# Characterisation of the bacterial flora associated with the grey field slug *Deroceras reticulatum* and assessment of its suitability as a target for biological control.

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# Abstract

The field slug *Deroceras reticulatum* is a major pest in UK agriculture and amidst growing concern and regulatory pressures surrounding chemical molluscicides, innovation is required to advance the current repertoire of slug controls. This study set out to investigate the bacteria associated with *D. reticulatum* to assess their importance to the slug and potential as a target for biological control. Slug gut bacterial isolates identified using the phenotypical API system (BioMérieux) and 16S rRNA gene sequencing, were mainly soil-dwelling organisms of the phyla Proteobacteria and Bacteriodetes some of which may be important in human or plant disease.

A ribosomal intergenic spacer analysis (RISA) was developed to study microbial communities in the slug gut. Slugs had an average species richness of 12 and comparing the bacterial communities in slugs from different locations yielded a mean similarity of 0.159 (Jaccard index) which was significantly lower than similarity indices of slugs collected within a single location (Mean Jaccard index 0.205, $p<0.001$, ANOVA). Cloning and sequencing of RISA bands common to slugs and slug eggs, but absent from the surrounding soil and plants identified bacteria for future investigation as potential beneficial symbionts.

Bacteria extracted from the slug gut were tested for sensitivity to 16 antibiotics and greatest inhibition of growth was observed for chloramphenicol, gentamicin and tetracycline. These antibiotics administered to slugs by feeding and injection caused a reduction in gut-associated bacteria in plate counts, and in bacterial 16S rDNA quantities estimated by real-time quantitative PCR. Field collected *D. reticulatum* has a large transient gut bacterial population which is reduced upon starvation to a low background level. No significant detrimental effect of antibiotic treatment on the fitness and survival of the slugs was seen, in some instances control slugs suffered greater mortality than slugs that had been injected with antibiotic. Slugs that died during bioassays had a significantly greater amount of bacterial 16S rDNA in their gut than slugs that were sacrificed as healthy individuals suggesting the presence of a bacterial pathogen. This study has found little evidence that a bacterial symbiont may exist and be important for optimal fitness and survival of *D. reticulatum*, but insight into slug associated bacteria will be valuable in the direction of future studies in this field.

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## Declaration

I hereby declare that the production of this thesis, presented for examination for the degree of PhD, was completed by myself and the work presented herein is my own, except where explicitly stated otherwise in the text.

This thesis has not been submitted, in whole or in part, for any other degree or professional qualification at this or any other institution.

**Peter Wilkinson**

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# Abbreviations

| | |
|---|---|
| %ID | Percentage identity |
| (A)RISA | (Automated) ribosomal intergenic spacer analysis |
| (T-)RFLP | (Terminal)-Restriction fragment length polymorphism |
| AMF | Arbuscular mycorrhizal fungi |
| ANCOVA | Analysis of covariance |
| ANOVA | Analysis of variance |
| API | Analytical Profile Index (BioMérieux) |
| a.s. | Active substance |
| BYDV | Barley Yellow Dwarf Virus |
| cfu | Colony forming units |
| CTAB | Cetyl trimethyl ammonium bromide |
| DAPG | 2,4-diacetyl-phloroglucinol |
| Defra | Department for the Environment, Farming and Rural Affairs, UK Government |
| DNA | Deoxyribonucleic acid |
| dNTP | Deoxynucleotide triphosphate |
| EDTA | Ethylenediaminetetraacetic acid |
| HGCA | Home Grown Cereals Authority |
| ICM | Integrated Crop Management |
| IPM | Integrated Pest Management |
| IPTG | Isopropyl beta-D-1-thiogalactopyranoside |
| ITS | Internal transcribed spacer |
| KOH | Potassium hydroxide |
| MRL | Maximum residue level |
| MSG | Metaldehyde Stewardship Group |
| OSR | Oilseed rape |

| | |
|---|---|
| OTU | Operational taxonomic unit |
| PCR | Polymerase chain reaction |
| PVPP | Polyvinylpyrrolidone |
| qPCR | Quantitative-polymerase chain reaction |
| rDNA | Ribosomal deoxyribonucleic acid |
| rRNA | Ribosomal ribonucleic acid |
| SAC | Scottish Agricultural College, Edinburgh |
| SDS | Sodium dodecyl sulfate |
| SDW | Sterile distilled water |
| SED | Standard error of difference |
| SEM | Standard error of the mean |
| SSCP | Single strand conformation polymorphism |
| T/DGGE | Temperature/Denaturing gradient gel electrophoresis |
| TRF | Terminal restriction fragment |
| tRNA | Transfer ribonucleic acid |
| UPGMA | Unweighted pair group method with arithmetic mean |

# Chapter 1

# Introduction

## 1.1 Why are Slugs a Problem?

Slugs are one of the most important pests in agriculture and horticulture. In Britain the species of greatest economic importance are *Deroceras reticulatum* (Müller, 1774), *Arion hortensis* (Férussac, 1819) and *Tandonia budapestensis* (Hazay, 1881) (Figure 1.1). *D. reticulatum,* the grey field slug, is widespread throughout temperate regions of the world and is currently the commonest and most destructive species in northern Europe (Port & Port, 1986; South, 1992; Salvio *et al.*, 2008). Many of the slugs of concern in areas of North America, New Zealand and Australia are introduced European species (Barker, 1979; Micic *et al.*, 2007). Slugs cause direct damage to a huge variety of crops and at virtually all stages of plant growth.


