
</tr>
<tr>
<td>25 ml</td>
<td>32.4</td>
<td>5027</td>
</tr>
<tr>
<td>50 ml</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>100 ml</td>
<td>20.0</td>
<td>6.0</td>
</tr>
</tbody>
</table>

S.E.D. for comparing numbers of infective juveniles = 1844.5 (24 D.F.).

Numbers of infective juveniles fell between week three and four in all flasks except the 50 ml unbaffled flasks, in which there were very few infectives at week three. Numbers of infective juveniles were highest at week three in the 50 ml baffled flasks, and numbers in these flasks were significantly greater than in all other flasks except than in the 25 ml baffled flasks. It can be seen (Table 6) that at week four, when the overall numbers of infective juveniles/ml had fallen, the percentage of the total nematodes which were infective juveniles had risen. This probably reflects greater production of infective juveniles and their ability to survive unfavourable conditions such as build of toxic waste products.

4.3.3.2. The effect of different inoculation levels on final nematode yield.

The total numbers of nematodes recorded throughout the experiment are shown in Figure 5. Total nematode numbers and numbers of infectives were transformed to square roots for analysis. Nematode numbers reached a plateau after three weeks for all treatments except for the highest inoculum level, whereas numbers of infective juveniles rose throughout the experiment. By day 20 flasks inoculated with 50 nematodes per ml had
significantly (P < 0.05) fewer nematodes than all other treatments and this persisted until the end of the experiment. There were no significant differences between other treatments.

Numbers of infective juveniles/ml are shown in Figure 6. At day 13 there were significant differences in the numbers of infective juveniles per ml between treatments. Flasks inoculated with 250 nematodes per ml had significantly (P < 0.05) more nematodes than flasks inoculated with 50, 170, or 330 nematodes per ml. From day 24 onwards the flasks inoculated with 50 nematodes per ml had significantly fewer infective juveniles (P < 0.05) than all other treatments. There were no other significant differences in numbers of infectives, indeed the mean final number of infectives/ml for all other treatments was remarkably similar (Fig. 6).

Figure 5. Nematode numbers/ml (all stages) over 28 days growth in liquid cultures inoculated initially with either 50, 110, 170, 230 or 330 nematodes/ml.
Figure 6. Numbers of infective juveniles/ml recorded between 7 and 28 days of growth in liquid cultures following inoculation with either 50, 110, 170, 230 or 330 nematodes/ml.

4.3.4. Storage Experiments.

4.3.4.1. Survival of nematodes at temperatures above room temperature.

The results of the first storage experiment are shown in Figure 7. All nematodes died within 2 hours at 35°C, and at 26°C and 29°C approximately half of the nematodes died during the 6 or 7 hours of the experiment. Many of the nematodes surviving at the end of the experimental period were only capable of infrequent, slight twitching movement, and while such worms were still recorded as being alive, it is unlikely that they would
be capable of infecting slugs. At the end of the experiment the nematodes were returned to 10°C where they showed no signs of recovery.

Figure 7. Nematodes surviving (%) during seven hours storage at 26, 29 and 35°C.

There was no true replication in this experiment, there being only one flask at each temperature, so the results were unsuitable for statistical analysis. However, it was apparent from the rapid death of nematodes at all temperatures in this experiment that Phasmarhabditis sp. does not survive well in aerated tap water at temperatures above 25°C. In this experiment the nematodes were rapidly heated from 10°C to the experimental temperature in a water bath and it is possible that thermal shock might have been responsible for some of the rapid mortality observed.

4.3.4.2. Survival of nematodes at temperatures below room temperature.

The numbers of living nematodes recorded at each temperature on each of the sampling dates were transformed to square roots for analysis of variance (Figure 8). Both time and temperature had highly significant (P<0.001) effects on the numbers of surviving nematodes and there was a highly significant (P<0.001) interaction between these two
variables. Exponential curves were fitted separately to the nematode counts from individual flasks. Equations for the curves took the form

\[ y = a + br^x, \]

where \( y \) is the square root of the number of surviving nematodes, \( a \) is the asymptote parameter, \( b \) is the range parameter, \( r \) is the rate parameter and \( x \) is the time in days.

![Figure 8. Numbers of live nematodes during two months storage in aerated artificial tap water at 5, 10, 15 or 22°C.](image)

The parameters \( a \), \( b \) and \( r \) were first estimated separately for each flask and models with these parameters accounted for 67\% to 98\% of the variation in nematode numbers with time.
The parameters \(a\), \(b\) and \(r\) were then subjected to analysis of variance. There were no significant effects of temperature on the parameters \(a\) and \(b\), but the rate parameter \((r)\) was influenced by temperature \((P<0.05)\) (Table 7). Thus, the dependence of nematode survival on time in this experiment was described by the function

\[y = 9.2 + 58.7r^4,\]

where the value of \(r\) at each temperature is shown in Table 7. Comparison of the values of \(r\) shows that \(r\) was significantly greater at 10°C than at 15°C \((P<0.05)\) and 22°C \((P<0.01)\). There were no other significant differences. This indicates that survival at 10°C was significantly better than at 15°C and 22°C. However it is clear from Fig. 8 that survival at 5, 10 and 15°C was similar, and better than at 22°C.

<table>
<thead>
<tr>
<th>Temperature</th>
<th>5°C</th>
<th>10°C</th>
<th>15°C</th>
<th>22°C</th>
<th>S.E.D. (8 D.F.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rate parameter (r)</td>
<td>0.9477</td>
<td>0.9708</td>
<td>0.9420</td>
<td>0.9223</td>
<td>0.01199</td>
</tr>
</tbody>
</table>

4.4. Discussion

The experiments in which Phasmorhabditis sp. was reared in xenic cultures do not represent the first time that this nematode was reared in vitro. Maupas (1900) reared \(P.\) hermaphroditis on rotten flesh for two years. However, this is the first time that this nematode has been cultured on artificial media. In the first experiment, in which Phasmorhabditis sp. was reared in xenic culture on three different media, there were no significant differences in nematode numbers between media, but best growth was seen on the slug medium, rather than the two media used for free living rhabditid nematodes. The slug medium was very rich, and was fortified with brain-heart infusion broth, a rich medium generally used for the cultivation of fastidious pathogenic bacteria of humans (Anon. 1982). Parasitic nematodes are adapted for life in an environment where they will be exposed to highly nutritious body fluids of the host organism, which in turn can support rapid growth of bacteria to serve as food for rhabditid nematodes. This is
reflected in the richness of the media used to cultivate entomopathogenic nematodes e.g. chicken offal, dog food and kidney based media.

Bedding (1984b) used 3 kg foam bags to mass produce different species of entomopathogenic nematodes. These 3 kg bags yielded an average of 1300 million Neoaplectana bibionis (=S. feltiae) infective juveniles per bag (=433 million /kg) which is more than four times higher than the maximum yields from the 1 kg bags used in this study. Bedding (1984b) reported that lower numbers of other species of Steinernema or Heterorhabditis which have larger infective juveniles than S. feltiae were achieved using this method. Bedding used media based on chicken offal rather than kidney in his studies and recommended different media for rearing Steinernema spp. and Heterorhabditis spp.

These media differences and the fact that Bedding’s (1984b) nematodes were grown in monoxenic culture with their preferred symbiotic bacteria might explain the higher yield he obtained. However, the foam chip culture method described for use with Phasmorhabditis sp. was satisfactory for producing sufficient numbers of nematodes for experimental work, and it is likely that yields of nematodes produced by this method could be increased by media development and by growing the nematodes in monoxenic culture with a specific bacterium known to be a good food source for the nematode (Section 7).

There is at present little published information regarding liquid cultivation of entomopathogenic nematodes; most work has been done to optimise commercial production systems and is hence confidential. Beucher and Popiel (1989) grew Steinernema feltiae with its bacterial symbiont in both shallow layers of liquid and large volumes. They found that in larger cultures aeration was growth limiting and found better nematode growth could be obtained by rotary shaking or by air-sparging the cultures. In the two experiments in which Phasmorhabditis sp. was grown in liquids, similar results were obtained. In general, baffled flasks gave better yields than unbaffled, and flasks containing smaller volumes of liquids tended to give better yields, probably because the greater surface area to volume ratio of the liquid allowed better aeration. However, this was not always the case because the baffled flasks containing 50 ml of liquid gave higher yields than baffled flasks containing 25 ml of liquid. The lower than expected yields in the baffled flasks containing 25 ml of medium were probably due to a reduced effect of the baffles, which did not extend right to the bottom of the flasks, and the 25 ml of liquid was only slightly agitated by them during rotation. The beneficial effects of the baffles
is not surprising, as baffles are known to improve oxygen absorption rate, mixing of nutrients and are better at producing an even distribution of experimental organisms in the culture (Freedman 1970).

In the second experiment no significant differences were found in final nematode yield when the flasks were inoculated with more than 100 worms/ml medium. This is lower than the 250 worms/ml maximum inoculation level for maximum yield found by Beucher and Popiel (1989) for growing *S. feltiae*. However both these initial inoculation levels are substantially lower than those used to inoculate commercial liquid fermentation systems, (J. Pearce, AGC. pers. comm.), and in future experiments it would be interesting to study the effects of much higher inoculation levels on final nematode yield.

It is not surprising that *Phasmorhabditis* sp. did not survive well in aerated tap water at temperatures above 25°C, since entomopathogenic nematodes are known to survive better when stored in aerated water at lower temperatures. Nematode metabolism is temperature driven, and a warm (20-30°C) environment increases metabolic activity and thus reduces viability and pathogenicity (Georgis 1990b). It is difficult to compare survival of *Phasmorhabditis* sp. in the experiment using lower temperatures with results reported for entomopathogenic nematodes. Bedding (1984b) recorded only 10% mortality in certain *Steinernema* species after four months storage at 1-2°C, but does not state the density at which they were stored. Survival of *Phasmorhabditis* sp. was not studied at 1-2°C because this temperature was thought to be lower than could be achieved during commercial storage or transport using standard refrigeration equipment. Georgis (1990b) states that in the post harvest period prior to formulation entomopathogenic nematodes can be stored in refrigerated aerated aqueous suspensions for between 6 and 12 months depending on the species and concentration but he does not state refrigerator temperature. In the experiment investigating survival of *Phasmorhabditis* sp. only 6% survival was seen after 2 months storage at 10°C (the temperature at which this worm survived best, although survival was not significantly better than at 5°C). While *Steinernema* spp. store best at very low temperatures, *Heterorhabditis* spp. tend to survive and maintain virulence best at temperatures of 10-15°C. (Poinar 1986, Georgis 1990b). More data would be needed to confirm the best temperature for survival of *Phasmorhabditis* sp., but in the absence of such data it may be best to store the nematode in aerated water at 10°C rather than at lower refrigerated temperatures.
It can be concluded from this series of experiments that Phasmorhabditis sp. can be grown in vitro on artificial media and, of great importance from a commercial point of view, can be grown in deep liquid cultures, using similar technology to that developed for use with entomopathogenic nematodes. Furthermore the dauerlarvae of this nematode survive when stored in aerated aqueous suspensions at low temperatures and it is possible that survival of Phasmorhabditis sp. at low temperatures may be increased by formulating the nematodes into a substrate that partially immobilises them (J. Jebb. AGC pers. comm.).
5. MINI- PLOT FIELD EXPERIMENTS USING XENICALLY CULTIVATED NEMATODES.

5.1. Introduction.

Mini-plot field experiments were done at an early stage in the project using xenically cultivated nematodes. Whilst it was appreciated that more basic research into the biology of the nematode would be needed to produce a reliable biological control agent, it was known that the nematodes when cultivated in this way were pathogenic to slugs under laboratory conditions and it was felt important to show that the nematode was capable of controlling slugs under field conditions. Full scale field trials are costly, time consuming and labour intensive, and were not felt to be appropriate at such an early stage in the project. A bed of mini-plots at Long Ashton Research Station had been used successfully for previous experiments (Glen, Milsom & Wiltshire 1986; Glen, Cuerdon & Butler, 1991) and these plots were used to assess the effectiveness of the nematode under conditions similar to the field.

Two mini-plot trials were done, the first was a simple experiment in June - July 1989 comparing slug damage to Chinese cabbage seedlings in untreated plots, plots treated with methiocarb pellets and plots treated with a single high dose of nematodes. The second trial started in October 1989 and investigated the effects of different doses of Phasmarhabditis sp. on slug damage to Chinese cabbage seedlings and wheat seeds, and compared this damage with that in untreated plots and plots treated with methiocarb pellets.

5.2. Materials and Methods

Both experiments were done in a series of 40 mini-plots containing a loam soil to a depth of 30 cm on a bed of coarse gravel. The plots were 70 cm x 70 cm (0.5 m²) x 30 cm deep and were separated by solid wood or concrete barriers extending from 5 cm above soil level to a depth of at least 20 cm. This was surmounted by a fence, 10 cm high of
0.8 mm woven copper mesh to act as a barrier to slug movement between plots (Moens et al. 1967). (Plate 16). Nematodes reared in xenic foam chip bag cultures were used in both experiments (see 4.2.2.)

Plate 16. Series of forty mini-plots at Long Ashton Research Station surmounted with copper fences to prevent slug movement between plots. Nine Chinese cabbage seedlings arranged in a 3 x 3 square are being planted in each plot.

5.2.1. First Mini-plot Trial.

After a previous experiment in autumn 1987 the plots had been emptied of soil and replaced with fresh non-sterilised soil. Thirty-six of the plots were populated with Deroceras reticulatum between March and June 1989. No slugs were added to the remaining 4 plots which were used as a measure of the slug population originally present in the fresh soil. Five field collected adults were added to each of the plots to be populated. These slugs had been kept in quarantine boxes for at least two weeks to ensure that they were not carrying any parasites when they were added. In addition thirty-four
laboratory reared (see 2.1.1.) neonate *D. reticulatum* were added to each throughout the three-month period. Thus, at the start of the experiment slugs at many stages of development were present.

The experimental design consisted of nine randomized blocks of four plots as shown in Figure 9. Each block consisted of two untreated plots, one plot treated with *Phasmorhabditis* sp. and one plot treated with methiocarb pellets.

Methiocarb pellets (Draza) were applied at the recommended field rate (5.5 Kg/ha = 0.275 g/plot). The pellets were weighed out for each plot separately and distributed evenly over the plots by hand. \(1.05 \times 10^6\) nematodes were drenched over each plot suspended in 900 ml of tap water using a watering can fitted with a rose. A further 100 ml of tap water was used to rinse the can out and then poured onto the plots. One litre of tap water was also added to the untreated and methiocarb-treated plots. The plots were irrigated from an overhead pipe as necessary to keep the surface of the soil moist throughout the course of the experiment.

At the start of the experiment young Chinese cabbage seedlings which had been grown in a glasshouse were planted out, nine seedlings in each plot arranged in a 3 x 3 square (plate 16). These were examined twice weekly and the amount of slug damage to each seedling was estimated to the nearest 5% (Plate 17).

Two weeks after planting, the seedlings in some of the untreated plots were completely destroyed, so remnants of the old seedlings were removed from all plots and new ones planted. This was repeated after a further two weeks. After two more weeks the experiment was finished (six weeks in total). Seedling damage was recorded twice weekly throughout the course of the experiment. The copper mesh barriers between plots in one of the treatment blocks (Block 9) became detached after the first four weeks, allowing slug movement between plots, so the results from these plots were ignored for the fifth and sixth weeks.

At the end of the experiment two soil samples 25 x 25 x 10 cm deep were taken from each plot of the remaining 8 blocks, one sample being taken from the middle and one from the south east corner of each plot. The samples were placed immediately in plastic tubs 35.5 cm in diameter, 18.5 cm deep, with three 2.0 cm diameter holes covered in 1
mm mesh at the base of the walls. The tubs were covered with wooden lids and transported to large wooden troughs in a soil flooding unit. The troughs were charged initially with a 2 cm depth of water, and a drip feed was introduced into each trough, which caused the water level in the samples to rise steadily until they were completely flooded after 8-10 days (Glen & Wiltshire 1986). Slugs, worms insects and other soil invertebrates were removed from the surface daily. Slugs were weighed so that effects on biomass as well as numbers could be monitored.

Plate 17. Chinese cabbage seedlings showing characteristic slug damage one week after planting. The upper plot in the photograph was a nematode treated plot and the lower plot was an untreated plot.
Figure 9. Plot plan for first mini-plot trial.

A = Untreated.
B = 0.275 g methiocarb pellets/plot.
C = $1.0 \times 10^6$ Phasmorhabditis sp./plot.
NONE = No slugs added.

Figure 10. Plot plan for second mini-plot trial.

A = $5.0 \times 10^3$ Phasmorhabditis sp./plot.
B = $1.5 \times 10^4$ Phasmorhabditis sp./plot.
C = $4.0 \times 10^4$ Phasmorhabditis sp./plot.
D = $1.0 \times 10^5$ Phasmorhabditis sp./plot.
E = $3.5 \times 10^5$ Phasmorhabditis sp./plot.
F = $1.0 \times 10^6$ Phasmorhabditis sp./plot.
G = Untreated.
H = 0.275 g methiocarb pellets/plot.
5.2.2. Second Mini-Plot Trial.

The mini-plots were emptied of soil in August 1989 at the end of the first mini-plot trial and the gravel beds were treated with "Jeyes Fluid" in order to kill any surviving nematodes. The plots were then filled with fresh loam soil and the copper barriers were repaired where necessary. The plots were re-populated with slugs during September and October 1989. Each plot initially received six adult, three juvenile field collected D. reticulatum and twenty one laboratory reared neonate D. reticulatum. Chinese cabbage leaves were added to the surface of plots before the start of the experiment as food for the slugs and these were replaced when necessary. The amount of slug damage to Chinese cabbage seedlings differed considerably between plots. Up to four extra adult D. reticulatum were added to plots with least damage so that the final number in each plot ranged from 30 to 34 slugs.

The experimental design consisted of five replicates of eight treatments arranged in randomized blocks (Figure 10). The treatments were:

a) Nematodes applied at $5 \times 10^3$/plot = $1 \times 10^8$/ha
b) Nematodes applied at $1.5 \times 10^4$/plot = $3 \times 10^8$/ha
c) Nematodes applied at $4 \times 10^4$/plot = $8 \times 10^8$/ha
d) Nematodes applied at $1 \times 10^5$/plot = $2 \times 10^9$/ha
e) Nematodes applied at $3.5 \times 10^5$/plot = $7 \times 10^9$/ha
f) Nematodes applied at $1 \times 10^6$/plot = $2 \times 10^{10}$/ha
g) Methiocarb pellets applied at 0.275 g/plot = 5.5 Kg/ha
h) Untreated control plots.

The highest dose of nematodes (treatment f) was the same as that used in the first mini-plot trial. The bottom dose was chosen to be equivalent to $1 \times 10^8$ nematodes per hectare, a dose well below that at which insect parasitic nematodes are used (Paul Rogers, AGC pers. comm), and the other four nematode doses were chosen to be at equal intervals on a logarithmic scale between these two extremes. Nematode suspensions were counted using a Peter's 1 ml nematode counting chamber and diluted to obtain the required
number of nematodes for each plot in 750 ml of tap water. Each plot was drenched with 750 ml of the appropriate suspension using a watering can fitted with a rose. A further 250 ml of water was used to rinse the can out and then poured onto the plots so that each plot received a total of 1 litre of water. One litre of tap water was also added to the methiocarb-treated and untreated plots. The methiocarb pellets were weighed out and distributed evenly over the plot surfaces by hand.

At the start of the experiment young Chinese cabbage seedlings were planted out, nine in each plot arranged in a 3 x 3 square as in the first mini-plot trial. These were examined at twice weekly intervals and the amount of slug damage to each seedling was estimated by eye to the nearest 5%. Remnants of the old seedlings were removed and new seedlings were planted at weekly intervals for the first four weeks. As the experiment progressed, the weather got colder and slug activity declined so that after the first four weeks it was only necessary to change the seedlings every two weeks.

Forty wheat seeds (cv. Avalon) were placed on the soil surface in two rows in each plot at the start of the experiment. These seeds were examined and the numbers showing slug damage in the differently treated plots were recorded at 4 and 7 days. The seeds were then immediately replaced by fresh seeds which were examined 7 days later. This procedure was repeated in subsequent weeks, but because of mice eating the seeds, data were obtained only for days 4, 7, 14, 42, and 53.
5.2.3. Meteorological Data.