**Figure 1.1.** Common pest slug species. A, Grey field slug, *Deroceras reticulatum*; B, Garden slug, *Arion hortensis*; C, Keeled slug, *Tandonia (Milax) spp.* Images P. Wilkinson (A), Dr. K. A. Evans, SAC (B,C).

Because they have a permeable integument, slugs are vulnerable to dehydration and hence have to be adaptable, spending much of their time seeking to preserve their moisture content by sheltering in cool, damp environments. Slugs are mostly active by night when temperatures are lower, retreating to shelter during the day. They favour dense vegetation and heavy, cloddy soils where the particle size is such to allow maximum contact between themselves and the soil to prevent moisture loss. Cracks are likely to form in heavy soils allowing slugs access below the surface to shelter. Slugs move deeper into the soil during warm, dry summer months and during particularly cold periods in winter and their surface activity is subsequently reduced. The slug life cycle, along with many aspects of slug behaviour and ecology is rather variable (Hunter & Symonds, 1971) and changes depending on climatic conditions, with *D. reticulatum* hatchlings taking over 1 year to first egg-laying at 5 °C but only 4.5 months at 18 °C (South, 1982). There are obvious peaks in slug activity

in the autumn and spring when conditions are more favourable (Glen *et al.*, 2006). *A. hortensis* and *T. budapestensis* become very inactive at 5 °C whilst *D. reticulatum* remains active at temperatures as low as 0.8 °C (Mellanby, 1961) posing a major threat to autumn-sown cereals which are slow to establish in the colder temperatures. Traditionally slug populations would be reduced considerably by harsh frosts during the winter but with current trends of climate change predicting warmer, wetter winters and hotter, drier summers, it is more difficult to say how slug populations will respond. Population models predict that the most favourable conditions for slugs will shift from the current areas of south-west England and Wales to northern Wales and western Scotland over the next 80 years (Willis *et al.*, 2006).

### *1.1.1 Slug damage in cereals*

Slug damage is particularly prevalent in wheat where slugs are amongst the three most important pests (Oakley & Young, 2000). Wheat seeds are at risk from grain hollowing (preventing germination) by slugs from the moment they are sown, and each slug is capable of killing up to 50 seeds (Glen *et al.*, 2006). Seedlings may be killed as slugs eat through the stems, or leaves may be shredded as slugs eat between the veins before or shortly after emergence (Figure 1.2C). Leaf damage is generally not so detrimental to yield in spring wheat as the plants quickly out-grow this risk 'window', however winter wheat is much more vulnerable as the plants grow more slowly in the cold and low-light conditions. Runham & Hunter (1970) briefly mention evidence that a small amount of leaf damage may stimulate the plant to tiller, actually having a positive effect on yield. Failure of wheat at establishment does not mean it is completely lost as the land can usually be re-drilled - the resultant extra costs are around 25% of the loss that would be incurred by the farmer if a crop was totally destroyed (Runham & Hunter, 1970). Barley seedlings are also eaten below the soil surface however slug damage in barley is not as significant as in wheat, possibly because it is generally sown earlier before slug activity has risen to critical levels. The potential cost of slug damage to the 1997/98 cereal crop in the UK was estimated at £5 million but actual damage came in at only half of this value, meaning the £4.3 million spent on control in this crop contributed to an overall loss of £1.8 million (Oakley & Young, 2000). Indeed, molluscicide usage varies considerably from season to season and depends largely upon autumn conditions influencing the time of sowing (Figure 1.3).

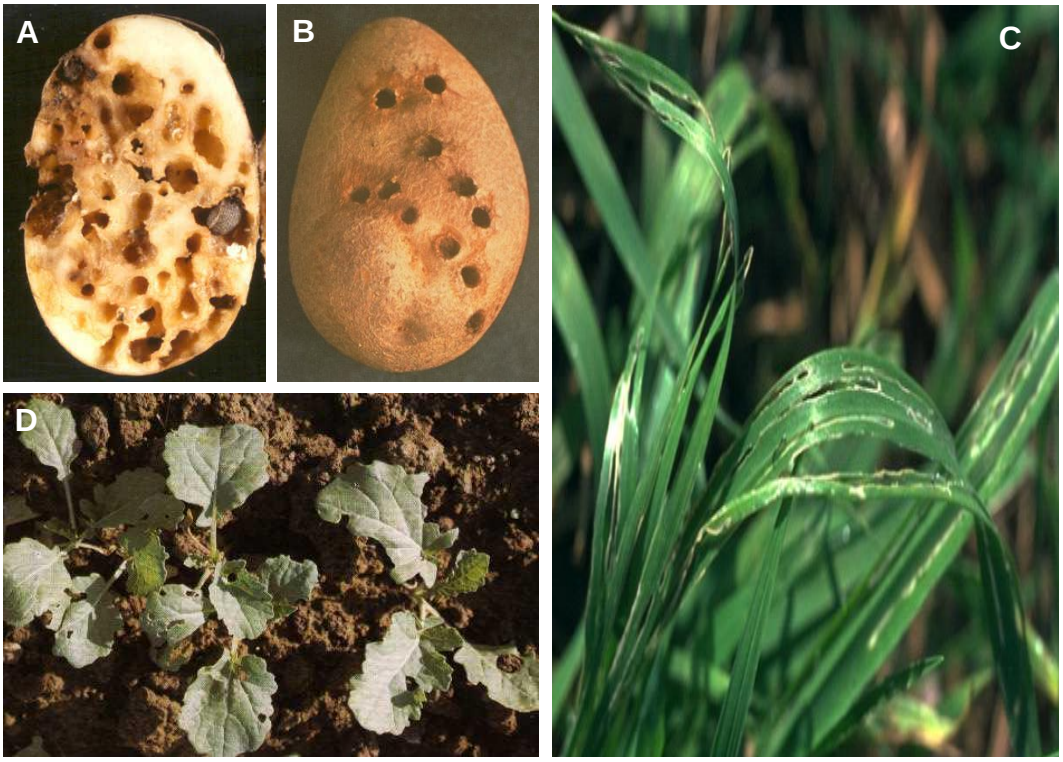



**Figure 1.2.** Examples of slug damage. A, B, hollowing of potato tubers; C, shredding of wheat leaves; D, damage to oilseed rape seedlings. Images Dr. K. A. Evans, SAC, and SAC Imagebank.

### *1.1.2 Slug damage in potatoes*

Although the above-ground parts of the potato plants can be subject to high levels of damage by slugs (Hunter *et al.*, 1968) it is hollowing of the tubers that is of greatest economical importance (Figure 1.2A,B). Slugs begin to feed on tubers from late summer until harvest, and later harvested crops are at greater risk, especially if the autumn is particularly wet (Port & Port, 1986). There are various consequences of slug damage in potatoes. Contamination is likely to result in the rejection of a whole consignment so grading lines must be run slower whenever slug damage is present, having knock-on economic effects (Beer, 1989). Stored potatoes are also at greater risk of secondary rots. Low quality potatoes destined for processing (greater than 10% damage) are worth around one fifth of the value per tonne of high quality potatoes for the pre-packed market (less than 5% damage), which equates to considerable losses when slug damage is severe (Beer, 1989). In potatoes, it is important that slug populations are sufficiently reduced before the tubers start to swell in late summer and slugs move underground where they will not encounter pellets (Meredith, 2003). These significant consequences of slug damage and because potatoes are at risk for a long time,

mean they often receive between two and three, and sometimes up to six applications (Beer, 1989).

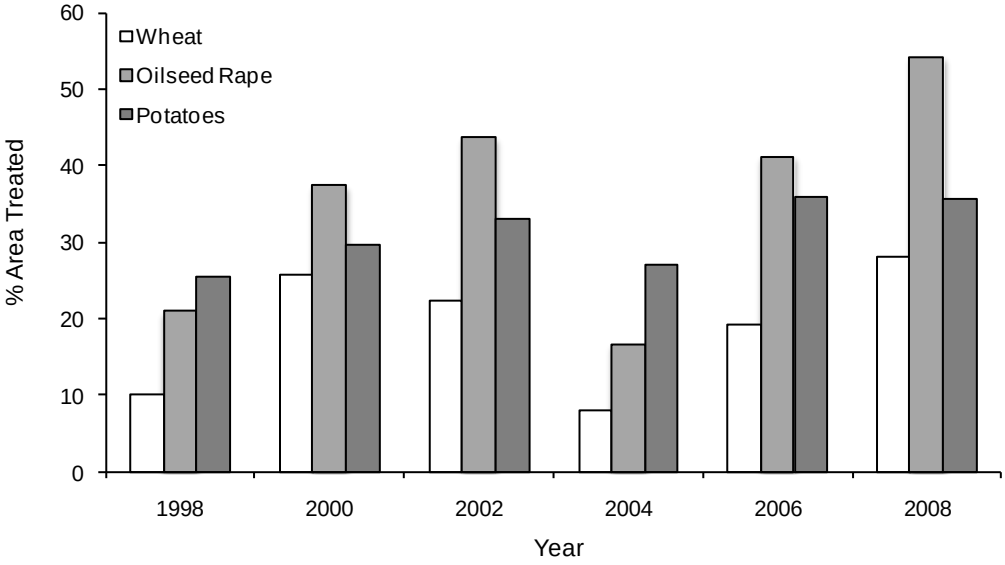



**Figure 1.3.** Percentage of the crop area treated with molluscicides for wheat, oilseed rape and potatoes in Great Britain between 1998 and 2008. Based on data from Garthwaite & Thomas (1998; 2000) and Garthwaite *et al.* (2002; 2004; 2006; 2008).

### *1.1.3 Slug damage in oilseed rape*

Oilseed rape is susceptible in the early stages of growth, with newly emerging seedlings at greatest risk (Figure 1.2D). Unlike winter cereals, the failure of establishment of winter oilseed rape has greater consequences as it may not be re-drilled (Glen & Moens, 2002). In reflection of this, the area of oilseed rape treated with molluscicides more than doubled from 20% of total area grown in 1998 to 54% in 2008 (Garthwaite & Thomas, 1998; Garthwaite *et al.*, 2008). Oilseed rape is a major crop in Europe, and the total area grown has seen a steady increase since the 1970s. More recently, government subsidies to encourage growth of rape as a biofuel have pushed this acreage even higher. In the 1980s, 'double low' cultivars were developed to reduce the levels of glucosinolates and erucic acid in the seeds in order to make the seed meal more palatable to livestock. Unfortunately this has also made the seedlings more palatable to slugs (Glen & Moens, 2002; Meredith, 2003).