Meterological data were recorded by the LARS meteorological station. The Mean daily air temperature was calculated as the mean of the maximum and minimum air temperatures. Daily rainfall was recorded throughout the first mini-plot experiment and for the first 6 weeks of the second mini-plot experiment. Air temperatures were felt to be more appropriate than soil temperatures measured at 10 cm depth because (a) the nematodes were applied to the soil surface and (b) the plots were raised above ground level and hence would be susceptible to changes in air temperature.

5.3. Results.

5.3.1. First Mini-Plot Trial.

Slug damage to Chinese cabbage seedlings is shown in Figure 11. Estimates of the percentage of each plant damaged by slugs were transformed to angles prior to analysis of variance.

Methiocarb pellets and Phasmorhabditis sp. significantly ($P < 0.001$) reduced the overall mean damage to Chinese cabbage seedlings for the whole experiment. At the first examination (four days after treatment) there was significantly ($P < 0.05$) more slug damage in plots treated with Phasmorhabditis sp. than in the plots treated with methiocarb but, as seedlings outgrew the initial damage, the difference between the nematode and methiocarb treated plots narrowed. By the third examination (Day 11), nematode treated plots showed less damage than methiocarb plots, but this difference was not significant.
Figure 11. Mean damage to Chinese cabbage seedlings for the differently treated plots in the first mini-plot trial.

After 17 days (first examination of the second planting of seedlings) the nematode treated plots had significantly (P < 0.05) less damage than methiocarb treated plots, and this persisted (P < 0.01) until the end of the experiment.

The figures in Figure 11 may be slight overestimations of slug damage as the plants were damaged by flea beetles during the experiment. While the small circular holes caused by this pest were readily distinguishable from slug damage, it would have been difficult to distinguish slug damage from flea beetle damage when individual holes made by flea beetles had merged together, or when small holes made soon after planting the seedlings...
had grown with the plant into large holes. Where there was doubt the damage was recorded as slug damage.

Three species of slugs were found in the soil samples, *Deroceras reticulatum*, *Deroceras caruanae* and *Boettgerilla pallens*. Only 2 *D. caruanae* were found overall, but 89 *B. pallens* were found, compared with 55 *D. reticulatum*. The *B. pallens* were presumably introduced into the plots with the soil or in some other way. The preferred diet of this slug is not known, but in laboratory tests it did not damage Chinese cabbage leaves during three weeks exposure without any alternative food source. It is therefore unlikely that this slug was causing damage to the seedlings in this trial. No *D. reticulatum* were found in the soil samples from the four plots to which none was added. This suggests that unlike *B. pallens* there were few or no *D. reticulatum* in the plots before the start of the experiment. However, some plants in these plots did suffer what appeared to be slug damage. (Table 8).

The numbers of *D. reticulatum* and *B. pallens* extracted from the differently treated plots are shown in Figure 12 and the biomass of the two slugs is shown in Figure 13. Both slug numbers and biomass were transformed to square roots prior to analysis. Analysis of variance of these results shows that both numbers and biomass of *D. reticulatum* were significantly lower in nematode treated plots than in untreated plots. No *D. reticulatum* were extracted from the nematode treated plots suggesting that this slug had been almost eliminated from these plots. Numbers of *D. reticulatum* were significantly reduced by methiocarb, but biomass was not significantly affected. Numbers of *B. pallens* were greatest in the untreated plots, biomass was greatest in the methiocarb-treated plots, and both numbers and biomass were least in the nematode-treated plots. However, there were no significant differences between treatments for this species.

Significantly more slugs (*P < 0.05*) were found in soil samples taken from the corner of the plots than the samples taken from the middle, probably because the corner sites provided better resting places.
Table 8. Mean percent slug damage in untreated plots with and without slugs added in the first mini-plot trial.

<table>
<thead>
<tr>
<th>Seedling Batch</th>
<th>Days after start of experiment</th>
<th>Mean percentage damage</th>
<th>Slugs added</th>
<th>No slugs added</th>
</tr>
</thead>
<tbody>
<tr>
<td>First</td>
<td>4</td>
<td>40.7</td>
<td>2.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>60.7</td>
<td>4.7</td>
<td></td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>56.2</td>
<td>5.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>56.2</td>
<td>1.8</td>
<td></td>
</tr>
<tr>
<td>Second</td>
<td>17</td>
<td>74.3</td>
<td>13.8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>87.6</td>
<td>18.3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>85.2</td>
<td>18.6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>27</td>
<td>85.2</td>
<td>18.4</td>
<td></td>
</tr>
<tr>
<td>Third</td>
<td>31</td>
<td>75.7</td>
<td>19.7</td>
<td></td>
</tr>
<tr>
<td></td>
<td>35</td>
<td>93.3</td>
<td>34.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>38</td>
<td>95.1</td>
<td>39.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>41</td>
<td>95.9</td>
<td>39.0</td>
<td></td>
</tr>
</tbody>
</table>

One species of worm, *Dendrobaena rubida* var *tenuis* was found in high numbers in the soil samples from the plots. Numbers of this worm were not significantly affected by *Phasmarhabditis* sp or methiocarb (Table 9).

Table 9. Square root numbers of *Dendrobaena rubida* var *tenuis* extracted per plot.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Untreated</th>
<th>Methiocarb</th>
<th>Nematodes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Square root No. worms extracted/plot</td>
<td>3.61</td>
<td>3.48</td>
<td>3.48</td>
</tr>
</tbody>
</table>

S.E.D. for comparing untreated with methiocarb or nematodes = 0.509 (22 D.F.)

S.E.D. for comparing methiocarb with nematodes = 0.588 (22 D.F.)
Figure 12. Numbers of *D. reticulatum* and *B. pallens* extracted per plot from the differently treated plots in the first mini-plot trial. (L.S.D. 1 for *D. reticulatum*, L.S.D. 2 for *B. pallens*, both for comparing untreated vs. methiocarb or nematodes, $P = 0.05$, 22 d.f.)

Figure 13. Biomass of *D. reticulatum* and *B. pallens* extracted per plot from the differently treated plots in the first mini-plot trial. (L.S.D. 1 for *D. reticulatum*, L.S.D. 2 for *B. pallens*, both for comparing untreated with methiocarb or nematodes, $P = 0.05$, 22 d.f.)
A large number of other soil invertebrates were removed from the soil samples but no single species was represented in sufficient numbers to permit statistical analysis. However, there were many dipterous larvae, in particular tipulid larvae, and many Turbellaria. Numbers of individuals in each of these three groups were transformed to square roots and subjected to analysis of variance. Results are shown in Table 10 (All dipterous larvae), Table 11 (tipulid larvae) and Table 13 (Turbellaria). There were no significant differences in the numbers of any of these groups between methiocarb-treated, nematode-treated and untreated plots.

Table 10. Mean square root numbers of all dipterous larvae extracted per plot.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Untreated</th>
<th>Methiocarb</th>
<th>Nematodes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Square root No. dipterous larvae extracted/plot</td>
<td>1.41</td>
<td>1.40</td>
<td>1.29</td>
</tr>
</tbody>
</table>

S.E.D. for comparing untreated with methiocarb or nematodes = 0.4 (22 D.F.)

S.E.D. for comparing methiocarb with nematodes = 0.46 (22 D.F.)

Table 11. Mean square root numbers of tipulid larvae extracted per plot.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Untreated</th>
<th>Methiocarb</th>
<th>Nematodes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Square root No. tipulid larvae extracted/plot</td>
<td>1.22</td>
<td>0.63</td>
<td>0.70</td>
</tr>
</tbody>
</table>

S.E.D. for comparing untreated with methiocarb or nematodes = 0.44 (22 D.F.)

S.E.D. for comparing methiocarb with nematodes = 0.51 (29 D.F.)
Table 12. Mean square root numbers of Turbellaria extracted per plot.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Untreated</th>
<th>Methiocarb</th>
<th>Nematodes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Square root No.</td>
<td>0.89</td>
<td>1.29</td>
<td>0.61</td>
</tr>
<tr>
<td>Turbellaria extracted/plot</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

S.E.D. for comparing untreated with methiocarb or nematodes = 0.284 (22 D.F.)

S.E.D. for comparing methiocarb with nematodes = 0.328 (22 D.F.)

5.3.2. Second Mini-Plot Trial.

5.3.2.1. Damage to Chinese cabbage seedlings.

The percentage damage by slugs to Chinese cabbage seedlings in the differently treated plots throughout the experiment is shown in Table 13. Percentages were transformed to angles for analysis of variance which revealed that time and treatment had significant (P<0.001 and P<0.01 respectively) effects on the amounts of slug damage and that there was a significant interaction between these two factors (P<0.001).

The damage to Chinese cabbage varied greatly throughout the experiment, with most damage occurring during the first three weeks of the experiment. These differences in slug damage on untreated plots could have been caused by changes in slug populations, and climatic conditions including air and soil temperatures, wind-speed, humidity and soil moisture, all of which are known to affect slug activity (Young & Port 1989).

In order to eliminate the effects of these variations, it was assumed that slug activity in the treated plots would be affected by climatic variables to the same extent as in untreated plots. Thus effects of different treatments on slug feeding was measured by calculating the percent reduction in damage in the treated plots compared to the untreated using Abbot's (1925) formula. These figures for percent reduction in damage were then related to nematode dose by means of regression analysis.
Table 13. Slug damage (%) to Chinese cabbage seedlings over time in second mini-plot trial.

<table>
<thead>
<tr>
<th>Days post treatment</th>
<th>Un-treated</th>
<th>Nematode Dose (Nematodes/hectare).</th>
<th>Methiocarb pellets 5.5 kg/ha</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1x10^8</td>
<td>3x10^8</td>
</tr>
<tr>
<td>4</td>
<td>91</td>
<td>92</td>
<td>89</td>
</tr>
<tr>
<td>7</td>
<td>96</td>
<td>94</td>
<td>92</td>
</tr>
<tr>
<td>11</td>
<td>99</td>
<td>94</td>
<td>94</td>
</tr>
<tr>
<td>14</td>
<td>99</td>
<td>95</td>
<td>94</td>
</tr>
<tr>
<td>18</td>
<td>66</td>
<td>37</td>
<td>26</td>
</tr>
<tr>
<td>21</td>
<td>83</td>
<td>58</td>
<td>37</td>
</tr>
<tr>
<td>25</td>
<td>29</td>
<td>29</td>
<td>21</td>
</tr>
<tr>
<td>28</td>
<td>51</td>
<td>40</td>
<td>27</td>
</tr>
<tr>
<td>33</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>39</td>
<td>11</td>
<td>9</td>
<td>13</td>
</tr>
<tr>
<td>42</td>
<td>26</td>
<td>21</td>
<td>24</td>
</tr>
<tr>
<td>46</td>
<td>13</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>49</td>
<td>33</td>
<td>29</td>
<td>30</td>
</tr>
<tr>
<td>55</td>
<td>68</td>
<td>79</td>
<td>74</td>
</tr>
<tr>
<td>70</td>
<td>58</td>
<td>57</td>
<td>34</td>
</tr>
<tr>
<td>74</td>
<td>24</td>
<td>20</td>
<td>13</td>
</tr>
<tr>
<td>77</td>
<td>51</td>
<td>44</td>
<td>34</td>
</tr>
<tr>
<td>81</td>
<td>64</td>
<td>60</td>
<td>48</td>
</tr>
<tr>
<td>84</td>
<td>72</td>
<td>72</td>
<td>56</td>
</tr>
<tr>
<td>88</td>
<td>24</td>
<td>29</td>
<td>14</td>
</tr>
<tr>
<td>91</td>
<td>39</td>
<td>42</td>
<td>25</td>
</tr>
<tr>
<td>95</td>
<td>57</td>
<td>56</td>
<td>42</td>
</tr>
<tr>
<td>98</td>
<td>67</td>
<td>65</td>
<td>53</td>
</tr>
<tr>
<td>103</td>
<td>13</td>
<td>13</td>
<td>12</td>
</tr>
</tbody>
</table>

Detailed analysis of these results concentrated on the first six weeks of the experiment since trends in damage reduction did not alter much after this period (Table 13) and because this period would be of most interest if the nematode was to be used as an...
inundative biological molluscicide. The readings taken on day 33, for which there was virtually no slug damage (Table 13) due to freezing temperatures, were not included. The experiment consisted of five replicate blocks of eight treatments and ideally analysis of variance would be done on the percent damage reduction figures from each block prior to regression lines being fitted to the mean percent damage reduction figures. Then the regression models could tested for lack of fit by calculating the F value for the ratio of the residual variance from the regression analysis (the lack of fit error) and the residual variance of the ANOVA done on the percent damage reduction figures (the pure error). However, there was much variation in slug damage in different plots of the same treatment and in some blocks where damage in the untreated plots was low some treatments had large negative % damage reduction figures (up to - 800 %) which were unsuitable for use in ANOVA. Thus regression lines were fitted to the mean damage reduction estimated over the experiment as a whole.

The percentage reduction in damage for all the nematode doses increased for the first three weeks and then levelled off (Figure 14). Thus non-linear regression analysis was used, and exponential models were fitted to the data. The relationship between slug damage over time for the different nematode doses was best described by the exponential function (Fig x);

\[ y = a - 46.86 \times 0.9188^d, \]

where y is the angular transformation of the % damage reduction, a is a constant which varies with dose (Table 14), and d is the number of days after treatment. This function describes damage reduction caused by the nematode rising as time progressed to an asymptote which differed for the six different nematode doses (Table 14).
Table 14. Values of the asymptote parameter (a) estimated for exponential models describing % damage reduction over six weeks for different nematode doses (see Fig 22).  

<table>
<thead>
<tr>
<th>Nematode dose</th>
<th>$1 \times 10^8$</th>
<th>$3 \times 10^8$</th>
<th>$8 \times 10^8$</th>
<th>$2 \times 10^9$</th>
<th>$7 \times 10^9$</th>
<th>$2 \times 10^{10}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Value of asymptote parameter a</td>
<td>34.82</td>
<td>40.60</td>
<td>44.57</td>
<td>50.42</td>
<td>48.42</td>
<td>54.00</td>
</tr>
</tbody>
</table>

This regression is highly significant ($P < 0.001$), and accounts for 49% of the variation in % damage reduction observed.

Figure 14. Relationship between % damage reduction to Chinese cabbage seedlings and time for the six nematode doses used in the second mini-plot trial. The % damage reduction over time caused by methiocarb pellets is also shown.
Figure 15. Relationship between mean % damage reduction to Chinese cabbage seedlings between days 18 and 42 and log. nematode dose in the second mini-plot trial.

It was not possible to describe the damage reduction figures more precisely than this, probably because there was much variation in the percent damage reduction recorded throughout the experiment. Nevertheless, since the effect of the nematode appeared to stabilised from 18 days onwards at all doses (Fig. 14), the mean percentage reduction in damage between days 18 and 42 for the different nematode doses was plotted against log nematode dose (Figure 15). This relationship was well described ($P < 0.05$) by an exponential curve which accounted for 78% of the variation in mean % damage reduction:
\[ y = 48.37 - 20400000 \times 0.133^n \]

where \( n = \log \) nematode dose/ha. This curve (Fig. 15) suggests that nematode efficacy tended to level off at or just above the third dose (8 x 10^8/ha).

It is difficult to directly compare the plant protection provided by methiocarb pellets with that given by the nematodes because the time at which the two agents gave maximum protection was different; methiocarb pellets gave most protection to plants at the first reading but this then fell over the six week study period, whereas protection given by the nematodes rose for the first three weeks and then stabilised until the end of the study period. Methiocarb gave high initial protection to Chinese cabbage seedlings with 62% damage reduction being observed at day 4 and protection given by methiocarb was significantly greater (\( P < 0.05 \)) than that given by all nematode doses until day 14. From day 18 onwards there were no significant differences in slug damage between methiocarb treated plots and any of the nematode treated plots, and this persisted until the end of the six week period of study.

5.3.2.2. Damage to wheat seeds.

The damage to wheat seeds recorded in the experiment is shown in Table 15. As stated in Section 5.2.2., because of mice eating the seeds satisfactory data is only available for days 4, 7, 14, 42 and 53. However, this period is similar to the period over which detailed analysis of damage to Chinese cabbage seedlings was done, and the data obtained from the Chinese cabbage seedlings suggests that the effect of nematode did not change much after this time. The percentage reduction in damage to wheat seeds was calculated in the same way as for the Chinese cabbage seedlings. There was insufficient data to fit curves describing the effect of different nematode doses over time as was done for Chinese cabbage seedlings.
Table 15. Slug damage (%) to wheat seeds over time in second mini-plot trial.

<table>
<thead>
<tr>
<th>Days post treatment</th>
<th>Un-treat</th>
<th>Nematode Dose (Nematodes/hectare)</th>
<th>Methiocarb pellets 5.5 kg/ha</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1x10^8</td>
<td>3x10^8</td>
</tr>
<tr>
<td>4</td>
<td>53</td>
<td>38</td>
<td>38</td>
</tr>
<tr>
<td>7</td>
<td>68</td>
<td>61</td>
<td>41</td>
</tr>
<tr>
<td>14</td>
<td>71</td>
<td>55</td>
<td>35</td>
</tr>
<tr>
<td>42</td>
<td>18</td>
<td>9</td>
<td>6</td>
</tr>
<tr>
<td>53</td>
<td>29</td>
<td>22</td>
<td>16</td>
</tr>
</tbody>
</table>

Negative exponential curves were fitted to the % damage reduction figures in relation to nematode dose for each of the five dates (Figure 16). The effect of different nematode doses on % damage reduction to wheat seeds was best described by the function:

\[ y = a - 2477667 \times 0.2459^n \]

where \( y \) is the % reduction in seed damage, \( a \) is a constant which is dependent on the number of days after application of nematodes, and \( n \) is the logarithm of the number of nematodes per hectare. This function describes parallel curves for each date rising steadily over the first three nematode doses (1 x 10^8, 3 x 10^8 and 8 x 10^9/ha) then approaching an asymptote (the same trend as seen in protection to Chinese cabbage seedlings).

The values of \( a \) at the five different sampling dates are shown in Table 16.

Table 16. Values of the asymptote parameter for the negative exponential models describing the effect of different nematode doses on % damage reduction to wheat seeds.

<table>
<thead>
<tr>
<th>No. days after treatment</th>
<th>4</th>
<th>8</th>
<th>15</th>
<th>43</th>
<th>54</th>
</tr>
</thead>
<tbody>
<tr>
<td>Value of asymptote parameter (a)</td>
<td>55.52</td>
<td>57.91</td>
<td>63.37</td>
<td>72.87</td>
<td>65.76</td>
</tr>
</tbody>
</table>

88
This regression is highly significant ($P < 0.001$) and accounts for 82 % of the variance in % damage reduction to wheat seeds. The asymptote parameters in Table 16 represent the theoretical maximum protection given by the nematodes on each day. These values suggest that the efficacy of the nematode increased between days 4 and 43 and then declined slightly by day 54. However, there was little difference in the efficacy of the nematodes between days 43 and 54 for all but the lowest two nematode doses ($1 \times 10^8$ and $3 \times 10^9$/ha).

Methiocarb had an immediate impact on slug damage causing a high % reduction in damage, which then declined over time. By 42 and 53 days after treatment the protection given by the all but the lowest two nematode doses ($1 \times 10^8$ and $3 \times 10^9$/ha) was similar to that given by methiocarb (Table 15).

![Graph](image)

Fig 16. Relationship between % damage reduction to wheat seeds and log. nematode dose recorded at days 4, 8, 15, 43 and 54 in the second mini-plot trials.
5.3.3. Meteorological Data.

The mean daily air temperature and daily rainfall throughout the first mini-plot experiment and for the first six weeks of the second mini-plot experiment are shown in Table 17.

Table 17. Mean air temperature and daily rainfall throughout the first mini-plot trial and for the first six weeks of the second mini-plot trial.

<table>
<thead>
<tr>
<th>Day</th>
<th>First mini-plot trial</th>
<th>Second mini-plot trial</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean air temperature(°C)</td>
<td>Mean rainfall (mm)</td>
</tr>
<tr>
<td>1</td>
<td>9.2</td>
<td>0.0</td>
</tr>
<tr>
<td>2</td>
<td>9.2</td>
<td>0.2</td>
</tr>
<tr>
<td>3</td>
<td>10.5</td>
<td>0.0</td>
</tr>
<tr>
<td>4</td>
<td>9.5</td>
<td>3.8</td>
</tr>
<tr>
<td>5</td>
<td>10.4</td>
<td>5.2</td>
</tr>
<tr>
<td>6</td>
<td>11.6</td>
<td>0.0</td>
</tr>
<tr>
<td>7</td>
<td>8.9</td>
<td>2.4</td>
</tr>
<tr>
<td>8</td>
<td>13.0</td>
<td>3.6</td>
</tr>
<tr>
<td>9</td>
<td>14.0</td>
<td>0.0</td>
</tr>
<tr>
<td>10</td>
<td>14.0</td>
<td>0.0</td>
</tr>
<tr>
<td>11</td>
<td>21.0</td>
<td>0.0</td>
</tr>
<tr>
<td>12</td>
<td>21.2</td>
<td>0.0</td>
</tr>
<tr>
<td>13</td>
<td>17.8</td>
<td>0.0</td>
</tr>
<tr>
<td>14</td>
<td>17.5</td>
<td>0.0</td>
</tr>
<tr>
<td>15</td>
<td>18.7</td>
<td>0.0</td>
</tr>
<tr>
<td>16</td>
<td>19</td>
<td>0.0</td>
</tr>
<tr>
<td>17</td>
<td>19</td>
<td>0.0</td>
</tr>
<tr>
<td>18</td>
<td>19.8</td>
<td>0.0</td>
</tr>
<tr>
<td>19</td>
<td>21.3</td>
<td>0.0</td>
</tr>
<tr>
<td>20</td>
<td>16.7</td>
<td>0.0</td>
</tr>
<tr>
<td>21</td>
<td>17.7</td>
<td>0.0</td>
</tr>
<tr>
<td>Day</td>
<td>First Mini-plot trial</td>
<td>Second mini-plot trial</td>
</tr>
<tr>
<td>-----</td>
<td>-----------------------</td>
<td>------------------------</td>
</tr>
<tr>
<td></td>
<td>Mean air temperature (°C)</td>
<td>Mean rainfall (mm)</td>
</tr>
<tr>
<td>22</td>
<td>16.4</td>
<td>0.0</td>
</tr>
<tr>
<td>23</td>
<td>14.3</td>
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<td>24</td>
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<td>25</td>
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<td>19.8</td>
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<td>27</td>
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<td>7.4</td>
</tr>
<tr>
<td>28</td>
<td>14.9</td>
<td>0.0</td>
</tr>
<tr>
<td>29</td>
<td>12.5</td>
<td>2.2</td>
</tr>
<tr>
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<td>16.2</td>
<td>1.8</td>
</tr>
<tr>
<td>31</td>
<td>15.6</td>
<td>0.0</td>
</tr>
<tr>
<td>32</td>
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<td>42</td>
<td>18.2</td>
<td>0.2</td>
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Table 17. (Continued) Mean air temperature and daily rainfall throughout the first mini-plot trial and for the first six weeks of the second mini-plot trial.
5.4. Discussion

It is clear from the results of both mini-plot experiments that the nematode is capable of protecting plants from slug damage under semi-field conditions. There was flea beetle damage on many of the Chinese cabbage seedlings and as stated in 5.3.1. in the few cases where it was not possible to distinguish this damage from slug damage, the damage was recorded as slug damage. Thus the results presented may underestimate the ability of the nematodes to protect Chinese cabbage seedlings. In the first experiment, it was encouraging that slug damage to Chinese cabbage seedlings was significantly less in nematode-treated than in untreated plots, even at the first examination four days after treatment, since slow action hinders the use of many biocontrol agents e.g. insect baculoviruses. The rapid activity of *Phasmorhabditis* sp. is probably due to feeding inhibition in slugs shortly after infection with *Phasmorhabditis* sp. rather than death of slugs (Section 3 and Section 9). The performance of the nematodes in comparison to methiocarb pellets is also encouraging. In the first experiment the plots were irrigated daily to provide suitable conditions for slug activity. This would also have protected the nematodes from desiccation. Dry soils are known to inhibit the activity of entomopathogenic nematodes (Sheltar, Suleman & Georgis 1988, Jackson, Todd & Wouts 1983). However, as slug activity is also restricted by dry conditions, the requirement of *Phasmorhabditis* sp. for soil moisture is less likely to restrict its use than is the case with entomopathogenic nematodes.

In this trial only one pest species of slug capable of damaging the bait plants was present (*D. reticulatum*) whereas in a field situation it is likely other species of slugs would be encountered (Glen 1989). Nevertheless, this nematode has been shown to have a wide host range (Section 3) so it seems likely that the nematode would provide similar protection to that seen in this trial in a field situation. The dose of nematodes used in the first mini-plot trial (1 x 10^6/plot = 2 x 10^10/ha) is very high and it is unlikely that such a dose could economically be applied to arable crops (Paul Rogers AGC pers. comm.). Poinar (1986) reviewed field trials using various entomopathogenic nematodes and none of the trials cited, in which the nematode dose is directly comparable with this trial (i.e. applied to the soil surface, not mixed in with potting compost or inoculated into trees) used a dose as high as in this trial. The highest dose in Poinar’s (1986) review was that
of that of Danilov (1974) in which a dose of $1.5 \times 10^6/m^2$ ($1.5 \times 10^{10}/ha$) was applied to
soil to control wireworms. In this trial 69-78% pest control was achieved.

Because the dose used in the first trial was so high the second experiment was done to
investigate the effect of lower nematode dose rates on slug damage but the data from the
second experiment was difficult to analyses. There was much variation in slug damage
in the untreated plots (C.V. = 85%). As stated in the results section, ideally regression
models would be fitted to the results of each of the five blocks separately which would
allow goodness of fit to be tested. However, the large amount of variation in slug damage
precluded the use of this method. The exponential models fitted to describe the
relationship between percentage reduction in slug damage and nematode dose can only
be regarded as giving the approximate form of the dose/response relationship.
Damage reduction improved over first three nematode doses and then approached an
asymptote. However the protection provided by the highest dose in the second trial, was
less than that given by the same dose in the first trial. (Approximately 50% damage
reduction after four weeks in the second trial compared with approximately 95% damage
reduction after four weeks in the first trial).

It is difficult to explain why the effect of the nematode in the second trial did not improve
beyond the 50% damage reduction level, when it had been demonstrated in the first trial
that the nematode was capable of giving much higher protection. However, variable
results have been characteristic of field trials using entomopathogenic nematodes.
Poinar (1986) in reviewing field trials suggested several reasons for differing degrees of
success in different field trials, including viability of the inoculum, inappropriate spraying
systems, temperature and moisture levels of the infection court and uneven distribution
of nematodes in the suspension applied to the infection court. Soil temperatures were not
directly measured in the mini-plots but air temperatures (probably closer to soil surface
temperatures in the mini-plot which were raised above the ground) were taken from the
nearby Long Ashton meteorology station. (Table 17). During the first mini-plot trial
(June-July) air temperatures were rising and were generally higher than in the second
experiment during the first six weeks of which air temperatures were falling. The
slightly higher temperatures in the first experiment might have led to greater nematode
activity, and hence better plant protection than in the second trial. Since the plots could
be irrigated by an overhead pipeline it is unlikely that nematode dehydration could have
reduced nematode activity in either trial. Also the method of nematode application was similar for both trials eliminating the possibility of inappropriate application methods resulting in the differences in plant protection between the two experiments.

One other possible explanation for the difference in plant protection provided by the nematodes in the two trials is that the nematodes might have been grown with different associated bacteria. The nematodes were grown in xenic foam-chip cultures (section 4.2.2) which were subcultured directly from bag to bag, and theoretically the bacterial mix in the bags should have been the same. However, no detailed comparisons of the bacterial mix in the cultures used for the first and second trial were made, and since the foam chip bag cultivation technique is susceptible to contamination (Friedman, 1990) it is possible that there were differences in the bacterial composition of the two nematode cultures. It has since been shown that monoxenic growth with different bacteria can have a significant effect on nematode pathogenicity (Section 9) and it is likely that different bacteria in a xenic mix could have the same effect.

While these factors might explain why the protection to plants given by the highest dose of nematodes was less in the second trial than in the first, they do not explain the lack of a dose response at doses above $8 \times 10^8$/ha. This phenomenon has been reported before in trials using entomopathogenic nematodes. Georgis and Gaugler (1991) in reviewing nematode field trials using a standard protocol found that *Heterorhabditis bacteriophora* under certain environmental conditions provided control of Japanese beetle larvae *Popillia japonica* as well as chemical control methods. However under these conditions they did not observe a dose response in trials using three nematode doses ($2.5 \times 10^9$, $7.5 \times 10^9$ and $1.25 \times 10^{10}$/ha). These doses fall within the range of the top four doses of the second mini-plot trial, the doses above which no increase in % damage reduction was observed (Fig 15). Georgis and Gaugler (1991) did not put forward any hypothesis to explain this puzzling lack of dose response.
6. ISOLATION, SELECTION AND IDENTIFICATION OF BACTERIA ASSOCIATED WITH PHASMORHABDITIS SP. FOR EXPERIMENTAL WORK

6.1. Introduction.

Phasmorhabditis sp., like all nematodes of the sub-order Rhabditina is a bacterial feeder. While certain rhabditid nematodes e.g. Caenorhabditis elegans can be grown in axenic culture, the generation time is usually much longer than in cultures with bacteria (Vanfleteren 1978). It is also known that growth rate and reproductive capacity of other nematode species can be greatly influenced by being grown in monoxenic culture with different bacteria (Sohlenius 1968; Andrew & Nicholas 1976; Poinar 1979; Grewal 1990). Furthermore, entomopathogenic nematodes of the genera Steinernema and Heterorhabditis carry pathogenic bacteria in their alimentary tract (section 1.5.6.) which are thought to be necessary for efficient host killing in these nematodes. Thus, it was felt important to investigate the relationship between Phasmorhabditis sp. and bacteria found associated with it, in order to optimise in vitro production and to understand the mechanism by which this nematode kills slugs. It should be noted that nematodes interact in many different ways with bacteria and different types of nematode/bacterium interactions have been reviewed by Poinar and Hansen (1986). In this thesis, however, the study of the relationship between Phasmorhabditis sp. and bacteria will concentrate on bacteria as a potential food source for the nematode and the possibility of the nematode acting as a vector for bacteria which are pathogenic to slugs.

In order to study the relationship between Phasmorhabditis sp. and different bacteria it was first necessary to isolate bacteria associated with the worms. Bacteria were isolated from within surface sterilised infective-juvenile nematodes, because if Phasmorhabditis sp. did vector a pathogenic bacterium it would be found in this nematode stage. Bacteria were isolated from within the swollen mantles of slugs infected with Phasmorhabditis sp. also with the aim of finding specific pathogenic bacteria. Bacteria were also isolated from dead slugs which were supporting growth of many thousands of nematodes and from thriving xenic foam chip cultures of nematodes. It was known in both these cases that
there were bacteria present which could promote prolific nematode growth, and it was hoped that individual species of bacteria capable of supporting such vigorous growth could be isolated and used in monoxenic in vitro cultures of Phasmorhabditis sp.


6.2.1. Isolation of Bacteria.

6.2.1.1. Isolation of bacteria from within nematodes.

In all investigations of the internal bacterial flora of nematodes, bacteria were isolated from within the infective dauerlarvae, because if Phasmorhabditis spp. did vector pathogenic bacteria they would be present in the infective stage. The infective larvae were surface sterilised by immersion in 0.1% (w/v) sodium ethylmercurithiosalicylate ('Thimerosal', Sigma Ltd) for 1 hour, then transferred to fresh Thimerosal for a further three hours. Bacteria were liberated from nematodes in one of two ways:

a.) Individual nematode larvae were transferred to a drop of sterile saline on a flame-sterilised microscope slide. The nematodes were then cut at several sites along the length of their bodies. The drop of saline complete with the dead nematode was then transferred using a sterile pasteur pipette to a 9 cm petri dish of nutrient agar where it was spread over the surface using an alcohol-flamed glass spreader.

b.) Many surface sterilised nematodes were suspended in 1 ml of sterile Ringer's solution which was transferred to a 5 ml teflon tissue homogeniser. The nematode suspension was ground for approximately 1 minute and then transferred to 9 ml of sterile nutrient broth. The broth was shaken vigorously and then 1 ml was transferred to another 9 ml of nutrient broth, making a ten fold dilution. A further four such ten-fold dilutions were made and then 0.1 ml aliquots of each dilution were placed onto each of three 9 cm plates of nutrient agar and spread using a glass spreader.

In some early attempts to isolate bacteria from within nematodes, agar plates had another layer of agar poured on top, immediately after the initial bacterial suspension had been spread over the surface. It was thought that this method might isolate micro-aerophilic
bacteria incapable of growth on ordinary spread plates. No such bacteria were isolated and this method was not used in later attempts.

6.2.1.2. Isolation of bacteria from xenic foam chip cultures

Foam chips containing nematodes and bacteria were taken from thriving xenic cultures using alcohol flamed forceps. Each chip was put into a tube containing 10 ml sterile nutrient broth and agitated. Serial dilutions of the resulting bacterial/nematode suspension were made and 0.1 ml aliquots of different dilutions were spread on nutrient agar plates and incubated. Before plating out, the dilutions were agitated and allowed to settle for approximately thirty seconds to allow nematodes to settle below the point at which the sample was taken, to stop live nematodes being transferred to the nutrient agar plates. 0.1 ml aliquots were then taken from the very top of the tube.

6.2.1.3. Isolation of Bacteria from Living Slugs Infected with Nematodes

*Phasmarhabditis sp.* infects and multiplies in the mantle region of slugs (see 3.3.1.) and bacteria were isolated from within this region. The mantle was first swabbed with dry cotton wool buds to remove as much slime as possible. The surface of the mantle was then swabbed with 70% (v/v) ethanol in an attempt to surface sterilise the slug, then a flame-sterilised mounted needle was used to pierce the mantle. Drops of fluid on the end of the needle were transferred directly to nutrient agar plates where they were spread using a glass spreader and incubated.

6.2.1.4. Isolation of bacteria from dead slugs.

Smears of tissue from dead slugs which had died following nematode infection and were covered in nematodes were suspended in nutrient broth using a bacteriological loop. Serial dilutions were made from this suspension and 0.1 ml aliquots spread onto nutrient agar plates and incubated.
6.2.2. Selection of individual species of bacteria following isolation.

Once agar plates had been inoculated with bacteria using any of the isolation methods described above they were incubated for 48 hours at 25°C after which time different bacterial isolates, as distinguished by colonial morphology, were selected and subcultured.

6.2.3. Identification of bacteria.

Three Analytical Profile Index (API, La Balme les Grottes 38390, Montalieu Vereieu, France) in-vitro diagnostic kits were used as aids to identifying bacteria. The API 20 B kit is a general kit of more use for differentiating isolates rather than species identification. This kit was used to test all isolates (Tables 20 & 21). The biochemical characteristics of Gram negative, non fermentative bacteria were further examined with the API 20 NE kit (Table 22) and those of Gram negative fermenting bacteria were investigated using the API 20 E kit (Table 23). Many additional tests were done on at least some of the bacteria to be identified (Tables 18 and 19). All tests used methods described by Cruickshank et al. (1975) and all tests were read after 48 hours incubation at 25°C unless otherwise stated. Additional tests were as follows:

Gram staining was done using Kopeloff and Beerman's (1922) modification in which the bacteria are decoloured with acetone. Bacterial cells retaining a deep violet colour were recorded as Gram positive, pink cell were recorded as Gram negative.

Motility was tested in a hanging drop preparation in sterile Ringer's solution.

Catalase production was tested by adding a drop of 1.5% (w/v) hydrogen peroxide to 24 hour colonies on nutrient agar. Effervescence was recorded as positive.

The ability to oxidise the redox dye tetramethyl-p-phenylene-diamine (the Oxidase test) was tested by transferring smears of colonies from overnight nutrient agar cultures using glass rods to filter paper soaked in fresh 1% (w/v) tetraethyl-p-phenylene dihydrochloride. An intense blue colour was recorded as positive.
Hugh and Leifson's (1951) twin tube test was used to distinguish between aerobic and anaerobic breakdown of a glucose. In this method two tubes containing glucose solution and pH indicator are inoculated with the test organism. One tube is left open and the other is covered with a layer of mineral oil to exclude atmospheric oxygen. A change from green to yellow of the pH indicator in the tube covered with oil was recorded as a positive fermentation reaction.

Metabolism of carbohydrates not included in the API kits, and gas production during fermentation of carbohydrates, were tested in universal bottles containing a Durham tube. Carbohydrates were filter-sterilised separately and added to the tubes at a final concentration of 1% (w/v). A change from purple to yellow of the Ph indicator was recorded as a positive metabolism reaction, and a positive reaction for aerogenic carbohydrate metabolism was recorded if a bubble of gas collected in the Durham tube.

Antibiotic sensitivity was tested using a disk inhibition method. Penicillin and Streptomycin disks were prepared by soaking Whatman antibiotic disks in an aqueous solution of antibiotic at 100 times the concentration per ml required in the disk. Commercially prepared novobiocin disks were used (Oxoid). Disks of the vibriostatic agent 2,4,-diamino-6-7 diisopropyl pteridine (O/129) were prepared by soaking Whatman antibiotic sensitivity disks in 0.1% (w/v) solution of O/129 in acetone. Disks were added to plates of Oxoid D.S.T. agar, lawned with one 'loopfull' of overnight broth culture of test bacterium. The plates were examined for zones of inhibition after 24 hours.

The ability of bacteria to produce fluorescent pigments was tested by viewing cultures under ultraviolet light (wavelength = 260 nm). Cultures were grown on medium A (King, Ward & Raney 1954) incubated at 30°C for one day then at room temperature for three days.

The ability of bacteria to produce lecithinases (phospholipases) was tested with egg yolk suspension in liquid medium (Hayward 1941).

The ability to grow at 41°C was tested by inoculating nutrient broth tubes with the test bacterium and incubating the tubes in a water bath for five days. Growth at 4°C was
tested by incubating nutrient broth cultures in an incubator at this temperature. Good growth was recorded if the medium was visibly turbid after 48 hours.

The ability to grow at different concentrations of NaCl was tested by preparing nutrient broth tubes by adding NaCl to obtain the desired final concentration (NB, standard nutrient broth contains 0.5% NaCl). Tubes were inoculated and incubated. Turbidity after 48 hours was recorded as a positive reaction.

6.3. Results.

6.3.1. Isolation of Bacteria.

A collection of over 150 bacterial isolates was taken from within nematodes, from slugs infected with nematodes, from slugs which had died following nematode infection, and from thriving xenic foam chip cultures using the methods described in section 6.2.1. In examining the bacterial flora taken from within surface-sterilised infective juveniles it was found that there was no one single species consistently carried in the gut, and xenic cultures, infected slugs and dead slugs supporting nematodes all had several different species of bacteria present, with no single species being consistently isolated. In the absence of any one obvious choice of bacterium to work with, it was decided to select a few isolates from the collection, since it was not possible to screen all these isolates for the ability to support nematode growth. At least one bacterium taken from each of the four isolation sites was screened. Thorough examination of the bacteria present in one xenic foam chip nematode culture revealed only three different bacterial isolates. All three of these were screened. Five other bacterial isolates were selected from the collection which had colonial morphologies distinct from one another and from the bacteria already selected, in order to reduce the possibility of screening the same species twice. The isolation source, code number and identification of these bacteria are shown in Table 24. in the summary at the end of this section.
6.3.2. Identification of bacteria

The results of all tests are shown in the following tables. Tables 18 and 19 give general characteristics (e.g. gram stain, shape etc.) and tests done in addition to the API tests. Tables 20 and 21 show the results for the API 20 B kits which were used on all bacteria. Table 22 shows the results for the non-fermenting Gram negative rods tested in the API 20NE kit, and Table 23 show the results for the two fermenting Gram negative rods tested using the API 20E kit.
Table 18. General characteristics and additional tests done on bacteria associated with *Phasmorhabditis* sp.