### *1.1.4 Slug damage in vegetables and soft fruits*

Slugs also affect vegetable crops such as such as Brussels sprouts, cabbage, lettuce, and carrots where damage reduces quality and value. A large percentage of the area of Brussels sprouts grown each year is affected by slugs which bore holes in the sprout buttons. In 2003, Brussels sprouts and turnips/swedes received the greatest molluscicide treatment of all vegetables grown in Scotland, with 62% and 81% of each crop treated respectively (Struthers & Snowden, 2003). Contamination of produce by slugs is important in cabbages, lettuces and peas, increasing the likelihood of rejection by the buyer. In high value crops such as lettuce, slug control is of great importance, not only to prevent grazing of the leaves, but also to avoid contamination of the produce by either dead or living slugs, their faeces or slug pellets. Slugs must be controlled before they move into the lettuce heads to feed or seek shelter. For this reason lettuce crops often get two or three applications of slug pellets throughout the 9-10 week growing period. Field trials in lettuce showed that a single application around four weeks after sowing was sufficient to protect the crop until harvest (Glen *et al.*, 1996). Strawberries, blackcurrants, flower bulbs and cut flowers are also victims of slug damage reducing their marketability.

### *1.1.5 Slugs as vectors of pathogens*

Slugs are implicated as possible vectors of plant pathogens and vertebrate parasites. Rose (1960) demonstrated that the sheep lungworm, *Cystocaulus ocreatus,* was transmissible by ingestion of infected *D. reticulatum* when grazing. The importance of this slug as an intermediate host of the parasite is emphasised by the ubiquitous nature of this species, as it is almost certain to be found wherever sheep are grazing. In 1964, Wester *et al*. recorded slugs as carriers of downy mildew (*Phytophthora phaseoli*) and found viable fungal spores in the mucus covering the slugs. In agreement to these findings, it has also been shown that slugs are able to transmit rot-causing pathogens in stored carrots and potatoes (Dawkins *et al.*, 1985; Dawkins *et al.*, 1986). In both experiments four slug species (*D. reticulatum, A. hortensis, Limax maculatus, T. budapestensis*) were allowed to feed on carrot and potato infected with liquorice rot (*Mycocentospora acerina*) and bacterial soft rot (*Erwinia carotovora*) respectively. Test slugs were later transferred to containers with a slice of healthy carrot or potato which was removed daily to check for development of infection. They concluded that after contact with infected potatoes, all four species of slug continued to transmit *E. carotovora* to healthy tissue for a further 42 days (when the experiment was terminated), during which period the bacterium was continually isolated from the mucus and

faeces of the slugs. In the case of the liquorice rot of carrots however, the transmission of the fungus *M. acerina* was less consistent in different slug species and the association between slug and pathogen only transient, so although a possible vector for the disease, it is unlikely to be of great importance in the field. Potato blight (*Phytophthora infestans*) may also be transmitted by slugs (Runham & Hunter, 1970). The parasitic tapeworms of domestic fowl, *Davainea proglottina* (*Platyhelminthes*) and *Syngamus trachea* (*Nematoda*) as well as the Lancet liver fluke (*Dicrocoelium dendriticum*) of domestic herbivores and parasitic trematodes (*Brachylecithum orfi*) of grouse all have slugs as intermediate hosts (Brown, 1933; Kingston & Freeman, 1959).

## 1.2 Current Controls

### *1.2.1 Chemical control of slugs*

The molluscicidal properties of metaldehyde were accidentally discovered in the 1930s. Until then, metaldehyde was used in solid fuel tablets and it was by chance that farmers in France noticed fuel tablets left by picnickers were surrounded by dead slugs (Godan, 1983). The effects of metaldehyde upon the slug are two-fold, acting as an irritant causing desiccation from excessive mucus production, and at high concentrations, as a fatal nerve toxin (Thomas, 1948). After contact with metaldehyde, slugs can lose up to 30% of their body weight through mucus production (Cragg & Vincent, 1952) but this condition can be remedied by washing the slug with water or by resting in a moist environment (Thomas, 1948). At lower concentrations, the neurotoxic property manifests itself as an anaesthetic, immobilising the slug from all but excessive stimuli, but it can recover under the above conditions. Metaldehyde therefore is less effective in cool, moist or humid conditions where slugs can quickly reverse this water loss (Godan, 1983). In conditions favourable to the slug, only 30% die after feeding on metaldehyde (Wedgwood & Bailey, 1988). Wedgewood & Bailey (1988) demonstrated that metaldehyde inhibits feeding by reducing the meal length, number of bites per meal, the bite size and by disruption of the bite cycle thus increasing the chance of a slug, a) ingesting a sub-lethal dose and b) subsequently recovering. The feeding suppressant affects appear to be due to disruption of the normal electrophysiology of the buccal ganglia (feeding motor neurones) producing uncoordinated mouthing movements (Mills *et al.*, 1989). Metaldehyde is a strong repellent in its pure form and only attractive at

very low concentrations (Godan, 1983) thus presenting a considerable problem in creating an effective formulation and it is this compromise that works against metaldehyde as a slug bait.

The other main chemical utilised in slug control is the carbamate compound methiocarb (another carbamate molluscicide, thiodicarb, was withdrawn in 2008). Carbamates in various forms were already in use as insecticides, herbicides and fungicides before they were investigated for mollusc control in the 1950s and 1960s. Methiocarb was first to see widespread use, with thiodicarb becoming available some time later in the 1980s (Meredith, 2003). Initial field trials by Martin *et al.* (1969) returned an 82.9% reduction in damage to winter wheat with a methiocarb application of 5.5 kg/ha compared to a 57.3% reduction achieved with a 31 kg/ha application of commercially available 4% metaldehyde pellets. Methiocarb is an acetylcholine esterase inhibitor, acting as both a stomach and contact poison (Kelly & Martin, 1989). Upon poisoning, slugs show an initial hyperactive and disorientated movement followed by loss of muscle tone, paralysis and death (Godan, 1983). Godan (1983) states that carbamates are not affected by environmental conditions and that their toxicity is even increased in high humidities, and although this is perhaps an overstatement, they are at least more effective in damp and cold weather than metaldehyde. Trapping experiments highlight differences in the susceptibility of different slug species to methiocarb, with *Arion* species appearing most susceptible (Kelly & Martin, 1989).

Molluscicides are incorporated into cereal-based pellets with commercial formulations usually containing between 1.5% and 4% of active ingredient of metaldehyde or 2-4% for methiocarb. Because molluscicides are applied in bait form, it is crucial that slugs easily and quickly find and feed on the pellets in preference to the crop. In cereals, pellets can be either broadcast on the soil surface or drilled as an admix with the seeds, however, a recent Defra and HGCA-sponsored Sustainable Arable Link Project recommends broadcast application soon after sowing as the most successful method (Glen *et al.*, 2006). In some seedbeds, admixed pellets will be unavailable to slugs which survive to attack emerging seedlings.

A summary of Central Science Laboratory (CSL, now Fera), annual pesticide usage survey data (Garthwaite & Thomas, 1996), shows a marked increase in the use of molluscicides from 1970 to 1995. During this period, the area treated with molluscicides saw a 67-fold increase from just over 12,000 ha in 1970 to nearly 812,000 ha in 1995. This corresponds to an increase in applied weight of active substance from 6.7 tonnes to 251.9 tonnes. Indeed from 1995 onwards, molluscicides use has continued to increase, with a peak of 486 tonnes

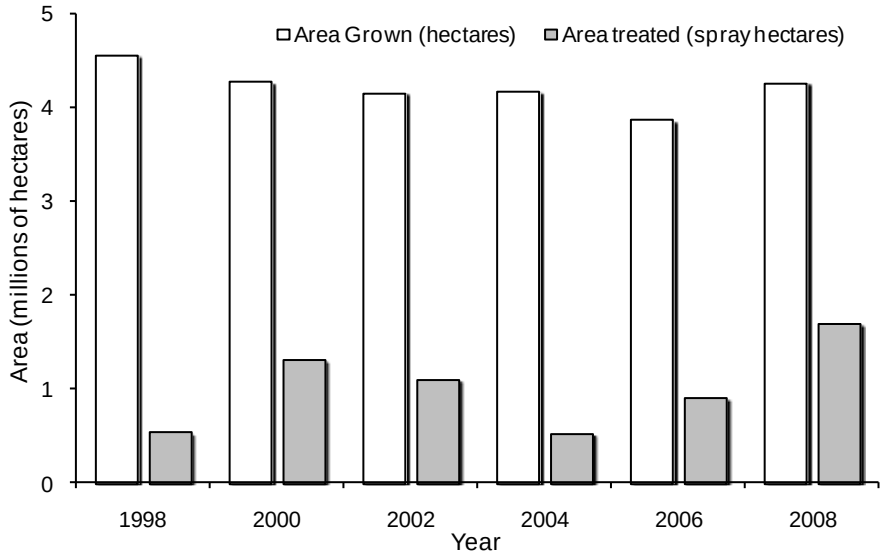



**Figure 1.4.** Molluscicide usage in arable crops in Great Britain from 1998 to 2008. Treated area refers to spray hectares for example one treatment applied to one hectare. A second application to the same hectare constitutes a second spray hectare. Based on data from Garthwaite & Thomas (1998; 2000)and Garthwaite et al. (2002; 2004; 2006; 2008).

of active substance applied to 1.7 million hectares in 2008 (Garthwaite *et al.*, 2008). Typically between 10% and 30% of UK grown cereals, up to 50% of oilseed rape and around 30% of potatoes are treated with molluscicides each year (Meredith, 2003) although these figures can vary substantially from one season to the next depending on the perceived slug threat (Figure 1.3). Arable crops account for the main molluscicide usage and in more recent years, the area of arable crops treated has fluctuated between around 0.5 million and 1.3 million hectares, with between 150 and 400 tonnes of active substances applied, despite relatively similar areas being grown (Figure 1.4). Garthwaite & Thomas (1996) estimated the cost of mollusc control (not including application costs or usage outside of agriculture) to be around £10 million in 1995 which reflects the perceived, if not real threat that slugs pose to UK agriculture alone. Currently, slug pellets cost on average £8 /ha for metaldehyde (Trigger, Allure, Metarex), and £15 /ha and £20/ ha for ferric phosphate (Sluggo, Sluxx) and methiocarb (Draza Forte, Decoy, Exit) respectively (Dr. K. A. Evans, SAC, Edinburgh. Personal communications). Based on 2010 prices for molluscicides, and revised application rates, the maximum 2008 application of molluscicides would have cost around £14.3 million. In France, where the warmer climate greatly favours slugs, the molluscicide market is valued around 3-fold that of the UK, the greatest in Europe (Meredith, 2003).

### *1.2.2 Metaldehyde residues in water*

Prior to 2007, water companies did not routinely test for the presence of metaldehyde in water courses. However new monitoring processes detected metaldehyde residues at levels greater than the 0.1 parts per billion threshold set for drinking water standards by the European Environment Committee (98/83/EC). Bristol Water were first to identify problematic levels of metaldehyde at a number of their treatment plants. Many other UK water companies later followed in these findings in 2008 (Drinking Water Inspectorate, 2009). In 2008, metaldehyde contamination was accountable for 100% of Bristol Water's water test failures (Bristol Water Plc, 2009). There is some correlation between metaldehyde spikes and rainfall events, but poor application was thought to be another possible cause. Although these levels were still several hundred times lower than those considered to be harmful by the Health Protection Agency, it was acknowledged by all parties that movements must be made to reduce levels in order to avoid a potential metaldehyde ban. Of particular concern is the fact that metaldehyde cannot be removed from water using conventional treatment processes including adsorption onto activated carbon or treatment with chlorine or ozone, as is the case with other pesticides so preventing contamination at source is key (UK Water, 2008; Bristol Water Plc, 2009).