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<td>-</td>
<td>-</td>
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</tr>
<tr>
<td>Shape</td>
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<td>rod</td>
<td>rod</td>
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<td>*</td>
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<td>*</td>
<td>++</td>
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<td>*</td>
<td>-</td>
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<td>*</td>
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<td>Diffusible fluorescent pigment produced</td>
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<td>Pathogenic when injected into slugs</td>
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*Sensitivity tests +++ = inhibition zone > 3cm
+++ = inhibition zone 1-3cm
+ = inhibition zone < 1cm
w = weak positive reaction * = not tested

102
Table 19. General characteristics and additional tests done on bacteria associated with *Phasmorhabditis sp.*

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<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Good Growth at 4°C</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>*</td>
<td>*</td>
<td></td>
</tr>
<tr>
<td>Growth at 42°C</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Growth in 4% NaCl</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>*</td>
<td>*</td>
<td></td>
</tr>
<tr>
<td>Growth in 7% NaCl</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>*</td>
<td>*</td>
<td></td>
</tr>
<tr>
<td>Growth in 8.5% NaCl</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>*</td>
<td>*</td>
<td></td>
</tr>
<tr>
<td>Growth in 10% NaCl</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>*</td>
<td>*</td>
<td></td>
</tr>
<tr>
<td>Growth at Ph 4.5</td>
<td>-</td>
<td>w</td>
<td>+</td>
<td>w</td>
<td>+</td>
<td>*</td>
<td>*</td>
<td></td>
</tr>
<tr>
<td>Gas from Glucose (Durham tube)</td>
<td>*</td>
<td>+</td>
<td>-</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td></td>
</tr>
<tr>
<td>Acid from Adonitol</td>
<td>*</td>
<td>*</td>
<td>-</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td></td>
</tr>
<tr>
<td>Acid from Trehalose</td>
<td>*</td>
<td>*</td>
<td>+</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td></td>
</tr>
<tr>
<td>Acid from Lactose</td>
<td>*</td>
<td>*</td>
<td>-</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td></td>
</tr>
<tr>
<td>Sensitivity to*</td>
<td>Penicillin G. 100 units</td>
<td>+++</td>
<td>++</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td></td>
<td>Streptomycin sulphate 100 units</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+++</td>
<td>+++</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td></td>
<td>0/129 (Vibriostat)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>±</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Novobiocin 5 µg</td>
<td>+</td>
<td>++</td>
<td>+</td>
<td>++</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Diffusible fluorescent pigment produced</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>*</td>
<td>*</td>
<td></td>
</tr>
<tr>
<td>Pathogenic when injected into slugs</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td></td>
</tr>
</tbody>
</table>

'Sensitivity tests +++ = inhibition zone > 3cm
++ = inhibition zone 1-3cm
+ = inhibition zone < 1cm
w = weak positive reaction.  * = not tested
Table 20. Results of API 20 B rapid diagnostic tests on bacteria associated with nematodes which were selected for use in nematode growth experiments. Strips were incubated at 25°C and were read after 48 hours.

<table>
<thead>
<tr>
<th>BACTERIUM</th>
<th>code number</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Gelatin liquefaction</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Nitrate reduction</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>$B$ Galactosidase</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Acid from</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saccharose</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Arabinose</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Mannitol</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Fructose</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Glucose</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Maltose</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Starch</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Rhamnose</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Galactose</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Mannose</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Sorbitol</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Glycerol</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Urease</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Indole</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Hydrogen Sulphide</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Voges Proskauer</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Simmons Citrate</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>
Table 21. Results of API 20 B rapid diagnostic tests on bacteria associated with nematodes which were selected for use in nematode growth experiments. Strips were incubated at 25°C and were read after 48 hours.

<table>
<thead>
<tr>
<th>BACTERIUM</th>
<th>code number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gelatin liquefaction</td>
<td>+</td>
</tr>
<tr>
<td>Nitrate reduction</td>
<td>-</td>
</tr>
<tr>
<td>B Galactosidase</td>
<td>+</td>
</tr>
<tr>
<td>Acid from Saccharose</td>
<td>+</td>
</tr>
<tr>
<td>Arabinose</td>
<td>+</td>
</tr>
<tr>
<td>Mannitol</td>
<td>+</td>
</tr>
<tr>
<td>Fructose</td>
<td>+</td>
</tr>
<tr>
<td>Glucose</td>
<td>+</td>
</tr>
<tr>
<td>Maltose</td>
<td>+</td>
</tr>
<tr>
<td>Starch</td>
<td>-</td>
</tr>
<tr>
<td>Rhamnose</td>
<td>-</td>
</tr>
<tr>
<td>Galactose</td>
<td>+</td>
</tr>
<tr>
<td>Mannose</td>
<td>+</td>
</tr>
<tr>
<td>Sorbitol</td>
<td>+</td>
</tr>
<tr>
<td>Glycerol</td>
<td>+</td>
</tr>
<tr>
<td>Urease</td>
<td>-</td>
</tr>
<tr>
<td>Indole</td>
<td>-</td>
</tr>
<tr>
<td>Hydrogen Sulphide</td>
<td>-</td>
</tr>
<tr>
<td>Voges Proskauer</td>
<td>-</td>
</tr>
<tr>
<td>Simmons Citrate</td>
<td>+</td>
</tr>
</tbody>
</table>

w = weak positive reaction
Table 22. Results of API 20 NE rapid diagnostic kits on Gram-negative, non-fermenting bacteria selected for nematode growth experiments. Strips were incubated at 25°C and were read after 48 hours.

<table>
<thead>
<tr>
<th>BACTERIUM</th>
<th>1</th>
<th>17</th>
<th>48</th>
<th>54</th>
<th>78</th>
<th>79</th>
<th>140</th>
<th>141</th>
</tr>
</thead>
<tbody>
<tr>
<td>NO₃ - NO₂</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>NO₃ - N₂</td>
<td>*</td>
<td>-</td>
<td>*</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Indole</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Acid from glucose</td>
<td>-</td>
<td>w</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Arginine dihydrolase</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Urease</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Escculin hydrolysis</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Gelatin hydrolysis</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>B Galactosidase</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Assimilation of</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucose</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Arabinose</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Mannose</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Mannitol</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>N. A. G.</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Maltose</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
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</tr>
<tr>
<td>Gluconate</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
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<td>+</td>
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</tr>
<tr>
<td>Caprate</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Adipate</td>
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<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Malate</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Citrate</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Phenyl acetate</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

* = Not tested.
Table 23. Results of tests using API 20 E rapid diagnostic tests for Gram negative facultatively anaerobic rod shaped bacteria. Strips were incubated at 25°C and read after 48 hours.

<table>
<thead>
<tr>
<th>BACTERIUM</th>
<th>code number</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>77</td>
</tr>
<tr>
<td>$B$ Galactosidase</td>
<td>-</td>
</tr>
<tr>
<td>Arginine dihydrolase</td>
<td>-</td>
</tr>
<tr>
<td>Lysine decarboxylase</td>
<td>-</td>
</tr>
<tr>
<td>Ornithine decarboxylase</td>
<td>-</td>
</tr>
<tr>
<td>Citrate utilisation</td>
<td>+</td>
</tr>
<tr>
<td>Hydrogen sulphide production</td>
<td>-</td>
</tr>
<tr>
<td>Urease</td>
<td>+</td>
</tr>
<tr>
<td>Tryptophane deaminase</td>
<td>+</td>
</tr>
<tr>
<td>Indole production</td>
<td>+</td>
</tr>
<tr>
<td>Voges Proskauer</td>
<td>-</td>
</tr>
<tr>
<td>Gelatin liquefaction</td>
<td>-</td>
</tr>
<tr>
<td>Acid from Glucose</td>
<td>+</td>
</tr>
<tr>
<td>Mannitol</td>
<td>+</td>
</tr>
<tr>
<td>Inositol</td>
<td>+</td>
</tr>
<tr>
<td>Sorbitol</td>
<td>-</td>
</tr>
<tr>
<td>Rhamnose</td>
<td>-</td>
</tr>
<tr>
<td>Sucrose</td>
<td>-</td>
</tr>
<tr>
<td>Melibiose</td>
<td>-</td>
</tr>
<tr>
<td>Amygdalin</td>
<td>-</td>
</tr>
<tr>
<td>Arabinose</td>
<td>-</td>
</tr>
</tbody>
</table>

* = Not tested.
6.3.3. Preliminary Identifications.

Bacterium 1. This is a Gram negative, non-fermentative bacterium capable of assimilating a wide range of carbohydrates and is a member of the family Pseudomonadaceae. The API 20NE profile of this bacterium was inconclusive and suggested this bacterium was either *Pseudomonas aeruginosa* or *P. fluorescens*. The inability of this bacterium to grow at 42°C suggests that this bacterium is *P. fluorescens*.

Bacterium 17. This bacterium is also a member of the family Pseudomonadaceae. The API 20NE profile for this bacterium fits *Pseudomonas paucimobilis* best with *Xanthomonas maltophilia* as next best choice. No members of the genus *Xanthomonas* are capable of growth at 5°C or below, thus the positive growth reaction at 4°C suggest that this bacterium is *P. paucimobilis*.

Bacterium 34. This large spore-forming Gram-positive rod is a member of the family Bacillaceae and being aerobic must be a species of *Bacillus*. No API profile was done on this bacterium, but the appearance of the spores (no lateral swelling of the sporangium) places this strain in *Bacillus* morphological subgroup 1. The positive lecithinase test suggest it is either, *B. anthracis*, *B. cereus* or *B. thuringiensis*. Microscopic examination revealed that there was no crystaline parasporal body as would be found in *B. thuringiensis*, and the isolation source and the positive motility reaction makes *B. anthracis* an unlikely identification. Thus it would seem that this bacterium is *B. cereus*.

Bacterium 48. This non-fermentative gram negative cocci/rod proved difficult to identify. Its API 20NE profile was not typical of any bacterium. A culture was sent to the National Collection of Industrial and Marine Bacteria (NCIMB) Aberdeen, who identified it as an atypical isolate of *Moraxella phenylpyruvica* from the family Neisseriaceae. The negative urease reaction of bacterium 48 is atypical of *M. phenylpyruvica* but the ability to produce urease can be lost after frequent laboratory sub-culture.

Bacterium 54. This bacterium is a member of the family Pseudomodaceae. The API 20NE profile suggested that this bacterium could be *Sphingobacterium spiritovorum*, *Pseudomonas vesicularis* or *Pseudomonas paucimobilis*, although bacterium 54 is atypical of all these bacteria in at least one test. The bacterium formed yellow colonies of nutrient
agar which is atypical of *P. vesicularis*, but typical of the other two bacteria. A positive growth reaction in 4% NaCl suggests that this bacterium is probably a strain of *Sphingobacterium spiritovorum*.

Bacterium 77 is a Gram negative, fermenting, oxidase negative rod shaped bacterium and is therefore a member of the family Enterobacteriaceae. Its API 20E profile is an excellent fit for *Providencia rettgeri*.

Bacteria 78 and 79 are both species of *Flavobacterium* (Gram negative, facultatively anaerobic rods, a genus of uncertain familial affiliation). The API 20 NE of profiles were good fits of *F. breve* (78) and *F. odoratum* (79).

Bacterium 83 is also a member of the Enterobacteriaceae. However, its API 20 E profile is inconclusive. It is similar to *Serratia marcesans* and *Serratia proteamaculans* although the positive arginine dihydrolase test would be atypical of both. The inability to grow at 41°C makes it most likely to be *S. proteamaculans*.

Bacterium 140 is a member of the Pseudomonadaceae. Its API 20 NE profile is an excellent fit of *Pseudomonas fluorescens*.

Bacterium 141 is also a member of the Pseudomonadaceae family. Its API 20 NE profile is identical to that of Bacterium 140, and therefore it is also *P. fluorescens*. However, it is worth noting that these two bacteria are distinguishable from one another in tests done using the API 20B Kit.

Bacteria 155 and 156 are Gram negative, fermenting, oxidase positive rods and are therefore members of the family Vibrionaceae. Bacterium 156 was identified by NCIMB as *Aeromonas hydrophila* with no atypical reactions. Bacterium 155 proved difficult to identify and was also sent to NCIMB. Its API 20 NE profile fits that of *Aeromonas hydrophila*. However, certain characteristics of bacterium 155 are atypical of this species including production of a brown diffusible pigment on nutrient agar, lack of motility, and its negative indole reaction. These three characteristics are all typical of *A. salmonicida*. Since *A. salmonicida* has previously only been isolated from fish, this
identification seems unlikely. Thus in this thesis Bacterium 156 will be called Aeromonas sp..

The isolation source, code number and identification of the bacteria to be used in the experiments described in sections 4, 5 and 6 are shown in Table 24.

**Table 24.** Isolation source, code number and identification of bacteria used in experiments investigating the relationship between bacteria and *Phasmorhhabditis* sp.

<table>
<thead>
<tr>
<th>Code number</th>
<th>Isolation Source</th>
<th>Identification</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Surface sterilised infective juvenile</td>
<td><em>Pseudomonas fluorescens</em></td>
</tr>
<tr>
<td>17</td>
<td>Surface sterilised infective juvenile</td>
<td><em>Pseudomonas paucimobilis</em></td>
</tr>
<tr>
<td>34</td>
<td>Surface sterilised infective juvenile</td>
<td><em>Bacillus cereus</em></td>
</tr>
<tr>
<td>48</td>
<td>Xenic foam chip culture</td>
<td><em>Moraxella phenylpyruvica</em></td>
</tr>
<tr>
<td>54</td>
<td>Xenic foam chip culture</td>
<td><em>Sphingobacterium spiritovorum</em></td>
</tr>
<tr>
<td>77</td>
<td>Xenic foam chip culture</td>
<td><em>Providencia rettgeri</em></td>
</tr>
<tr>
<td>78</td>
<td>Xenic foam chip culture</td>
<td><em>Flavobacterium breve</em></td>
</tr>
<tr>
<td>79</td>
<td>Xenic foam chip culture</td>
<td><em>Flavobacterium odoratum</em></td>
</tr>
<tr>
<td>83</td>
<td>Xenic foam chip culture</td>
<td><em>Serratia proteamaculans</em></td>
</tr>
<tr>
<td>140</td>
<td>Slug which had died following nematode infection</td>
<td><em>Pseudomonas fluorescens</em></td>
</tr>
<tr>
<td>141</td>
<td>Mantle of a field-collected nematode-infected slug</td>
<td><em>Pseudomonas fluorescens</em></td>
</tr>
<tr>
<td>155</td>
<td>Slug which had died following nematode infection</td>
<td><em>Aeromonas sp.</em></td>
</tr>
<tr>
<td>156</td>
<td>Surface sterilised infective juvenile</td>
<td><em>Aeromonas hydrophila</em></td>
</tr>
</tbody>
</table>
7. GROWTH WITH KNOWN BACTERIA

7.1. Introduction.

It has been shown for certain free living rhabditid nematodes (Sohlenius 1968; Andrew & Nicholas 1976; Grewal 1990) and insect parasitic rhabditid nematodes (Poinar & Thomas 1966, Poinar 1979), that different species of bacteria do not support nematode growth and reproduction equally well. Differences in the efficacy of any one bacterium as a food source for a nematode are attributable to its attraction, ingestion and digestion, and the presence or absence of toxic metabolic products (Poinar & Hansen 1986). While growing the nematodes in xenic culture was a satisfactory method for producing large numbers of nematodes for laboratory studies, the bag cultures gave inconsistent yields, possibly as a result of changes in the proportions of different species of bacteria taking place on subculturing. Growing a nematode with a single bacterial species in monoxenic culture forms a system designed to provide the nematode with a bacterial food under somewhat more controlled conditions. Such a system would be more likely to achieve the consistent and predictable yields required in a commercial production system. Thus, it was felt necessary to investigate growth of Phasmorhabditis sp. in monoxenic cultures with different bacteria to determine the suitability of different bacteria as a food source for Phasmorhabditis sp. with the aim of selecting a suitable bacterium/nematode combination to form the basis of a commercial production system.

It was also felt important to raise Phasmorhabditis sp. in monoxenic culture with different species of bacteria to investigate the importance of nematode-associated bacteria in causing disease in slugs. It was hoped to select several bacteria which could support monoxenic nematode growth so that different nematode/bacterium combinations could be bioassayed against slugs.

Having isolated many species of bacteria associated with nematodes (Section 6), a method of axenising nematodes was developed and attempts were made to grow the nematodes in monoxenic and gnotobiotic cultures on nutrient agar. After poor results were obtained on this medium a second experiment was done to compare monoxenic and gnotobiotic growth on nutrient agar with that on kidney agar. A new method of applying the nematodes to the agar plates was tested in which only half the plate was lawned with bacteria and it was found that this method not only promoted better nematode growth than when the bacteria were grown over the entire surface of the plate, it also showed which
plates contained bacterial contamination. Once this satisfactory method of screening the ability of bacteria to support monoxenic nematode growth had been developed, two experiment were done to screen different bacteria.

7.2. Materials and Methods.

7.2.1. Axenisation of nematodes.

To achieve axenic, monoxenic and gnotobiotics nematode cultures it was first necessary to obtain nematodes free from all contaminating bacteria. The reproductive tract of female nematodes is generally sterile (Poinar & Hansen, 1976) and thus L1 juveniles immediately after hatching are sterile. Dougherty (1960) recommended a method in which gravid female nematodes are killed in H₂O₂ and suspended on wire mesh in antibiotic solution where the eggs hatch within the adults and the juveniles escape into the solution below. This method was used in an attempt to axenise Phasmorhabditis sp., but eggs hatched within the adult, juveniles were unable to escape and died within the adult. It was therefore necessary to devise a method in which the adults were kept alive while they "gave birth" to juveniles.

The method used, a modification of Grewal's (1990) method for axenising Caenorhabditis elegans, was as follows. Nematodes were grown in vivo in D. reticulatum kept individually in plastic universal tubes with the bottoms blacked out with insulating tape (see 2nd tube bioassay, Section 3), which had been inoculated with large numbers of nematodes. The slugs were left in the tubes for approximately one week after death until many hundreds of nematodes were growing on the dead slug. The dead slug was dipped in Ringer's solution to remove nematodes, then gravid adult nematodes were transferred individually, using a bristle from a test-tube brush mounted on a needle, to a sterile watch glass containing a bactericide, 0.02% (w/v) ethyl mercurithiosalicylate ("Thimerosal" Sigma), where they were left overnight at 10°C. During this time eggs hatched within the adults and the juveniles were released. The following day juveniles were transferred by pipette to centrifuge tubes filled with 10 ml of quarter strength Ringer's solution containing 500 units per ml of both streptomycin sulphate and penicillin G. The juveniles were kept in this solution for a further 24 hours at 10°C. They were then concentrated by gentle centrifugation (50 x G for 10 minutes), collected from the bottom of the tube,
re-suspended in fresh sterile quarter strength Ringer's solution and spun down again. The re-suspension and centrifugation was repeated once more to remove any traces of antibiotics. The larvae were then placed in sterile watch glasses where they could then be handled individually using micro-pipettes made by drawing out dropping pipettes in a bunsen burner flame to a width of approximately 0.1 mm.

7.2.2. Preparation of kidney agar plates.

An aqueous suspension of 3.5% homogenised pigs kidney, 2.5% yeast extract, 3% beef dripping and 2% Agar Technical No. 3 (Oxoid)(% w/v) was prepared and autoclaved at 121°C. This was poured into 3 cm petri-dishes while still liquid and allowed to solidify.

7.2.3. Monoxenic, Gnotobiotic, Axenic and Xenic Growth Experiments.

7.2.3.1. Experiment 1: monoxenic, gnotobiotic, axenic and xenic nematode growth on nutrient agar.

In this experiment, growth of *Phasmorhabditis* sp. on axenic plates of nutrient agar was compared with growth on three different bacterial isolates (*Providencia rettgeri, Flavobacterium breve* and *Flavobacterium odoratum*) both individually and in all possible gnotobiotic combinations. These three bacteria had been isolated from a thriving xenic foam chip culture. Growth of nematodes on nutrient agar plates inoculated with a xenic mix of bacteria taken from a thriving xenic foam culture was also tested in this experiment. The experiment consisted of nine treatments (Table 25), with ten replicate plates of each treatment. The bacterial cultures were initiated by inoculating nutrient broth with the bacterium or bacteria required taken from stock cultures, and the xenic mix of bacteria was obtained by adding foam chips from a xenic culture to nutrient broth. All nutrient broth cultures were incubated overnight at 25°C. The following day 0.025 ml of each bacterial culture was spread across the whole surface of each of ten 30 mm nutrient agar plates using a flame-sterilised glass spreader. Ten axenic L1 *Phasmorhabditis* sp. obtained by the method described in 2.1.3. were placed in the centre of each plate. Plates were incubated at 15°C for two weeks then nematodes were counted by direct microscopic observation. This was done by removing the petri dish lid and replacing it with a lid which had previously been marked with a counting grid.
7.2.3.2. Experiment 2: comparison of monoxenic, gnotobiotic, axenic and xenic growth on nutrient agar with growth on kidney agar.

This experiment was similar to experiment 1, but nematodes were grown on both nutrient agar and kidney agar. The bacterial treatments were: axenic, *Pr. rettgeri*, *F. breve*, *F. odoratum*, a mixture of all three bacteria and xenic plates. Twenty plates were used for each bacterial treatment; ten of nutrient agar, and ten of kidney agar. Plates were inoculated with bacteria and nematodes, incubated, and counted after two weeks as in experiment 1. The cultures were incubated for a further week and counted as appropriate by either direct microscopic examination or by flooding off the nematodes into a known volume of water and counting the nematodes in the resulting suspension.

7.2.3.3. Experiment 3: monoxenic and xenic growth on nutrient agar and kidney agar on plates half lawned with bacteria.

This experiment again compared nematode growth on kidney agar and nutrient agar but with a slight change in bacterial species and methodology. The bacterial treatments were xenic, *Pr. rettgeri*, and *F. odoratum* (as in previous experiments), and *Pseudomonas fluorescens* (isolate no. 141) isolated from a field-collected slug infected with *Phasmorhabditis* sp. No axenic plates, without added bacteria, were included in this experiment as nematodes did not grow on these plates in experiments 1 and 2. In this experiment one half of the surface of each 30 mm petri dish was streaked with one bacteriological loopful of overnight broth culture of the required bacterium (Plate 18). The 10 axenic juvenile nematodes were then added at the edge of the petri dish in the half without bacteria, so that nematodes had to move at least 15 mm across a bacteria-free surface before reaching the test bacterium. After one week the plates were examined for bacterial contamination and plates showing bacterial contamination in the half where the nematodes had been added were discarded. (see Plate 18). Plates were examined again after two weeks and the nematodes counted by direct microscopic observation of the plates. After three weeks a final estimate of nematode numbers was made by flooding the plates with a known volume of water and counting the nematodes in the resulting suspension.
Plate 18. Two 3 cm kidney plates with only half the plate lawned with bacteria as used in experiments 3, 4, and 5. The plate on the right shows where bacterial contamination has been brought onto the plate with the nematode inoculum.

7.2.3.4. Experiment 4. screening of bacteria for the ability to support monoxenic nematode growth.

This experiment had ten different bacterial treatments (Table 27). Three bacteria (Pseudomonas fluorescens (1A), Ps. paucimobilis (17), Bacillus cereus (34), were isolated from within surface sterilised infective juvenile nematodes taken from xenic cultures (Section 6) and four (Moraxella phenylpyruvica (48), Shigobacterium spiritovorum (54), Providencia rettgeri (77) and Serratia proteamaculans (83)) had been taken from thriving xenic cultures. One bacterium, Aeromonas sp.(155), had been isolated from nematodes taken from a field-collected slug which had died following nematode infection and one bacterium, Ps. fluorescens (141), had been isolated from a living nematode-infected slug. Xenorhabdus nematophilus, a symbiont of
entomopathogenic nematodes of the genus *Steinernema*, was also screened. This bacterium was supplied by Jeremy Pearce (AGC Littlehampton).

All cultures were grown on kidney agar, and the bacteria were streaked on only one half of the plate, since experiment 3 suggested that this method favoured growth. Plates were incubated at 15°C. After one week, plates showing bacterial contamination in the "clean" half were discarded. The plates were counted after two weeks by direct microscopic examination. After three weeks, plates were counted by flooding nematodes off the agar as described previously. On several plates nematodes had burrowed down into the agar and could not be removed by washing. In these cases the agar was melted, diluted and poured into a petri dish placed on a counting grid and the total number of nematodes in the agar counted. A count of the proportion of live nematodes was made from the corresponding liquid suspension so that the number of live nematodes in the agar could be estimated. The results were transformed to logarithms for statistical analysis.

7.2.3.5. Experiment 5: screening of bacteria for the ability to support monoxenic nematode growth.

The method was identical to that used in Experiment 4. Seven bacteria were used with six replicate plates per treatment. *Pr. rettgeri* (77), which had supported good growth in previous experiments, and *Ps. paucimobilis* (17), which had not supported growth in previous work, were both screened. *Flavobacterium breve* (78), which had been found in high numbers in xenic foam-chip bag cultures was also included. This bacterium had not supported monoxenic growth in previous experiments with bacteria lawned over the entire surface of the petri-dish (Experiment 2), but had not been screened with only part of the petri dish lawned. Two bacteria associated with slugs which had died following nematode infection were screened, *Aeromonas hydrophila* (156) and *Pseudomonas fluorescens* (140). Two bacteria which had supported good monoxenic growth of the free living nematode *Caenorhabditis elegans* were included: *Acinetobacter calcoaceticus* and *Pseudomonas fluorescens*, both of which were obtained from P.S. Grewal, HRI Littlehampton.
7.3. Results

7.3.1. Monoxenic, Gnotobiotic, Axenic and Xenic Growth Experiments.

7.3.1.1. Experiment 1: monoxenic, gnotobiotic, axenic and xenic nematode growth on nutrient agar.

The results for this experiment are shown in Table 25.

Table 25. Mean numbers of nematodes, both live and dead, after two weeks in monoxenic, gnotobiotic and xenic culture with different bacteria on nutrient agar. (Bac 77 = Pr. rettgeri, Bac 78 = Fl. breve, Bac 79 = Fl. odoratum.)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>axenic</th>
<th>77</th>
<th>78</th>
<th>79</th>
<th>77&amp;78</th>
<th>77&amp;79</th>
<th>78&amp;79</th>
<th>77&amp;78&amp;79</th>
<th>xenic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean nematode numbers</td>
<td>1</td>
<td>38</td>
<td>4</td>
<td>3</td>
<td>13</td>
<td>6</td>
<td>3</td>
<td>15</td>
<td>139</td>
</tr>
</tbody>
</table>

As the vast majority of nematodes had died before they were examined after two weeks, the numbers in Table 25 represent both live and dead nematodes visible on the plates. Xenic plates supported better nematode growth than all other treatments. Adult nematodes could be seen on plates of all treatments except axenic, demonstrating that the nematodes were capable of at least some growth on all three bacteria. Second generation juveniles were seen only in the xenic plates, the monoxenic plates with Pr. rettgeri, and the gnotobiotics plates with Pr. rettgeri and Fl. breve, or with Pr. rettgeri, Fl. odoratum & Fl. breve. This suggests that Pr. rettgeri was the only one of the three bacteria tested which could support nematode reproduction under the experimental conditions. After three weeks all nematodes had died, except on one of the xenic plates which had >1000 nematodes. No growth was observed on the axenic plates but they gave an indication of the level of background contamination: 3 out of the 10 axenic plates had become contaminated.
7.3.1.2. Experiment 2: Comparison of monoxenic, gnotobiotic, axenic and xenic growth on nutrient agar with that on kidney agar.

The results of this experiment are shown in Table 26. Numbers quoted are the numbers of live nematodes only and are thus not directly comparable with Table 25. Data were transformed to logarithms for statistical analysis.

Nematode growth did not occur on the axenic plates of nutrient agar or kidney agar in this experiment, but these plates gave an estimate of the level of background contamination, (approximately 20%). It was not possible, however, to distinguish which of the plates lawned with bacteria had become contaminated and hence were not monoxenic.

After two weeks the only nutrient agar plates on which nematode reproduction had occurred were the xenic plates (Table 26). However, on kidney medium, nematode growth and reproduction occurred on xenic plates and on plates treated with *Pr. rettgeri*. More nematodes were present on kidney plates with *Pr. rettgeri* than on xenic kidney plates, but this difference was not significant. There was no significant difference in nematode numbers between kidney agar xenic plates and nutrient agar xenic plates.

After three weeks the numbers of live nematodes on nutrient agar xenic plates had fallen significantly (*P* < 0.05) (Table 26). On kidney agar, nematode numbers on xenic plates and those treated with *Pr. rettgeri* had risen significantly (*P* < 0.01), and there were significantly (*P* < 0.01) more nematodes on kidney plates treated with *Pr. rettgeri* than on xenic kidney plates.
Table 26. Mean nematode numbers after two and three weeks of growth on nutrient agar or kidney agar in axenic, monoxenic, xenic or gnotobiotics culture with different bacteria. Numbers of nematodes were transformed to logarithms for statistical analysis. (mean log numbers are shown in brackets.)

<table>
<thead>
<tr>
<th>Bacterium</th>
<th>Week 2</th>
<th>Week 3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Nutrient agar</td>
<td>Kidney agar</td>
</tr>
<tr>
<td>Axenic</td>
<td>2 (0.232)</td>
<td>2 (0.270)</td>
</tr>
<tr>
<td>Pr. rettgeri</td>
<td>10 (0.938)</td>
<td>380 (2.043)</td>
</tr>
<tr>
<td>Fl. breve</td>
<td>0 (0.000)</td>
<td>2 (0.126)</td>
</tr>
<tr>
<td>Fl. odoratum</td>
<td>0 (0.000)</td>
<td>12 (0.740)</td>
</tr>
<tr>
<td>Pr. rettgeri + Fl. breve + Fl. odoratum</td>
<td>5 (0.732)</td>
<td>7 (0.865)</td>
</tr>
<tr>
<td>Xenic</td>
<td>50 (1.643)</td>
<td>80 (1.879)</td>
</tr>
</tbody>
</table>

(S.E.D. for comparing log. nematode numbers = 0.204, 207 D.F.)

7.3.1.3. Experiment 3: Monoxenic and xenic growth on nutrient and kidney agar on plates half lawned with bacteria.

The results of this experiment are shown in Table 27. After two weeks no nematodes had reproduced on any of the bacteria grown on nutrient agar plates apart from the xenic cultures. On kidney agar, nematodes had grown on all three bacteria tested as well as on the xenic mix. There were no significant differences in nematode numbers between any of the treatments on kidney agar, but there were significantly ($P < 0.001$) more nematodes on kidney agar xenic plates than on nutrient agar xenic plates. After three weeks the numbers of nematodes had increased significantly ($P < 0.001$) on all individual bacteria and on the xenic mix of bacteria on kidney plates. Nematode numbers had also increased significantly ($P < 0.01$) on xenic nutrient agar plates. On kidney medium all three monoxenic cultures had significantly ($P < 0.001$) more nematodes than the xenic plates but were not significantly different from each other. Xenic cultures on kidney medium in turn had significantly ($P < 0.001$) more nematodes than xenic cultures on nutrient agar.
Table 27. Mean numbers of nematodes after two and three weeks growth on nutrient agar or kidney agar in xenic culture or monoxenic culture with different bacteria. Numbers were transformed to logarithms for statistical analysis. (Log. transformed data are shown in brackets.)

<table>
<thead>
<tr>
<th>Bacterium</th>
<th>Nutrient Agar</th>
<th>Kidney Agar</th>
<th>Nutrient Agar</th>
<th>Kidney Agar</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pr. rettgeri</td>
<td>1 (0.100)</td>
<td>300 (2.338)</td>
<td>1 (0.010)</td>
<td>44350 (4.538)</td>
</tr>
<tr>
<td>Fl. odoratum</td>
<td>0 (0.075)</td>
<td>300 (2.408)</td>
<td>0 (0.000)</td>
<td>41800 (4.597)</td>
</tr>
<tr>
<td>Ps. fluorescens</td>
<td>0 (0.111)</td>
<td>300 (2.393)</td>
<td>0 (0.000)</td>
<td>43750 (4.633)</td>
</tr>
<tr>
<td>(141)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Xenic</td>
<td>160 (1.912)</td>
<td>400 (2.568)</td>
<td>300 (2.434)</td>
<td>6000 (3.697)</td>
</tr>
</tbody>
</table>

(S.E.D. for comparing log nematode numbers = 0.147, 96 D.F.)

7.3.1.4. Experiment 4: Screening of bacteria for the ability to support monoxenic nematode growth.

The results of experiment 4 are shown in Table 28. After two weeks there were significant differences in nematode numbers between treatments (Table 28) with plates treated with Pr. rettgeri having the greatest numbers of nematodes. No nematodes had reproduced on Ps. paucimobilis, or X. nematophilus and reproduction had taken place on only one plate treated with B. cereus. Intermediate growth between these two extremes was seen in the remaining five treatments. After three weeks, plates treated with Pr. rettgeri still had the greatest numbers of nematodes, but numbers were not significantly greater than on plates treated with Serratia proteamaculans, Moraxella phenylpyruvica, Aeromonas sp. or Ps. fluorescens (141). Nematode numbers on other bacteria were significantly less than at least some of these five species (Table 28). No nematodes had reproduced on plates treated with X. nematophilus, Ps. paucimobilis, or the axenic plates and nematodes had reproduced on only one plate treated with B. cereus. This plate was examined for contamination but none was found.
Table 28. Numbers of Phasmorhabditis sp. after two and three weeks growth in monoxenic culture with different bacteria. Data were transformed to logarithms for statistical analysis.

<table>
<thead>
<tr>
<th>Bacterium</th>
<th>Week 2</th>
<th>Week 3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>numbers</td>
<td>log.</td>
</tr>
<tr>
<td>X. nematophilus</td>
<td>0</td>
<td>0.000</td>
</tr>
<tr>
<td>Ps. paucimobilis</td>
<td>0</td>
<td>0.043</td>
</tr>
<tr>
<td>B. cereus</td>
<td>1</td>
<td>0.125</td>
</tr>
<tr>
<td>Ps. fluorescens (1)</td>
<td>170</td>
<td>2.183</td>
</tr>
<tr>
<td>Sp. spiritovorum</td>
<td>75</td>
<td>1.528</td>
</tr>
<tr>
<td>M. phenylpyruvica</td>
<td>60</td>
<td>1.699</td>
</tr>
<tr>
<td>S. proteamaculans</td>
<td>520</td>
<td>2.456</td>
</tr>
<tr>
<td>Ps. fluorescens (141)</td>
<td>700</td>
<td>2.769</td>
</tr>
<tr>
<td>Aeromonas sp.</td>
<td>250</td>
<td>2.257</td>
</tr>
<tr>
<td>Pr. rettgeri</td>
<td>1160</td>
<td>3.055</td>
</tr>
</tbody>
</table>

S.E.D. for comparing log. nematode numbers = 0.204, 128 D.F.

7.3.1.5. Experiment 5: Screening of bacteria for the ability to support monoxenic nematode growth.

The results of Experiment 5 are shown in Table 29. After two weeks there were significantly (P<0.05) fewer nematodes on plates treated with A. hydrophila or Ps. paucimobilis than on all other treatments but there were no other significant differences between treatments. After three weeks Pr. rettgeri and Pseudomonas fluorescens (139) supported best growth of Phasmorhabditis sp., but not significantly better than Ps. fluorescens (140). Nematode numbers on other bacteria were significantly less than at least one of these three bacteria. Numbers of nematodes in this experiment were lower than expected (after three weeks Pr. rettgeri yielded a mean of 14,400 nematodes per plate compared with 86,300 in experiment 4). This might be a result of differences in
kidneys used to make the medium. It is also interesting to note that while *Ps. paucimobilis* supported no growth and development in experiment 4, it did support limited growth in experiment 5.

Table 29. Numbers of nematodes after two and three weeks growth in monoxenic culture with different bacteria in experiment 5. Nematode numbers were transformed to logarithms for statistical analysis.

<table>
<thead>
<tr>
<th>Bacterium</th>
<th>Week 2</th>
<th>Week 3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Number</td>
<td>log.</td>
</tr>
<tr>
<td><em>Ps. paucimobilis</em></td>
<td>2</td>
<td>0.106</td>
</tr>
<tr>
<td><em>A. hydrophila</em></td>
<td>9</td>
<td>0.093</td>
</tr>
<tr>
<td><em>F. breve</em></td>
<td>200</td>
<td>2.128</td>
</tr>
<tr>
<td><em>Ps. fluorescens (140)</em></td>
<td>100</td>
<td>1.682</td>
</tr>
<tr>
<td><em>Acinetobacter calcoaceticus</em></td>
<td>75</td>
<td>1.855</td>
</tr>
<tr>
<td><em>Pr. rettgeri</em></td>
<td>250</td>
<td>2.261</td>
</tr>
<tr>
<td><em>Ps. fluorescens (139)</em></td>
<td>200</td>
<td>2.128</td>
</tr>
</tbody>
</table>

S.E.D. for comparing log nematode numbers = 0.1598 (63 D.F.)
In this first investigation of the ability of different bacteria to support monoxenic growth of *Phasmorhabditis* sp., sixteen different isolates of bacteria out of the collection of more than 150 isolates were screened and out of this selection all bacteria except *X. nematophilus* supported at least some growth and reproduction of nematodes in at least one of the experiments on some of the plates. The growth and reproductive capacity of this nematode differed considerably when grown on different bacteria. These findings suggest that the bacterial food requirements of *Phasmorhabditis* sp. are similar to other bacteriophagous nematodes: the free living nematodes *Caenorhabditis elegans* (Andrew & Nicholas 1976, Grewal 1990), *Rhabditis terricola*, *Diplogaster nudicapitatus*, *Mesodiplogaster biformis* (Sohlenius 1968); a nematode found associated with earthworms, *Rhabditis maupasi* (Sohlenius 1968); and the insect parasitic nematode *Steinernema glaseri* (Poinar 1979). Grewal (1990) grew *C. elegans* on nine different bacteria found associated with the nematode in mushroom compost and found that although all nine supported growth, there were large differences in reproductive capacity of the nematode when grown on different bacteria. Poinar (1979) reported that *S. glaseri* was capable of growth on three different species of bacteria.

This series of experiments was intended solely as a means of identifying a range of bacteria that could be used to support growth of *Phasmorhabditis* sp. in monoxenic culture and to choose bacteria/nematode combination for testing in bioassays against slugs (Section 9). It was not designed to investigate interactions between bacteria and nematodes when grown together in monoxenic culture. However, these results indicate that factors other than species of bacterium appeared to influence growth and reproduction of *Phasmorhabditis* sp. suggesting that interactions between bacteria, nematodes and the growth medium may determine whether or not nematodes can grow and reproduce. None of the individual bacterial species tested supported growth and reproduction of *Phasmorhabditis* sp. on nutrient agar, although all of the bacteria grew well on this medium. In experiment 1 much better growth was seen in nutrient agar plates lawned with the xenic mix of bacteria than on gnotobiotic plates containing all three of the bacteria which had been isolated from this mix. One possible explanation for the much better growth of the xenic plates is that the xenic mix of bacteria contained a species of bacterium other than *Pr. rettgeri*, *Fl. breve* and *Fl. odoratum* which had not
been isolated in previous investigations. This explanation seems unlikely in light of the thoroughness of the examination of the bacterial flora of the thriving xenic culture, unless the bacterium had extremely fastidious nutritional or environmental requirements. Another possibility is that nutrients from the kidney-impregnated foam chips which were initially added to the nutrient broth to raise the xenic inoculum, were transferred with the 0.025 ml of broth inoculum. These nutrients might have been present in sufficient amounts to promote extra growth in the xenic plates. The results of experiment 2 in which bacteria incapable of supporting nematode growth on nutrient agar did support growth on kidney agar would support the latter hypothesis. However, the lack of nematode growth on nutrient agar in these two experiments cannot be attributed to any absolute nutrient deficiency in the medium because in previous work (see 4.3.1) *Phasmorhabditis* sp. grew well in xenic culture on this medium. This may imply that only certain bacteria are capable of synthesising nutrients necessary for nematode growth on nutrient agar. Similar interactions between medium and bacteria have been observed for *Rhabditis maupasi*. Sohlenius (1968) found that *Escherichia coli* could support good monoxenic growth of *Rhabditis maupasi* on Nigon’s agar, but not on nutrient agar. This work also indicates that nematode growth may be influenced by the way in which bacteria and the nematodes are introduced into the culture. *Fl. odoratum*, which supported no nematode reproduction in Experiment 2, in which the bacteria were lawn over the entire surface of the plate, supported vigorous growth in Experiment 3, where the bacteria were lawn over one half of the plate, and the nematodes were introduced into the other half.

Bacteria are known to produce nematicidal metabolic products (Bergmann & Van Duuren 1959). In studying nematode growth and reproduction of *Heterorhabditis* sp. in monoxenic culture with its symbiotic bacterium *Xenorhabdus luminescens* Ehlers, Stoessel and Wyss (1990) reported cultures dying without obvious cause. They put forward the hypothesis that the nematodes were capable of metabolising toxic bacterial waste products, but the nematodes had to reach a certain threshold population in relation to the numbers of bacteria to achieve this effect. They suggested that the balance between the growth rates of the bacteria and the nematodes was of prime importance. It is possible that this hypothesis could in part explain the results presented here. All the bacteria used in these experiments were capable of rapid and prolific growth on nutrient agar. However it is possible that the lower nutrient content of this medium compared to kidney medium
would slow down nematode growth and possibly upset this balance between nematode and bacterial growth rates required to maintain nematode growth.