Metaldehyde stewardship by farmers is now encouraged and the Metaldehyde Stewardship Group (MSG) comprising the principal active ingredient manufacturer Lonza, and several slug pellet formulators was set up in 2008 to promote this cause. They introduced best-practice guidelines in 2009 which were updated again in 2010. Currently growers are advised to use the minimum amount of active substance (a.s.) possible and to observe maximum application rates of 210 g metaldehyde a.s./ha and a maximum total dose rate of 700 g a.s./ha/year (Metaldehyde Stewardship Group, 2010). In addition, farmers should avoid run-off into water courses by not applying metaldehyde pellets when heavy rain is forecast or when field drains are flowing. The guidelines also set out a minimum six metre application distance from water courses to prevent pellets entering water accidentally. Care should be exercised when storing and transporting metaldehyde pellets, loading and cleaning applicators and equipment and disposable overalls should be worn to prevent residues entering water when clothes are washed. Pellet manufacturers have also taken steps to improve packaging to minimise the risk of spillage, and all parties in the supply chain are urged to take heed of correct handling and transportation procedures, highlighting the seriousness of the situation and potential wide-ranging economic impacts a ban could have.

A survey of farmers commissioned by the MSG in 2009 revealed that 80% of respondents believed their business would be unsustainable without metaldehyde slug pellets, with more than 90% agreeing that crop production would suffer as a result.

Water companies perceive metaldehyde residues as a severe problem as they strive to meet European Standards for drinking water quality. Dealing with the presence of metaldehyde in raw water was described as "the biggest current challenge" in Bristol Water's Water Quality Report for 2007 (Bristol Water Plc, 2008) and their 2009 business plan listed the potential unplanned investment costs associated with metaldehyde mitigation as a key uncertainty in their management of risk. They estimate these costs at £120 million for the installation of reverse osmosis treatment facilities and additional £5 million per year running costs which would inevitably lead to significant price increases to consumers (Bristol Water Plc, 2009). Water companies are currently working with growers in river catchments to try to minimise metaldehyde entry into water courses to avoid these costs. In a position statement issued in 2010, The Environment Agency (UK) called for a sustainable solution that protects drinking water, aquatic and terrestrial wildlife and the economic viability of farming communities affected (Environment Agency, 2010). They also acknowledge that stewardship holds no guarantee of successfully reducing metaldehyde residues in water and recommends that alternative measures should be ready for implementation during 2011 should this initiative not be effective.

### *1.2.3 Cultural control of slugs*

Slug control by cultural methods relies on altering the way in which a crop is grown, by cultivating the land differently, sowing at different times or growing different varieties so as to reduce the risk or extent of damage to the crop. In coarse, cloddy seedbeds, slugs can move easily through the soil where they can eat seeds or seedlings they encounter. Such soils also offer plenty of moist cracks and crevices for slugs to shelter in. There are several advantages to cultivating the soil to a fine tilth. Ploughing has the effect of drying the soil out and exposing slugs to the air and risk of death by desiccation or predation, whilst the mechanical action of cultivation can directly kill slugs (Hunter, 1967). The fine soil particles prevent slugs accessing newly sown seeds until the seedlings break the surface, by which time the risk of attack is lower (Glen *et al*., 2006; Hunter, 1967). Compaction of the soil to consolidate the seedbed and further remove airspace enhances this effect. Wheat seeds can

also be protected by increasing the depth to which they are drilled - current recommendation states a depth of 4-5 cm to be optimal for a cloddy seedbed (Glen *et al*., 2006). Deeper drilling can also improve germination because of the increased soil moisture. Cultural control measures do also have their disadvantages however and perhaps the most prominent are the associated expenses of extra cultivations. In the mid 1980s, an application of molluscicide pellets was around £5 /ha whilst extra cultivations cost £6 /ha and increasing the wheat seed rate by 20% to compensate for anticipated slug damage would add about £8 /ha (Port & Port, 1986) so it is clear that molluscicides were more favourable from a financial point of view. Multiple cultivations can result in heavy soils becoming over-compacted, impeding root growth (Runham & Hunter, 1970). Hunter (1967) states that cultivation affects *D. reticulatum* to a lesser extent than *A. hortensis* or *T. budapestensis* because it is generally more active and has a high reproductive capacity so can survive where it is prevented from sheltering below the soil surface.

Selection of less susceptible crop varieties may also afford a degree of protection against slug damage. In reality though, there are few crops which possess such varietal differences, potatoes being about the only exception, with Maris Piper being regarded as one of the most susceptible whilst for example, King Edward and Pentland Dell are considerably more resistant (Winfield *et al.*, 1967; Blake, 2009). It is not clear whether this differing susceptibility is due to the potatoes being less attractive to slugs, or if there is a chemical response which deters the slug after feeding has commenced. It is likely that the latter seems more plausible given the opportunistic feeding habits of slugs, and a number of studies have suggested possible answers, with total protein content, and variations in starch, glycoalkaloid and phenolic acid content, as well as structural features of the tuber skin all being put forward (Port & Port, 1986). As mentioned in section 1.1.3, reduced levels of erucic acid and glucosinolates in modern 'double-low' cultivars of oilseed while being useful properties for human and animal consumption, actually increase the risk of slug damage (Glen & Moens, 2002; Meredith, 2003). This is an area where breeding of new varieties or genetic modification could provide potential solutions.

Lifting potatoes early can reduce damage to potatoes, as Runham & Hunter (1970) report a 10-fold increase in damage during September, suggesting the decline of food on the surface, an increase in size and numbers of slugs during the autumn, wetter weather, or biochemical changes in the maturing tuber as possible causes. Planting at different times can ensure that the crop's stage of greatest vulnerability does not coincide with periods of high slug activity,

for example, drilling winter cereal varieties later in the autumn when the weather is colder and slug numbers lower. This is risky however because if it does not get cold enough, slug numbers may not be significantly reduced, and the crop will be vulnerable as colder temperatures delay germination and increase susceptibility to attack by wheat-bulb fly (*Delia coarctata*), while *D. reticulatum* will remain active (Runham & Hunter, 1970). If the crop is deemed to be at too high a risk, drilling could be delayed until spring when establishment will be quicker. The way that crop residue is dealt with after harvest has an impact upon slug populations. Since the burning of stubble was banned in the 1990s, more crop residues have increased food for the slugs, providing shelter and keeping the soil moist. One way to reduce this problem is to plough crop residues into the soil soon after harvest and although this has little effect on slug numbers, damage to the subsequent crop is reduced (Glen *et al.*, 1984).

### *1.2.4 Biological control of slugs*

Parasitic nematodes are the only commercially available biological control for slugs. The rhabditid nematode *Phasmarhabditis hermaphrodita* was found to parasitise *D. reticulatum* at IACR Long Ashton in 1988 (Wilson *et al.*, 1993), and is capable of infecting and killing all pest species of slugs (Glen *et al.*, 2000). Infective (dauer) juvenile nematodes exhibit a chemotactic and chemokinetic response to slug mucous, actively seeking out slugs in the soil (Rae *et al.*, 2006; Hapca *et al.*, 2007). They enter the shell cavity of the slug, below the posterior dorsal surface of the mantle. Here they grow into hermaphrodite adults and begin to reproduce, causing a characteristic swelling of the mantle eventually resulting in the death of the host slug. The nematodes continue to reproduce and feed on the host cadaver and when this food source is depleted, new dauer juveniles will move off into the soil where they can infect other slugs (Wilson *et al.*, 1993). Associations have been documented between *P. hermaphrodita* and many species of bacteria and Wilson *et al*. (1995) suggested *Moraxella osloensis* as most suitable for monoxenic mass-production of the nematode. Tan and Grewal (2001) studied this association with *M. osloensis* in more detail and showed that the pathogenicity of *P. hermaphrodita* was dependent upon the number of viable bacteria carried by them. They concluded that this bacterium alone was capable of killing slugs and that *P. hermaphrodita* acted merely as a vector of *M. osloensis*. *M. osloensis* co-injected with antibiotics into the shell cavity of *D. reticulatum* resulted in mortality at the same rate as when *M. osloensis* alone was injected, providing evidence of toxin-mediated pathogenicity as a toxin may have been produced prior to killing of the bacteria by addition of antibiotics. This theory is further supported by the finding that 24 hour cultures were non-pathogenic

whilst pathogenicity increased between 40 hour and 60 hour cultures suggesting a build up in toxin concentration to pathogenic levels with time. An *et al.* (2008) hypothesised that a number of *M. Osloensis* genes that are up-regulated during slug infection may encode virulence factors.

The first commercial formulation to use nematodes was released in the UK in 1994 under the trade names Nemaslug® and Nemaslug® Xtra, produced by Becker-Underwood (Glen *et al.*, 1994). *P. hermaphrodita* has been shown to reduce slug damage in a variety of arable, vegetable and high value crops (Wilson *et al.*, 1994; Wilson *et al.*, 1995; Speiser *et al.*, 1996; Ester *et al.*, 2003; Ester *et al.*, 2003; Grubisic *et al.*, 2003). The formulation is mixed with water and applied to the soil as a spray or drench. Applications of nematodes in field trials show a strong and rapid reduction in crop damage, despite relatively little impact on slug numbers (Wilson *et al.*, 1993). This effect appears to be due to a strong feeding inhibition soon after contact with the nematodes, followed by a much slower mortality (Glen *et al.*, 2000). In this study, feeding activity and mortality were directly related to nematode dose and time after exposure. The optimum temperature for nematodes is 17 °C but they can survive in temperatures as low as 5 °C (Glen *et al.*, 1996). Larger slugs appear to show reduced mortality compared to smaller individuals, but this should not be too large a problem as most pest species are relatively small (Speiser *et al.*, 2001). Where larger slug species are present, timing of nematode applications to coincide with more vulnerable juvenile life stages is advised (Glen *et al.*, 1996). Nematodes do not survive desiccation and should be applied to moist soil. If this is not possible, shallow incorporation into the soil after application is beneficial (Wilson *et al.*, 1996). Iglesias (2001) conducted field trials in Northern Spain on leaf beet and lettuce and concluded that under these hot and dry conditions several applications at lower rates were more effective at reducing damage than a single application at the standard rate. Iglesias (2001) also observed that manure applied prior to treatment of soil with nematodes rendered them ineffective, suggesting that this could be due to interference with their ability to find slug hosts (likely to utilise chemoreception as in other entomopathogenic nematodes), the attraction of slugs from surrounding areas or increased feeding of those slugs already present.