The superior growth of nematodes on plates in which only half the surface was lawned with bacteria might be attributable to diffusion of bacterial toxins into the bacteria free half of the plate and away from the nematodes feeding on the bacteria in the lawned half. For bacterial isolates which supported no growth on plates entirely lawned with bacteria but did support growth on plates with only one half of the surface lawned with bacteria it is possible that the diffusion of toxins gave the nematodes time to increase to "detoxifying" population levels before the toxins had reached a lethal concentration.

These explanations can only be regarded as speculative and the supposed bacterial toxins would need to be purified and characterised for use in experiments to confirm their importance.

The observed interactions between nematode growth and bacterial species, medium, and the way the nematodes and bacteria were introduced to one another means that these results should be treated with caution. Bacteria which support best growth of nematodes on a petri dish of kidney medium half lawned with bacteria might not support the best growth for example in bulk liquid fermentation systems using a totally soluble medium. It would be unwise to select just one bacterium from these experiments for further development of in-vitro production. Rather a selection of bacteria which supported good nematode growth should be screened at least in the early stages of liquid fermentation development.
8. EFFECTS OF BACTERIA SUPPORTING GROWTH OF PHASMORHABDITIS SP. ON THE SLUG DEROCERAS RETICULATUM.

8.1. Introduction

Investigations into bacteria associated with Phasmorhabditis sp. (Section 6.) suggest that there is not only one bacterium mutualistically associated with this nematode. As a wide range of bacteria appear to be retained within the gut of the dauer larvae, it was decided to investigate the possibility that Phasmorhabditis sp. may be able to vector pathogenic bacteria into slugs. This section describes a series of experiments in which bacteria associated with Phasmorhabditis sp. were injected into D. reticulatum to determine which, if any of these bacteria were pathogenic to this slug.

Experiments 1 and 2 were done at an early stage of the project, before detailed examination of nematode-associated bacteria and before the ability of different bacteria to support nematode growth had been investigated. A bacterium was found which was pathogenic to D. reticulatum. It was at first thought that this bacterium might have been a mutualistic associate of Phasmorhabditis sp. similar to those seen in entomopathogenic nematodes, but failure to isolate this bacterium consistently, and the inability of this bacterium to support monoxenic growth of Phasmorhabditis sp. suggested that this was not the case.

At a later stage in the project, it was decided to screen all the bacteria found associated with Phasmorhabditis sp. which supported good monoxenic nematode growth (Section 7.) for pathogenicity against D. reticulatum.

8.2. Materials and Methods

8.2.1. Injection of slugs

Slugs were first anaesthetised by filling their culture boxes with CO₂ from a gas cylinder and leaving them for fifteen minutes (Henderson 1969). The slugs were injected with overnight exponential phase nutrient broth cultures of different bacteria using an oven-sterilised all-glass micro-applicator syringe fitted with a hypodermic needle. The syringe
was then mounted in an Arnold hand applicator (Burkard, London) (Plate 19) and known volumes of bacterial cultures were injected into various parts of the body of *D. reticulatum*.

Plate 19. Arnold Hand Micro-Applicator fitted with an all glass syringe.

8.2.2. Injection Experiments.

8.2.2.1. Experiment 1. Injection of a mixed culture of nematode-associated bacteria into slugs.

Nematodes taken from a number of infected slugs were washed in sterile water, ground with a pestle and mortar, then transferred to a tube of nutrient broth and incubated at 25°C overnight. 10 µl of the resulting mixed bacterial suspension was then injected into 15 *D. reticulatum* through the dorsal surface of the mantle. A further 15 slugs were similarly injected with 10 µl of sterile nutrient broth. The slugs were kept in plastic boxes and examined after 10 days.

The mix of bacteria was separated into individual species by making serial 10 fold dilutions of the broth culture and spreading 0.1 ml aliquots of the dilutions onto nutrient agar plates.
The plates were incubated at 25°C and species were distinguished by their colonial morphology.

8.2.2.2 Experiment 2: Injection of individual species of bacteria taken from a pathogenic mix.

The four bacterial species taken from the mixed culture used in experiment 1 were all assayed against *D. reticulatum* by injection through the mantle. Only two micro-applicator all-glass syringes were available at this stage in the project and it was not possible to assay all four bacteria at once in a balanced experimental design because time did not permit cleaning and oven sterilisation of the syringes between injecting one bacterium and another. Thus the results presented are the cumulative results of five experiments. At least twenty slugs were injected with each bacterium. In all cases untreated slugs, and slugs injected with sterile nutrient broth were included as controls.

8.2.2.3. Experiment 3: Injection of a pathogenic bacterium into different sites of *D. reticulatum*.

*Aeromonas hydrophila* was injected into three sites in *D. reticulatum* (Figure 17).

![Figure 17. Transverse section through *D. reticulatum* showing the shell cavity (A), the mantle cavity (B) and body cavity (C) into which bacteria were injected. (Modified from Runham and Hunter 1970)
Slugs were injected 1) into the shell cavity through the dorsal surface of the slug, 2) into the mantle cavity through the dorsal surface and below the shell, and 3) through the tail of the animal into the rear of the body cavity (the rear end of the body cavity was chosen to avoid puncturing the gut and allowing escape of gut bacteria into the haemocoel). Nutrient broth culture of *A. hydrophila* or 5 µl of sterile nutrient broth was injected at one of these three sites, or left untreated. Thus the experiment consisted of seven treatments (Table 31), with 16 slugs per treatment. The slugs were then kept in boxes with four slugs per box and fed Chinese cabbage leaves.

8.2.2.4. Experiment 4: First experiment screening bacteria for pathogenicity to *D. reticulatum*.

5 µl of overnight broth cultures of *Moraxella phenylpyruvica*, *Providencia rettgeri*, *Serratia proteamaculans*, *Pseudomonas fluorescens* (141) and *Aeromonas hydrophila* were injected into the body cavity of *D. reticulatum*. Slugs injected with sterile nutrient broth or left untreated were included so that the experiment consisted of seven treatments (Table 32). Thirty slugs were used per treatment. After being injected, slugs were kept in plastic boxes, with 10 slugs in each box.

8.2.2.5. Experiment 5: Second experiment screening bacteria for pathogenicity to *D. reticulatum*.

This experiment was similar to Experiment 4 but used different bacteria. 5 µl of overnight broth cultures of *Pseudomonas fluorescens* (140), *Aeromonas* sp., *Ps. fluorescens* (141) or *A. hydrophila* (156) were injected into the body cavity of *D. reticulatum*. Untreated slugs and slugs injected with sterile broth were included as controls. Twenty slugs were used for each treatment. Following injection the slugs were kept in plastic boxes as in previous experiments.
8.2.2.6. Experiment 6: Third experiment screening bacteria for pathogenicity to *D. reticulatum*.

5 µl of overnight broth cultures of *Flavobacterium breve*, *Flavobacterium odoratum*, *Ps. fluorescens* (141) and *Aeromonas hydrophila* were injected into the body cavities of *D. reticulatum*. Untreated slugs and slugs injected with sterile broth were included as controls. Twenty slugs were used per treatment.

8.2.2.7. Experiment 7: Comparison of injection of nutrient broth cultures of pathogenic bacteria and sterile filtrates of these cultures.

Twenty *D. reticulatum* were injected into their body cavities with 5 µl of overnight broth culture of *A. hydrophila* or *Ps. fluorescens* (140). The remainder of the two broth cultures were then centrifuged at 250 x G for 20 minutes to settle out bacterial cells. The supernatant was then removed and filtered through a cellulose acetate filter (2µm pore diameter) to ensure sterility. Twenty *D. reticulatum* were injected with 5 µl of the *A. hydrophila* culture extract and 20 were injected with 5 µl of the *Ps. fluorescens* extract. Slugs injected with 5µl of sterile nutrient broth and untreated slugs were included as controls. Following injection the slugs were kept in plastic boxes as in previous experiments.

8.2.2.8. Experiment 8: Dose response relationship for *D. reticulatum* injected with different doses of the pathogenic bacterium *Ps. fluorescens* (140).

Four doses of this bacterium were prepared by making three 100 fold serial dilutions of an overnight nutrient broth culture of *Ps. fluorescens* (140). 5 µl of the original suspension and 5 µl of each of the three one hundred fold dilutions were injected into the body cavity of twenty *D. reticulatum*. The number of viable bacteria in the suspension was counted by making serial 10 fold dilutions of the culture in nutrient broth, and then spreading 0.1ml of the lower dilutions onto each of five 10 cm nutrient agar plates. These plates were then incubated for 48 hours at 25° C. After this time bacterial colonies were counted on plates for the dilutions producing plates containing between 100 and 400 colonies. The mean number of colonies per plate at this dilution was estimated and the
number of colony forming units (cfu) in the original suspension was calculated by multiplying this number by the dilution factor. *D. reticulatum* were injected with 5 µl of one of four doses of bacteria into their body cavities (twenty slugs per dose).

8.2.3. Ingestion Experiments

8.2.3.1. Experiment 9: The effects of ingestion of different bacteria on *D. reticulatum*.

The experiment consisted of 8 treatments (Table 37) and used 20 *D. reticulatum* per treatment. The slugs were starved for two days prior to the start of the experiment. The test bacteria were overnight cultures grown on nutrient agar plates. The bacteria were spread onto 5 mm discs of Chinese cabbage cut out with a cork-borer, then the discs were put into 9 cm petri-dishes lined with moist filter paper and one *D. reticulatum* was added to each dish. The petri dishes were then kept in plastic boxes lined with damp absorbent cotton wool to maintain high humidity. After 24 hours the leaf discs were examined. The percent of each leaf disc eaten was estimated for each slug. The numbers of dead slugs was then recorded after 5 and 8 days.

8.2.3.2. Experiment 10: Effects on *D. reticulatum* of ingestion of four bacteria.

The experimental methods were identical to experiment 7 except that after one day any slugs which had not consumed the original leaf disc were discarded. All slugs which had eaten the entire leaf disc were then transferred to petri-dishes and the numbers of dead slugs recorded after 6 and 9 days.
8.3. Results.

8.3.1. Injection experiments.

8.3.1.1. Experiment 1: Injection of a mixed culture of nematode-associated bacteria into slugs.

This experiment was done at an early stage of the project before any work on monoxenic cultivation of the nematode, as an initial test of the pathogenicity to slugs of bacteria found in nematode-infected slugs. After 10 days, eight out of the fifteen slugs injected with the bacterial mix had died whereas none of the slugs injected with sterile broth had died. This difference in mortality is highly significant (P<0.001) showing that the bacterial mix was probably pathogenic when injected into slugs.

The bacterial mix was found to contain four different species of bacteria.

8.3.1.2. Experiment 2: Injection of individual species of bacteria taken from a pathogenic mix.

The results are shown in Table 30.

Table 30. Percent mortality in slugs at 6 and 10 days following injection with different bacteria or sterile nutrient broth. (Numbers of slugs per treatment shown in brackets)

<table>
<thead>
<tr>
<th>Days</th>
<th>Untreated (55)</th>
<th>Sterile Broth (50)</th>
<th>Bacterium A (20)</th>
<th>Bacterium B (75)</th>
<th>Bacterium C (20)</th>
<th>Bacterium D (25)</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>20</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>10</td>
<td>4</td>
<td>4</td>
<td>5</td>
<td>49</td>
<td>0</td>
<td>5</td>
</tr>
</tbody>
</table>

It can be seen from the table that Bacterium B (later identified as A. hydrophtila 156) was the only bacterium that caused mortality greater than the untreated slugs. $X^2$ tests comparing mortality in the untreated slugs with mortality in the slugs injected with bacterium B showed that this bacterium caused mortality significantly (P<0.001) higher than in the untreated slugs by day 10, when 49% of slugs treated with this bacterium had
died. The surviving slugs treated with this bacterium all appeared healthy at 10 days, whereas the majority of slugs which had died had shown swellings similar to those seen in nematode-infected slugs so the experiment was ended.

8.3.1.3. Experiment 3: Injection of a pathogenic bacterium into different sites in the body of *D. reticulatum*.

The aim of this experiment was to determine whether *Aeromonas hydrophila*, known from experiment 2 to be pathogenic when injected into the slug's mantle, was still pathogenic when injected into different parts of the slug's body. The result of this experiment are shown in Table 31. *Aeromonas hydrophila* appeared to cause mortality in *Deroceras reticulatum* when injected at all three sites, but this was only significantly ($P<0.05$) higher than mortality in untreated slugs in the case of slugs injected into their body cavities. Injection with sterile nutrient broth did not harm the slugs. By day 5 all slugs injected with bacteria into their body cavities had died whereas 56% and 63% of slugs injected with bacteria into the mantle cavity or shell cavity respectively had died. From this experiment it was concluded that the body cavity was the best site for injecting bacteria in order to test their pathogenicity.

Table 31. % Mortality of slugs up to 9 days following injection with 5 µl of *Aeromonas hydrophila* or 5 µl of nutrient broth at different sites. (16 slugs per treatment)

<table>
<thead>
<tr>
<th>Day</th>
<th>Untreated</th>
<th>Shell Cavity</th>
<th>Mantle Cavity</th>
<th>Body Cavity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Bacteria</td>
<td>Broth</td>
<td>Bacteria</td>
<td>Broth</td>
</tr>
<tr>
<td>3</td>
<td>6</td>
<td>19</td>
<td>0</td>
<td>25</td>
</tr>
<tr>
<td>5</td>
<td>6</td>
<td>63</td>
<td>0</td>
<td>56</td>
</tr>
<tr>
<td>9</td>
<td>19</td>
<td>63</td>
<td>0</td>
<td>69</td>
</tr>
</tbody>
</table>
8.3.1.4. Experiment 4: First experiment screening bacteria for pathogenicity to *D. reticulatum*.

In this experiment overnight broth cultures of four bacteria which had supported good monoxenic growth of nematodes (see Section 7) were injected into the body cavity of slugs to determine if they were pathogenic to *D. reticulatum*. These were *Moraxella phenylpyuvica* (48), *Providencia rettgeri* (77), *Serratia proteamaculans* (83) and *Pseudomonas fluorescens* (141). *A. hydrophila* (156), a bacterium previously shown to be pathogenic, was also used. The results are shown in Table 32. None of the four bacteria which had supported good nematode growth was pathogenic to *D. reticulatum*. Only *A. hydrophila* caused mortality significantly (PP <0.001) higher than in the untreated slugs.

Table 32. Percentage mortality of slugs during ten days following injection with 5 µl of nutrient broth or cultures of different bacteria. (30 slugs per treatment)

<table>
<thead>
<tr>
<th>Day</th>
<th>Untr-</th>
<th>Broth</th>
<th>S. prot. (83)</th>
<th>Pr. rett. (77)</th>
<th>Ps. fluo. (141)</th>
<th>M. phen. (48)</th>
<th>A. hyd. (156)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>0</td>
<td>3</td>
<td>3</td>
<td>0</td>
<td>7</td>
<td>0</td>
<td>43</td>
</tr>
<tr>
<td>5</td>
<td>3</td>
<td>3</td>
<td>7</td>
<td>0</td>
<td>10</td>
<td>3</td>
<td>57</td>
</tr>
<tr>
<td>7</td>
<td>3</td>
<td>7</td>
<td>10</td>
<td>3</td>
<td>10</td>
<td>3</td>
<td>73</td>
</tr>
<tr>
<td>10</td>
<td>10</td>
<td>13</td>
<td>13</td>
<td>10</td>
<td>13</td>
<td>7</td>
<td>83</td>
</tr>
</tbody>
</table>

8.3.1.5. Experiment 5: Second experiment screening bacteria for pathogenicity to *D. reticulatum*.

The results of this experiment are shown in Table 33. Both *A. hydrophila* and *Pseudomonas fluorescens* (140) caused significant mortality in *D. reticulatum* (P <0.001). Slugs injected with *Aeromonas* sp. (155) or *Ps. fluorescens* (141) did not show significant mortality.
Table 33. Percentage mortality of slugs in the week following injection with 5 µl of nutrient broth or nutrient broth cultures of different bacteria. (20 slugs per treatment)

<table>
<thead>
<tr>
<th>Day</th>
<th>Un-treated</th>
<th>Sterile broth</th>
<th>A. hydrophila (156)</th>
<th>Aeromonas sp. (155)</th>
<th>Ps. fluorescens (140)</th>
<th>Ps. fluorescens (141)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>0</td>
<td>0</td>
<td>25</td>
<td>0</td>
<td>85</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>0</td>
<td>0</td>
<td>50</td>
<td>5</td>
<td>85</td>
<td>0</td>
</tr>
<tr>
<td>7</td>
<td>5</td>
<td>0</td>
<td>70</td>
<td>5</td>
<td>95</td>
<td>0</td>
</tr>
</tbody>
</table>

8.3.1.6. Experiment 6: Third experiment screening bacteria for pathogenicity to D. reticulatum.

The results of this experiment are shown in Table 34. It can be seen that neither of the two test bacteria caused mortality significantly higher than that seen in the slugs injected with nutrient broth. *P. fluorescens* (140) as in experiment 5 caused high mortality in slugs.

Table 34. Percentage mortality in slugs up to 8 days following injection with nutrient broth and three different bacteria (20 slugs per treatment).

<table>
<thead>
<tr>
<th>Days</th>
<th>nutrient broth</th>
<th>Fl. breve (78)</th>
<th>Fl. odoratum (79)</th>
<th>Ps. fluorescens (140)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>10</td>
<td>25</td>
<td>0</td>
<td>75</td>
</tr>
<tr>
<td>6</td>
<td>10</td>
<td>25</td>
<td>5</td>
<td>100</td>
</tr>
<tr>
<td>8</td>
<td>15</td>
<td>25</td>
<td>10</td>
<td>100</td>
</tr>
</tbody>
</table>

8.3.1.7. Experiment 7: Comparison of injection of nutrient broth cultures of pathogenic bacteria and sterile filtrates of these cultures.

The aim of this experiment was to determine whether the pathogenic action of *Ps. fluorescens* (140) and *A. hydrophila* (156) relied on viable cells actively invading the
slug, or whether it was a results of pre-formed toxins or enzymes present in the nutrient broth cultures injected into the slugs. The results of this experiment are shown in Table 35. The slugs injected with the sterile extract of both cultures showed no mortality whereas slugs injected with live cells of the two bacteria both showed mortality significantly higher than in the controls ($P<0.001$). This indicates that pathogenicity depends on viable cells of the bacteria being introduced into the slugs.

**Table 35.** Percentage mortality of slugs in the week following injection with 5 µl of nutrient broth, broth cultures of different bacteria, or sterile filtrates of different bacterial cultures (20 slugs per treatment).

<table>
<thead>
<tr>
<th>Days</th>
<th>Un-treated</th>
<th>Broth</th>
<th>A. hydrophila (156)</th>
<th>Ps. fluorescens (140)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>cells</td>
<td>cells</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>filtrate</td>
<td>filtrate</td>
</tr>
<tr>
<td>3</td>
<td>5</td>
<td>5</td>
<td>55</td>
<td>100</td>
</tr>
<tr>
<td>5</td>
<td>5</td>
<td>5</td>
<td>95</td>
<td>100</td>
</tr>
<tr>
<td>7</td>
<td>5</td>
<td>5</td>
<td>95</td>
<td>100</td>
</tr>
</tbody>
</table>

8.3.1.8. Experiment 8: Dose response relationship for *D. reticulatum* injected with different doses of the pathogenic bacterium *Pseudomonas fluorescens* (140).

The results of this experiment are shown in Table 36. It can be seen from this table that all the doses of bacteria had caused mortality in slugs by day 10 except the lowest dose. (1 cell per slug). At day 3 there was high mortality in slugs treated with the top dose of bacteria but mortality in slugs treated with the second highest dose was not significantly different from mortality in the slugs treated with sterile broth. By day 5, however, there was 100% mortality of slugs treated with the top two doses. These results suggest that the disease in slugs caused by *P. fluorescens* (140) is of an invasive infection type, the slugs being killed when the bacteria reach sufficiently high numbers. Lower inoculations of the bacteria can still be lethal, but the bacteria take longer to reach lethal levels.
Table 36. Percentage mortality of slugs 3, 6 and 10 days after injection with different doses of bacterium 140 or sterile nutrient broth (20 slugs per treatment).

<table>
<thead>
<tr>
<th>Bacteria/slug</th>
<th>0</th>
<th>1</th>
<th>100</th>
<th>10,000</th>
<th>1,000,000</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 3</td>
<td>0</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>75</td>
</tr>
<tr>
<td>Day 6</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Day 10</td>
<td>20</td>
<td>15</td>
<td>65</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

8.3.2. Ingestion Experiments.

8.3.2.1. Experiment 9: The effects of ingestion of different bacteria on *D. reticulatum*.

In this experiment a wide selection of the bacteria used in the above experiments were fed to slugs to determine whether they were pathogenic to slugs when ingested. The results of this experiment are shown in Table 37. There was higher mortality in slugs fed with *Pr. rettgeri* (77) than for slugs fed leaf discs without bacteria, but this difference was not significant. The results were difficult to interpret because many of the slugs fed *Pr. rettgeri* which died had not consumed any of the original leaf disc covered with the test bacterium. Because of this it was felt necessary to re-test *Pr. rettgeri* even though mortality of slugs fed this bacterium was not significantly higher than the untreated slugs.
Table 37. Percentage mortality of slugs over 8 days following ingestion of different bacteria. (20 slugs per treatment)

<table>
<thead>
<tr>
<th>Bacterium</th>
<th>Day 5</th>
<th>Day 8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>M. phenylpyruvica (48)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Pr. rettegeri (77)</td>
<td>10</td>
<td>30</td>
</tr>
<tr>
<td>F. breve (78)</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td>F. odoratum (79)</td>
<td>5</td>
<td>15</td>
</tr>
<tr>
<td>S. proteamaculans (83)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Ps. fluorescens (140)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Ps. fluorescens (141)</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td>A. hydrophila (156)</td>
<td>5</td>
<td>5</td>
</tr>
</tbody>
</table>

8.3.2.2. Experiment 10. Effects on D. reticulatum of ingestion of four bacteria.

This experiment was a repeat of experiment 7 but with only four species of bacteria. The results of this experiment are shown in Table 38. There were no significant differences in mortality between treatments demonstrating that Pr. rettegeri was not pathogenic to D. reticulatum following ingestion.

Table 38. Percentage mortality of slugs 6 and 9 days after ingestion of different bacteria, (20 slugs per treatment).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Day 6</th>
<th>Day 9</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>0</td>
<td>15</td>
</tr>
<tr>
<td>Pr. rettegeri</td>
<td>4</td>
<td>7</td>
</tr>
<tr>
<td>S. proteamaculans</td>
<td>21</td>
<td>26</td>
</tr>
<tr>
<td>Ps. fluorescens (141)</td>
<td>0</td>
<td>20</td>
</tr>
</tbody>
</table>
8.4. Discussion.

In experiment 1, although it was clear that *A. hydrophila* was pathogenic to slugs when injected into the mantle, the bacterium caused only ≤50% mortality. One possible explanation for this relatively low mortality compared with later experiments was that the bacterium was being introduced to slightly different sites. Dissection of nematode infested slugs revealed that the nematodes multiply around the shell of the slugs (See section 3.3.2.), separated from the organs in the mantle cavity by membranous tissue. It is possible that in this experiment the bacteria had been introduced into the shell cavity on some occasions but at other times the bacteria were being injected lower into the mantle cavity or body cavity of the slugs. However in experiment 2 mortality of ≥60 - 70% was found when *A. hydrophila* was injected into either the mantle cavity or the shell cavity, whereas 100% mortality was seen following injection of this bacterium into the body cavity. It was thus decided to screen bacteria for pathogenicity by injection into the body cavity because this was considered to be the most sensitive test, even though this is not the site at which the nematodes multiply.

Two bacteria were identified as being pathogenic to *D. reticulatum* in this series of experiments: *Aeromonas hydrophila* and a strain of *Pseudomonas fluorescens* (141). It should be noted that while these bacteria were lethal to slugs when injected, neither was pathogenic when ingested or on skin contact, which must have happened in the ingestion experiments, and they should therefore be referred to as opportunistic pathogens.

It is a tribute to the slug’s immune mechanisms that out of the 8 bacterial isolates screened only two were found to be pathogenic, considering the high doses of bacteria used. The inoculation rate of bacteria was only estimated for *Ps. fluorescens* (140), (10⁹ cells/slug) in one experiment (experiment 6). However, all the bacteria grew well in nutrient broth, and the overnight cultures of bacteria used to inject the slugs were all of similar turbidity (by eye judgement) so it seems likely that inoculation dose for other bacteria would not be too dissimilar. The adult *D. reticulatum* used were not weighed but typical weights for the size of slug used would be between 300-400 mg (C.W. Wiltshire pers comm.) which would thus equate to a bacterial dose of approximately 3 x 10⁹ cell/Kg of animal.
As mentioned in section 1.2, there is little published information regarding bacterial pathogens of slugs or on molluscan anti-bacterial defense systems. Phagocytic cells are thought to be important in molluscan immune systems (Cheng et al, 1969) and bacterial agglutinating haemolymph proteins have been demonstrated in the snail Helix aspersa (Prokop et al, 1968). It is noteworthy that one of the opportunistic pathogens (A. hydrophila) has been implicated as causing disease in molluscs before. Dean, Mead & Northey (1970) found Aeromonas liquefaciens (=A. hydrophila) associated with a disease in the giant African snail Achatina fulica. The disease was characterised by leucodermia-like lesions, and it was from these lesions that the bacterium was isolated. Dean et al. were not able to transmit this disease to healthy snails by either feeding the bacterium, or applying it to the surface of snails, and they were not able to explain the mode of transmission in nature. The authors did not mention if nematodes or any other parasites were found associated with the diseased slugs. It seems possible that A. hydrophila is also an opportunistic pathogen of this snail, and is a secondary infection which follows invasion with some other pathogen or parasite. A disease with identical symptoms is also found in pest slugs in India. Field observations suggest that this disease is capable of reducing populations of snails (Raut & Ghose 1977) and slugs (Raut & Mandal 1986). It is difficult to speculate on whether or not Phasmorhabditis sp. would be capable of vectoring the pathogenic strain of P. fluorescens (140) into slugs and thus increasing the pathogenicity of the nematode. Certainly the results of experiments 5, in which it was shown that it was viable cells of the bacterium which were pathogenic rather than a toxin produce during rapid growth in rich media, and the results of experiment 6 in which 65% mortality of slugs was seen at 10 days following injection with 100 bacterial cells, suggest that this bacterium causes an invasive infection type disease which does not need many viable cells to kill slugs, although the slugs die more rapidly if injected with higher doses. Quantitative data on the retention of Ps. fluorescens has not yet been obtained, but it is quite likely that if more than one nematode entered the slug they would deliver 100 viable bacterial cells or more. However, it must be realised that the interactions between the bacteria, the nematodes and the slug immune system in vivo will be complicated. With no knowledge of these interactions the only way to determine whether Phasmorhabditis sp. can vector this pathogenic bacterium into slugs, and thus act more effectively at killing slugs than Phasmorhabditis sp carrying non-pathogenic bacteria, is by bioassay of the nematode/bacterium complex (Section 9).
9. BIOASSAY OF MONOXENIC NEMATODES

9.1. Introduction.

In section 7 it was shown that the nematode could be grown in monoxenic culture with several different bacteria, and it was shown in section 8 that one of the bacteria capable of supporting nematode growth was pathogenic when injected into slugs. In order to test the hypothesis that the pathogenicity of *Phasmorhabditis* sp. is dependent on bacteria it was decided to compare the pathogenicity to *Deroceras reticulatum* of nematodes monoxenised with different bacteria. The bacteria selected for use in monoxenic bioassays were the pathogenic strain of *Pseudomonas fluorescens* (140) and four of the five bacteria which gave best growth in the first monoxenic growth screening experiment: *Providencia rettgeri*, *Pseudomonas fluorescens* (141), *Serratia proteamaculans* and *Moraxella phenylpyruvica*.

Nematodes grown in monoxenic foam chip cultures with all five of these bacteria were tested in bioassays for their effects on *D. reticulatum*, both feeding inhibition and mortality.


9.2.1. Method of bioassay

The method of bioassay used plastic boxes containing soil with 10 slugs per box as recommended by Stroud (1990), which have been previously described in section 3. For each monoxenic combination, five doses of nematodes were used (15,000, 23,000, 35,000, 55,000, and 75,000 infectives per box) together with an untreated control. The nematodes were counted and the appropriate number suspended in 50 ml of tap water which was distributed evenly over the surface of 440 g dry weight of coarse soil aggregates which had been previously soaked with 80 ml tap water. The aggregates were put in the box layer by layer and the slugs placed between the middle layers. The slugs were left in the boxes of soil for five days after which the cells were dismantled and the numbers of dead slugs recorded. Surviving slugs were individually transferred to 9 cm petri dishes lined with moist filter paper each containing a 3 cm disc of Chinese cabbage.
At three day intervals slugs were examined and the numbers of dead individuals were recorded. The leaf discs were removed and the percentage eaten was estimated by eye to the nearest 5% so that feeding inhibition could be assessed. The percent mortalities were corrected for mortality in the control using Abbott’s (1925) formula and feeding results were expressed as percentage feeding inhibition as was done for the second mini-plot trial (see section 5.3.2).

9.2.2. Bioassay of monoxenic nematodes reared in foam chip cultures.

Nematodes were axenised (6.2.1.) and then grown in monoxenic culture in 3 cm kidney agar plates half-lawned with bacteria (6.2.3.3.) with each of the five different bacteria selected for use in monoxenic assays. After three weeks the discs of agar containing nematodes and bacteria were removed and used to inoculate 250 ml foam chip flasks (4.2.2). After a further three weeks the contents of the flasks were tested for monoxenicity and harvested. In this way nematodes were successfully reared in monoxenic culture with all five bacteria. The nematodes were washed and stored in aerated tap water. When counts were made prior to use in bioassay only infective juveniles were counted. Nematode yields from monoxenic foam chip cultures varied considerably between and within different bacterial treatments and also many monoxenic foam chip flasks were found to be contaminated on harvest; contaminating bacteria may have entered the flasks with the disk of agar culture used to inoculate them. Thus it was not possible to produce sufficient numbers of monoxenic nematodes grown on the five different bacteria to set up simultaneous bioassays using all five monoxenic combinations. Therefore the experiment was not done as a single balanced experiment but as a series of replicate bioassays using identical methods, each replicate consisting of five nematode doses and an untreated control with one box of slugs per dose (i.e. 60 slugs). The different monoxenic nematodes were bioassayed in this way as and when they were available in sufficient numbers. The numbers of slugs used per dose at the end of the series of bioassays were:

- *M. phenylpyruvica* 50 slugs per dose
- *Pr. rettgeri* 70 slugs per dose
- *S. proteamaculans* 50 slugs per dose
- *Ps. fluorescens*(140) 30 slugs per dose
- *Ps. fluorescens*(141) 70 slugs per dose.
Slugs of similar size were available from the same site at LARS throughout the year and at least three replicate assays were done for each monoxenic combination on different dates. For these reasons it was assumed that the time of year at which the experiment was done would not affect the results.

9.2.3. Bioassays of monoxenic nematodes reared in liquid cultures at AGC Littlehampton.

9.2.3.1. First experiment using nematodes produced in liquid culture.

Monoxenic cultures of Phasmorhabditis sp. grown in liquid monoxenic cultures with two of the bacteria used above, *M. phenylpyruvica* and *Pr. rettgeri*, were bioassayed against slugs. These two bacteria had supported best nematode growth in experiments at AGC Littlehampton to investigate the ability of different bacteria to support monoxenic growth in liquids. The nematodes were harvested, stored on foam chips without medium, and posted to LARS in a cool box.

The bioassay method was as described in section 8.2.1. Three replicate boxes of ten slugs were used for the untreated control and each of the five doses for both batches of monoxenic nematodes. (i.e. thirty slugs per dose, 180 slugs per monoxenic nematode). The two monoxenic combinations were bioassayed simultaneously using the same batch of slugs. At the end of the experiment 10 slugs were randomly selected from the 17 surviving slugs treated with 75,000 nematodes monoxenised with *Pr. rettgeri*. These slugs were dissected and examined for the presence of nematodes.

8.2.3.2. Second experiment using nematodes produced in liquid culture.

This was a repeat of the first experiment using nematodes produced in liquids but using different batches of the two monoxenic nematodes which had been grown at Littlehampton under identical conditions, and had been posted to LARS in the same way as those used in experiment 1.
9.3. Results

9.3.1. Bioassays of nematodes reared in monoxenic foam chip cultures.

The feeding inhibition data and the % mortality data for slugs treated with different doses of the five different monoxenic nematodes measured at 8, 11 and 14 days after exposure to the nematodes are shown in Figures 18 and 19. There was no evidence for a dose response in either slug mortality or feeding inhibition for nematodes monoxenised with Pr. rettgeri, S. proteamaculans or Ps. fluorescens(140). However for M. phenylpyruvica and Ps. fluorescens(141) there was a clear dose response with increasing slug mortality or feeding inhibition with increasing nematode dose. For both of these bacteria, the dose response relationship for mortality was most clear when measured at day 14, by which time many nematode infected slugs had died. Conversely, the feeding inhibition dose response was most clear at the first reading on Day 8 (i.e. measuring the amount of food the slugs had consumed between days 5 and 8). At later dates, particularly day 14, many of the slugs treated with higher doses of nematodes had died, and the feeding inhibition for these doses, based on the feeding of very small numbers of slugs did not follow the general trend (Fig 18 and 19). For these reasons it was decided to make detailed analysis of feeding inhibition measured at day 8 and mortality measured at day 14. Day 14 mortality and day 8 feeding inhibition data used for detailed analysis for nematodes grown in monoxenic culture with the five different bacteria are shown in Figure 20 and Figure 21 respectively.

Because of the low numbers of slugs used per dose in each replicate, the data was not suitable for analysis of variance and mortality data were analysed as follows. The proportion of slugs which had died was calculated for each dose for nematodes monoxenised with the different bacteria. For each type of monoxenised nematode, the doses were ranked from 1-5 based on the proportion of slugs which had died at each dose and the data for all monoxenic nematodes were then was analysed using Friedman’s test (Campbell 1989).
Figure 18. Percentage slug mortality and feeding inhibition measured at 8, 11 and 14 days following treatment with different numbers of nematodes grown in monoxenic culture with *Moraxella phenylpyruvica*, *Providencia rettgeri* or *Serratia proteamaculans*. 
Ps. fluorescens (140) mortality

Ps. fluorescens (140) feeding inhibition

Ps. fluorescens (141) mortality

Ps. fluorescens (141) feeding inhibition

Figure 19. Percentage slug mortality and feeding inhibition measured at 8, 11 and 14 days following treatment with different numbers of nematodes grown in monoxenic culture with *Pseudomonas fluorescens* (140) or *Pseudomonas fluorescens* (141).

This test showed that there were significant ($P<0.01$) differences in mortality in relation to dose for the five monoxenic nematodes. Feeding inhibition was analysed using analysis of variance. Differences in feeding inhibition in relation to dose for the five monoxenic nematodes were highly significant ($P<0.001$). These analyses revealed no significant differences in either mortality or feeding inhibition between the two different monoxenic pathogenic nematodes, those monoxenised with *M. phenylpyruvica* and *Ps. fluorescens* (141).
Figure 20. Percentage slug mortality measured 14 days after treatment with different numbers of nematodes grown in monoxenic culture with all five bacteria (M. phenylpyruvica, Pr. rettgeri, S. proteamaculans, Ps. fluorescens (140) and Ps. fluorescens (141). Data were analyzed using Friedman's test.

Figure 21. Percentage feeding inhibition of slugs measured 8 days after treatment with different numbers of nematodes grown in monoxenic culture with all five bacteria (M. phenylpyruvica, Pr. rettgeri, S. proteamaculans, Ps. fluorescens (140) and Ps. fluorescens (141). Data were analyzed using ANOVA.
It can be seen from Figures 20 and 21 that slug mortality and feeding inhibition increased linearly with log nematode dose for nematodes monoxenised with *M. phenylpyruvica* and *Ps. fluorescens*(141), but there was no such response for nematodes monoxenised with *Pr. rettgeri*, *S. proteamaculans* or *Ps. fluorescens*(140). To further investigate the relationship between nematode dose and slug mortality and feeding inhibition for the pathogenic nematodes monoxenised with *M. phenylpyruvica* and *Ps. fluorescens* (141), logistic curves were fitted to the mortality data recorded at day 14 and the feeding inhibition data recorded at day 8 for the nematodes monoxenised with these two bacteria. Curves took the form:

\[
y = \frac{100}{1 + (x^{-b})(\exp(-m)^{-b})}
\]

Where \( y \) = the % mortality in slugs (or % feeding inhibition), \( x \) = the number of nematodes per assay box, and \( b \) and \( m \) are constants. The curves for mortality are shown in Figure 22 and those for feeding inhibition are shown in Figure 23.

The relationship between nematode dose and mortality for nematodes monoxenised with *M. phenylpyruvica* was best described by the function

\[
y = \frac{100}{1 + (x^{-3.84})(\exp(-4.4578)^{-3.84})}
\]

which is highly significant (\( P<0.01 \)) and accounts for 78% of the variation in slug mortality. This function estimates the ED\(_{50}\) (the dose of nematodes needed to kill 50% of slugs by day 14 in this bioassay system) at 28,700 with a standard error (S.E.) of 4870.
Figure 22. Logistic curves fitted to describe the dose response relationship between % slug mortality and numbers of nematodes per assay box measured at day 14 for nematodes monoxenised with *M. phenylpyruvica* or *Ps. fluorescens* (141).

Figure 23. Logistic curves fitted to describe the dose response relationship between % feeding inhibition of slugs and numbers of nematodes per assay box measured at day 8 for nematodes monoxenised with *M. phenylpyruvica* or *Ps. fluorescens* (141).
The relationship between slug mortality and nematode dose for nematodes monoxenised with *Ps. fluorescens* was best described by the function:

\[
y = \frac{100}{1 + (x^{-1.903})(\exp(-4.713)^{-1.905})}
\]

which is also highly significant (*P* < 0.01) and accounts for 56% of the variation in slug mortality. This function estimates the ED50 to be 51,700 with S.E. 13,900, not significantly different from that estimated for nematodes monoxenised with *M. phenylpyruvica*.

The relationship between feeding inhibition of slugs and nematode numbers for nematodes monoxenised with *M. phenylpyruvica* was best described by the function:

\[
y = \frac{100}{1 + (x^{-3.191})(\exp(-4.557)^{-3.191})}
\]

This was highly significant (*P* < 0.01) and accounted for 82% of the variation in feeding inhibition. This estimated the ED50(f) (The number of nematodes needed to cause a 50% reduction in feeding by slugs between days 5 and 8 using this bioassay) to be 36100 with S.E. 5120.

The equivalent relationship for nematodes monoxenised with *Ps. fluorescens* (141) was

\[
y = \frac{100}{1 + (x^{-2.3})(\exp(-4.736)^{-2.3})}
\]

which was highly significant (*P* < 0.001) and accounted for 95% of the variation in feeding inhibition. The ED50(f) was estimated at 54400 with S.E. 4375 which is significantly (*P* < 0.05) greater than the estimate for nematodes monoxenised with *M. phenylpyruvica*.

A dose response was evident in all replicates of assays using nematodes monoxenised with *M. phenylpyruvica* or *Ps. fluorescens* whereas all replicates of nematodes monoxenised with *Ps. fluorescens* (140), and *S. proteamaculans* showed no dose response.
monoxenised with *Pr. rettgeri* the nematodes appeared to be highly pathogenic. Figure 24 shows % mortality in slugs in the seven replicate assays using nematodes monoxenised with *Pr. rettgeri*, the atypical replicate can easily be distinguished. The nematode cultures for all seven replicates were tested for monoxenicity but no contamination was found in any.

![Graph showing percentage mortality of slugs](image)

**Figure 24.** Percentage mortality of slugs measured at day 14 in seven replicate experiments investigating the dose response relationship for slugs treated with different doses of nematodes monoxenised with *Pr. rettgeri* showing one atypical replicate (rep. 1).

9.3.2. Bioassays using monoxenic nematodes grown in liquid cultures at AGC Littlehampton.

9.3.2.1. First experiment using nematodes produced in liquid culture.

The relationships between slug mortality and nematode dose for both nematodes monoxenised with *M. phenylpyruvica* or *Pr. rettgeri* are shown in Figure 25. Non-linear regression analysis was used to fit Logistic curves, as described in 9.3.1., to the mortality
data. The relationship between nematode dose and slug mortality at day 14 for nematodes monoxenised with *M. phenylpyruvica* was best described by the function

\[
y = \frac{100}{1 + (x^{-3.06}) (\exp(-4.5726)^{-3.06})}
\]

which is highly significant \((P<0.01)\) and accounts for 70 \% of the variation in slug mortality. This function estimates the ED50 to be 37400 with S.E. 7130.

It can be seen from fig 25. that there was no evidence of a dose response for nematodes monoxenised with *Pr. rettgeri* and it was not possible to fit this type of logistic curve to the slug mortality data obtained for nematodes monoxenised with *Pr. rettgeri* because the residual variance of the best curves exceeded the variance of the mortality. The feeding inhibition response measured at eight days for the two different monoxenic nematodes showed the same trend (Figure 26). Again a logistic function:

\[
y = \frac{100}{1 + (x^{-2.976}) (\exp(-4.2813)^{-2.976})}
\]

described the relationship between nematode dose and feeding inhibition well for nematodes monoxenised with *M. phenylpyruvica* \((P<0.001)\) and accounted for 91 \% of the variation in feeding inhibition. The ED50(f) was estimated to be 19100, with S.E. 2220. There was no evidence of increased feeding inhibition by slugs with increasing nematode dose for nematodes monoxenised with *Pr. rettgeri* (Fig 26), and it was not possible to fit a logistic curve to this data.

Only one out the 10 slugs from the boxes treated with 75000 nematodes monoxenised with *Pr. rettgeri* which were dissected and examined for the presence of nematodes was found to have any nematodes in the shell cavity. This slug contained many juveniles (Stages L1 and L2) but only one adult nematode, suggesting that the infection had probably been initiated by just one infective juvenile. This suggested that the nematodes grown on *Pr. rettgeri* in this experiment were failing to find or enter slugs.
Figure 25. Dose response relationship between % slug mortality measured at day 14 and nematode dose for nematodes monoxenised with *M. phenylpyruvica* or *Pr. rettgeri* in the first experiment using nematodes produced in liquid at AGC Littlehampton.

Figure 26. Dose response relationship between % feeding inhibition measured at day 8 and nematode dose for nematodes monoxenised with *M. phenylpyruvica* or *Pr. rettgeri* in the first experiment using nematodes produced in liquid at AGC Littlehampton.
9.3.2.2. Second experiment using nematodes produced in liquid culture.

This experiment used identical methods to experiment 1 but used different batches of nematodes monoxenised with *M. phenylpyruvica* or *Pr. rettgeri* which had been grown under identical conditions at AGC Littlehampton. The relationships between slug mortality and feeding inhibition and nematode dose of these two differently monoxenised nematodes are shown in Figures 27 and 28 respectively. While the methods used in the production of the nematodes and the bioassay were identical, the results were quite different. In this experiment both nematodes monoxenised with *M. phenylpyruvica* and *Pr. rettgeri* caused slug mortality and feeding inhibition. Both batches of nematodes were checked for monoxenicity and no contaminating bacteria were found.

Logistic curves were fitted to the mortality data and feeding inhibition data for nematodes monoxenised with both bacteria (Figures 27 and 28).

The relationship between slug mortality and nematode dose for nematodes monoxenised with *M. phenylpyruvica* was best described by the function:

\[
y = \frac{100}{1 + (x^{-7.65})(\exp(-4.2764)^{-7.65})}
\]

which is highly significant (*P* < 0.001) and estimates the *ED₅₀* to be 18897 with S.E. 1656 which is significantly lower (*P* < 0.05) than the *ED₅₀* estimated for nematodes monoxenised with *M. phenylpyruvica* in the first experiment using nematodes grown in liquid cultures.

The function describing the dose mortality relationship for nematodes monoxenised with *Pr rettgeri* was:

\[
y = \frac{100}{1 + (x^{-3.84})(\exp(-4.4578)^{-3.84})}
\]

which again was highly significant (*P* < 0.001) and accounted for 77.5% of the variation in slug mortality. The estimated *ED₅₀* was 28698 with a S.E. of 4874. Which is not significantly different from the estimate for *M. phenylpyruvica* in this experiment.
The feeding inhibition dose response for \textit{M. phenylpyruvica} was best described by the function:

\[ y = \frac{100}{1 + (x^{-7.65})(\exp(-4.28)^{-7.65})} \]

This is highly significant ($P < 0.001$) and accounts for 90\% of the variation in feeding inhibition. The ED$_{50}$ was estimated to be 18894 with S.E. 1654.

The feeding inhibition dose response for \textit{Pr. rettgeri} was best described by the function

\[ y = \frac{100}{1 + (x^{-4.394})(\exp(-4.536)^{-4.394})} \]

This is highly significant ($P < 0.001$) and accounts for 99\% of the variation in feeding inhibition. The ED$_{50}$ was estimated at 34353 with S.E. 1253. This ED$_{50}$ is significantly ($P < 0.001$) higher than the estimate for nematodes monoxenised with \textit{M. phenylpyruvica} in this experiment.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure27.png}
\caption{Dose response relationship between \% slug mortality measured at day 14 and nematode dose for nematodes monoxenised with \textit{M. phenylpyruvica} or \textit{Pr. rettgeri} in the second experiment using nematodes produced in liquid at AGC Littlehampton.}
\end{figure}
**Figure 28.** Dose response relationship between % feeding inhibition measured at day 8 and nematode dose for nematodes monoxenised with *M. phenylpyruvica* or *Pr. rettgeri* in the second experiment using nematodes produced in liquid at AGC Littlehampton.

### 9.4. Discussion

The bioassays of nematodes grown in monoxenic foam chip cultures clearly showed that growing the nematodes in monoxenic culture with different bacteria can significantly alter the observed activity of the nematodes against slugs. It is difficult from these results alone, however, to suggest what the cause of the different activities might be. One possible explanation is that there are differences in the pathogenicity of different bacteria to slugs; nematodes enter the slug and release cells of the bacterium on which they were grown, and certain bacteria on release, are more likely to kill the slugs and/or provide better conditions for nematode growth resulting in toxic products leading to the death of the slugs. However, the experiments described in section 8 showed that of the bacteria used for monoxenic culture of nematodes, only *Ps. fluorescens* (140) is capable of killing slugs on its own when introduced into the slug’s haemocoel. In the monoxenic bioassays, nematodes monoxenised with *Ps fluorescens* (140) had no pathogenic effect on slugs. It
is also worth noting that the nematodes which were pathogenic in the monoxenic bioassays, those monoxenised with _M. phenylpyruvica_ and _Ps. fluorescens_ (141) were carrying bacteria which were not pathogenic to slugs, even when injected directly into the slug’s haemocoel in very high numbers, suggesting that unlike entomopathogenic nematodes, it is the nematode that is of prime importance in the killing of slugs and not the bacterium. It is possible, however, that differences could exist in the pathogenicity of bacteria, but these difference lie in the way in which the bacteria interact with nematodes when in the slugs shell cavity and that these differences could not be detected in the experiments described in section 8.

Another possibility is that it is the number of bacteria that are carried into the slug that is important rather than which species of bacteria they are; if no bacteria were carried into the slugs the bacterial multiplication necessary to allow nematode growth could not occur. Poinar (1979) grew _Neoaplectana_ (=_Steinernema_ glaseri) in monoxenic culture with its own symbiotic strain of _X. nematophilus_, with the symbionts of other entomopathogenic nematodes, and with _Alcaligenes faecalis_, _Proteus_ (=_Providencia_ rettgeri) and _Pseudomonas aeruginosa_, and stated that no bacterial cells were retained in _S. glaseri_, except for when it was grown on its own symbiotic strain of _X. nematophilus_. Also, Dunphy, Rutherford and Webster (1985) showed that _Steinernema glaseri_ was more virulent when grown on certain subspecies of _Xenorhabdus nematophilus_ than on others and found that virulence correlated well with the proportion of infective juveniles which retained bacteria. However, while no detailed quantitative study has been done of the ability of _Phasmorhabditis_ sp. to retain different bacteria, bacterial cells have been isolated from within surface sterilised infective juvenile nematodes grown in monoxenic culture with all five bacteria used in these bioassays, with no obvious differences in the numbers of cells isolated from each monoxenic nematode. Detailed quantitative studies of bacterial retention would be needed to confirm whether or not this is the case, but present observations, coupled with the fact the nematodes will probably take bacteria into the slug on their cuticles (Poinar 1979) make this explanation for the failure of certain monoxenic nematodes to cause disease seem unlikely.

Another possibility is that for some reason the host finding ability of nematodes is in some way impaired or altered if the nematodes are grown on different bacteria. This possibility, although difficult to explain, is certainly supported by the fact that in the first bioassay using nematodes grown in monoxenic liquid cultures with _Pr. rettgeri_, only one out of the 10 slugs examined from the boxes treated with the highest dose of nematodes
was found to have any nematodes in the shell cavity. This slug contained many juveniles (Stages L1 and L2) but only one adult nematode, suggesting that the infection had been initiated by just one infective juvenile. The infective juveniles monoxenised with *Pr. rettgeri* used in this assay appeared no different from those monoxenised with *M. phenylpyruvica* used in the same assay and both monoxenic nematode types appeared equally active when observed in the counting chamber immediately prior to use in the bioassay. The fact that the nematodes monoxenised with *Pr. rettgeri* did not find the slugs suggests that either these worms had lost the ability to seek out slugs, or that they became quiescent when applied to the soil and did not actively seek out slugs. *Steinernema feltiae* has been shown to remain at the point of application when applied to soil (Moyle & Kaya 1981, Georgis & Poinar 1983) and it has been suggested that nematodes become quiescent in soil in the absence of a host (Ishibashi & Kondo 1986). It is difficult to imagine how being grown in monoxenic culture with different bacteria could induce quiescence in nematodes when applied to the soil. Ishibashi & Kondo (1986) found that quiescence in *S. feltiae* was more pronounced when nematodes were applied to non-sterile soil rather than to steam-sterilised soil which suggest that micro-organisms in the nematode's immediate environment can affect quiescence. Certain chemical substances are known to activate steinernematid nematodes (Ishibashi & Kondo 1990) and these have been used to increase the performance of nematodes in field trials (Ishibashi, Choi & Kondo 1987). It is possible that certain bacteria produce activating chemicals which could increase the activity of the nematodes when applied to soil.

The fact that nematodes monoxenised with *Pr. rettgeri* gave inconsistent results is puzzling. Biochemical test using API strips were done with *Pr. rettgeri* taken from both the pathogenic and non-pathogenic batches of nematode produced on this bacterium and no differences were found. There were no differences in the colonial morphology of the two isolates (J. Pearce pers. comm.). It is noteworthy that nematodes monoxenised with *Pr. rettgeri* have been used in more replicate assays than any other monoxenic nematode combination, if those assays using nematodes produced in liquids are included. It is possible that if the other bacteria/nematode combinations were used in many more replicate assays, similar inconsistencies would be found.

Nematodes monoxenised with *M. phenylpyruvica* appeared to be the most consistently pathogenic to *D. reticulatum* of the five nematode/bacterial combinations tested in this series of bioassays. Nematodes monoxenised with *Ps. fluorescens* (141) were almost as
pathogenic as those monoxenised with *M. phenylpyruvica*. Un-reliability could not be tolerated in a commercial product, and while two nematode/bacterium combinations were reliably pathogenic in this limited series of bioassays, it would certainly be advisable to investigate the reasons for differences in pathogenicity between different batches of nematodes monoxenised with *Pr. rettgeri*. 
10. GENERAL DISCUSSION

In this thesis experimental results have been discussed in each individual section. This general discussion will summarise progress and needs for future work.

10.1. The relationship between bacteria and nematodes.

This study shows that Phasmorhabditis sp. feeds on bacteria both in vitro and in-vivo. Microscopic examination of fluid taken from the shell cavity of infected slugs reveals that bacteria are present, and while it is not known whether the nematodes could complete their life cycle feeding on slug haemolymph without bacteria, a bacterial food source seems to be the norm (as would be expected in a rhabditid nematode). It has yet to be established whether the bacteria in the shell cavity are carried into the slug in the nematode gut, as are cells of Xenorhabdus in entomopathogenic nematodes; or on the nematode body surface; or if they are bacteria associated with the slug which infect opportunistically following entry of the nematodes. However, it would seem that Phasmorhabditis sp. does not vector a specific pathogenic bacterium as do entomopathogenic nematodes. Reasons for this belief are:

1. The failure to isolate any one species of bacterium consistently from infected slugs, or infective juvenile nematodes (Section 1).

2. The infective juveniles have no anatomical adaptation for carrying bacteria similar to the intestinal vesicles (Bird & Akhurst 1983) seen in entomopathogenic nematodes (Paul Richardson pers. comm.).

3. The time until death following nematode infection is very much longer in Phasmorhabditis sp. (usually between 7-21 days) than in entomopathogenic nematodes in which the highly pathogenic cells of Xenorhabdus once released kill the insect host usually within 48 hours.

4. Slugs lack a rigid cuticle. Such a cuticle is thought to be important in development of entomopathogenic nematodes because it maintains a closed environment, free from contaminating bacteria. Poinar (1989) in reviewing non-insect hosts of entomopathogenic

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nematodes states that nematodes can penetrate certain non-insect hosts but do not complete their life cycle because breakdown of the host body wall exposes the nematodes to the environment and contaminating bacteria. The slug body wall is soft and breaks down completely very shortly after death (See Plate 10). Many soil bacteria multiply on the corpse and thus a nematode parasite of slugs cannot afford to be fastidious in its bacterial food requirements. Such a parasite would have to be adapted to feeding on a wide range of bacteria, as is the case in Phasmorhabditis sp.

The reasons for the significant differences in pathogenicity to slugs of nematodes grown on different bacteria are not known. Clearly more research is needed to establish the underlying causes of the failure of certain monoxenic nematodes to kill slugs.

10.2. The potential of Phasmorhabditis sp. as a biological control agent of slugs

Gaugler (1981) considered that entomopathogenic nematodes of the genera Steinernema and Heterorhabditis possess virtually every attribute of an "ideal" biological control agent. Characteristics which make these nematodes particularly suitable for commercial use as biological insecticides include:

1. They are considered to be safe, and are thus (like all nematodes) exempt from pesticide registration schemes.
2. They have a broad host range.
3. They are easily mass produced in vitro.
4. They have a durable infective stage which is amenable to formulation.
5. They have high virulence and kill the host rapidly within 48 hours.

The preliminary examination of Phasmorhabditis sp. suggest that this nematode too, shares many of these characteristics. Being a nematode it would not need to be registered, it kills a wide range of slug species, a property essential in a biological molluscicide, it can be reared in-vitro in liquids and is thus likely to be suitable for bulk fermentation and it too forms a durable infective larva. Although Phasmorhabditis sp. does not cause rapid death of the host in the same way as entomopathogenic nematodes, it does strongly inhibit feeding by slugs shortly after infection. In the first micro-plot trial significantly less slug damage was recorded in Chinese cabbage seedlings in plots treated with nematodes than
in untreated plots at the first reading, only four days after application. A crop protection agent needs only to stop the host from damaging the crop and rapid death of the host is not essential.

The potential of *Phasmorhabditis* sp. is further increased by the fact that slugs are pests which are only poorly controlled by chemical means and hence represent a market where biological control might succeed.

However, entomopathogenic nematodes, more so than certain other biological control agents (e.g. *Bacillus thuringiensis*) are still expensive to produce and command a high price. Thus their use in developed countries is at present restricted to high value crops, e.g. glasshouse ornamentals and mushrooms. The main markets in the U.K. for slug control are in low value arable crops, winter wheat, maincrop potatoes and oilseed rape. At present there are no biological control agents for pests commercially available for use on arable crops. Nevertheless, production and formulation costs for entomopathogenic nematodes have fallen in recent years and there is much scope for reducing them further (Friedman 1990). Thus it is possible that *Phasmorhabditis* sp. could be used commercially in the future as a biological molluscicide against slugs in arable crops. Until then *Phasmorhabditis* sp. could be used in higher value crops in which slugs are a problem.

Lettuce, cannot be commercially produced without protection with molluscicides (Symondson 1989). Also organic vegetables command high prices and by nature cannot be treated with chemical molluscicide. Slugs are particularly troublesome in organic lettuce production and many organic growers have abandoned this crop because of slug damage (Peacock & Norton 1990).

It is also possible that *Phasmorhabditis* sp. could be used in a "semi-classical" way. This would involve introducing relatively small numbers of nematodes into a field with the aim of building up a nematode population capable of suppressing slugs and protecting future crops. For example it might be possible to treat fields of oilseed rape with the nematode with the hope that the nematode would stop the build-up of slug populations associated with this crop, and thus protect the following winter wheat crop. Much data would be needed on the efficacy of this approach before farmers would be likely to apply a crop protection agent to a crop in which the perceived threat of the pest is low.
10.3. Future Work.

Research on entomopathogenic nematodes has become increasingly widespread throughout the world over the last decade (Gaugler and Kaya 1990). However, recent review articles considering these worms from the view of parasitologists (Homminick and Reid 1990) and with respect to their use as biological control agents (Ehler 1990) show that there is much work that needs to be done before these nematodes can be used to give consistently reliable and predictable control of plant pests. This study represents the first assessment of *Phasmorhabditis* sp. as a potential biological control agent, and its rather limited scope leaves a wealth of research that remains to be done into the biology, ecology, behaviour, and physiology of *Phasmorhabditis* sp.. However, certain research priorities both fundamental and applied can be identified.

Fundamental research into the nematode which must be done soon includes:

1. **Taxonomy:** populations with and without males present should be examined using molecular biological techniques to determine whether or not *P. hermaphrodita* and *P. neopapillosa* are indeed different species, and, their actions against slugs should be assessed individually, with a range of populations of each tested for pathogenicity to slugs.

2. **Bacterial associations:** more work must be done to determine how growth in monoxenic culture with different bacteria affects the pathogenicity of the worms to *D. reticulatum* and to see if differences in pathogenicity apply to all species of slugs.

3. **Nematode response to environmental conditions:** All bioassays have been done in clay soils moistened with 30% (w/w) water at 10°C, a temperature typical of soil temperatures at the time of sowing winter wheat. Studies on the tolerance and behaviour of *Phasmorhabditis* sp. in relation to temperature, soil moisture, and soil texture would provide information relevant to the applied aspects of using the nematode.

4. **Natural epizootics:** Valuable information could also be gained by studying natural infestations of slugs by *Phasmorhabditis* sp..
Applied research into the use of the nematode which will need to be done to make commercialisation possible includes:

1. Monoxenic growth in liquids: other bacteria capable of supporting monoxenic growth of *Phasmarhabditis* sp. in liquid cultures should be identified and nematodes produced in this way must be bioassayed against slugs.

2. Formulation: nematode survival has been assessed only in aerated tap water. However, it will be necessary to develop formulations in which *Phasmarhabditis* sp. can survive, and which are compatible with growers' needs. Formulations used for entomopathogenic nematodes could be tried initially, but further research could lead to better formulations for use in arable crops.

3. Application: the dose range at which the nematode is effective and the optimum timing and method of nematode application to different crops will need to be determined. Information obtained from bioassays may provide useful information but ultimately these factors will have to be determined in the field.

4. Host range: this study has shown that *Phasmarhabditis* sp. can kill several species of pest slugs but it is not known whether this nematode can kill snails. Many snails are known to be pests of crops, and also certain snail species act as vectors for human diseases (Godan 1983).

5. Effects on non-target organisms: The anatomy and physiology of other soil invertebrates such as arthropods and earthworms are very different from those of slugs, and thus it seems unlikely that *Phasmarhabditis* sp. would kill beneficial organisms. Nevertheless it is important to assess the impact of this nematode on non-target organisms, especially soil invertebrates, in the field.

10.3 Conclusions

From the results of this study it can be concluded that *Phasmarhabditis* sp. has considerable potential as a biological control agent of slugs, although in the short term its use may be restricted initially to higher value crops, which represent only a small section
of its potential market. While it might be possible to develop a product using only the information presented in this thesis and further applied research into the production, formulation and application of the nematode, it would be wise to do this in parallel with fundamental research on the biology of the nematode. The precise taxonomy of the nematode is unclear and environmental tolerance is unknown. Furthermore the relationships between the nematode and bacteria are not yet fully understood, and the activity against slugs of Phasmorhabditis sp. grown in monoxenic culture with one particular species of bacterium has been inconsistent. Further research into the relationship between Phasmorhabditis sp. and different bacteria might not only explain the observed differences in pathogenicity and thus improve predictability of control, but also provide information relevant to improving nematode activity in the field, thus reducing its production costs.
11. REFERENCES


Kennedy, P.J. (1988). The use of polythene barriers to study the long term effects of pesticides on ground beetles (Carabidae; Coleoptera) in small-scale field experiments. In: Environmental Effects of Pesticides (Ed Greaves et al.) BCPC Monograph No. 40. British Crop Protection Council, Thornton Heath, UK.