The main disadvantage of *P. hermaphrodita* as a slug control is its financial cost at around £110 per hectare, but this remains a relatively new product and if continued optimisation of the fermentation process can help to improve production economics then hopefully it can become more affordable in the future. Wilson *et al.* (1999) showed that *D. reticulatum* and

*Arion ater* were capable of detecting infective juvenile nematodes and avoiding soil treated with them, although the mechanism of this detection is not known. They suggest that it may be possible to treat only the soil surrounding crops and so reduce the amount and cost of the application, but this would only be feasible where plants are widely spaced and does not take into account slugs in the untreated soil that would be effectively 'fenced in' at the base of the plants. Due to the high cost of nematode mediated control of slugs, to date it has seen most use in horticultural applications and on high value crops. As long term effects on slug populations are small, and *P. hermaphrodita* are specific to slugs, wildlife that utilise slugs as a food source are thus protected. Control of slugs with nematodes provides a powerful tool for integrated crop management, where they can work side-by-side with natural slug predators and cultural controls.

## 1.3 Non-target Effects of Chemical Molluscicides

The non-target effects of molluscicide usage have been the subject of much debate. A number of studies have investigated possible detrimental effects of contact with molluscicidal pellets on earthworms (Brieri *et al.*, 1989; Wellmann & Heimbach, 1996) and carabid beetles (Purvis & Bannon, 1992) however, the extent of these effects is extremely variable and dependent upon various species-specific, behavioural, agronomic and environmental factors.

### *1.3.1 Earthworms*

Earthworms are beneficial to soil nutrient status and structure. Many earthworms remain primarily underground, but *Lumbricus terrestris* is particularly active on the surface around its burrow, continually transporting plant litter underground where it is incorporated into the soil. Earthworms thus play an important role, actively recycling nutrients and improving topsoil carbon content (Brieri *et al.*, 1989). Worm casts are rich in nutrients in an easily-exchangeable form and the walls of their burrows are lined in rich organic nutrients, providing channels for root growth and water infiltration, important factors in plant establishment. That said, it is unlikely that changes in earthworm populations will have an instant impact on crop yield as any effects may be masked by factors such as weather and fertiliser use, but farmland with large earthworm populations may experience more stable long-term yields (Brieri *et al*., 1989).

Brieri *et al.* (1989), assessed the affects of metaldehyde and methiocarb pellets on the fitness, survival, behaviour and activity of *L. terrestris*. They found that the worms were unaffected by metaldehyde (Dose equivalent to approx 440 pellets/$m^2$ and 4400 pellets/$m^2$), and analysis of the metaldehyde content of excreta and worm tissue, revealed amounts of metaldehyde proportional to the pellets applied, suggesting that they can tolerate large quantities of this molluscicide. Metaldehyde pellets were readily and quickly drawn down into the burrow. The authors pointed out the possible effect of this activity on slug control in the field, but this could be beneficial if the worms may make the pellets more accessible to slugs that remain mostly underground – an aspect of slug control of particular importance in potato fields (Section 1.1.2). Worms exposed to methiocarb presented above-average body weight loss and appeared to be weakened. This is most likely explained by starvation, caused by a massive reduction in activity observed after the worms' initial contact with the pellets. It is not clear whether this is caused by the physiological effects of, or behavioural changes in response to the poison.

Wellmann & Heimbach (1996) pointed out that the pellet concentrations used by Brieri *et al* were not representative of field application rates. In this trial they found that detrimental effects on *Lumbricus terrestris* were only apparent at 10-fold application (400 pellets/$m^2$) rates, and normal rate (40 pellets/$m^2$) results did not differ significantly from controls. They conclude that little effect can be expected on earthworm populations in the field when molluscicides are applied at the recommended rates.

### *1.3.2 Carabid beetles*

Carabid beetles are abundant on arable land throughout the world and are widely considered an important beneficial invertebrate family. There are around 350 carabid species in the UK and more than 40,000 worldwide (Kromp, 1999). They differ greatly in their habitats, feeding preferences and activity patterns so the threat posed by pesticides to these insects will depend upon the developmental stage, the species and the time of year relative to both the life-cycle of the beetles and the agricultural calendar. As polyphagous predators, they are potentially useful biocontrol agents of insect pests and have been shown to eat (primarily) the eggs and larvae of a vast number of species including aphids, wheat midge (*Sitodiplosis moselana*), cabbage root fly (*Delia radicum*), onion maggot (*Delia antiqua*), Colorado potato beetle (*Leptinotarsa decemlineata*), carrot weevil (*Listronotus oregonensis*), rape blossom beetle (*Meligethes anaeus*), and cereal leaf beetle (*Oulema melanopus*). A comprehensive

review of this subject is available (Kromp, 1999). Slugs can form a significant part of the diet of some carabid beetles and *Pterostichus melanarius* in particular is a well documented slug predator although no obligate slug predators have been identified (Symondson *et al.*, 1996; Bohan *et al.*, 2000; Symondson *et al.*, 2002). Symondson (2002) reported positive relationships between yearly beetle population growth and slug abundance and suggest that beetles can have a significant impact on slug population growth from year to year, but not between months within a year. Thus any non-target effects of molluscicides upon carabid beetles may be having an adverse effect on a valuable biological control and has obvious implications for integrated pest management (IPM).

Carabid beetles are not only threatened by pesticide sprays, but as largely opportunistic scavengers, are also at risk of harmful interactions with slug pellets broadcast on the soil surface. A number of species have been well studied in relation to the threat posed by slug pellets and/or their predation of slugs. *Pterostichus madidus* attack and feed for longer on dead *D. reticulatum* and, of living slugs, scavenging beetles may target those that have been exposed to sub-lethal concentrations of molluscicides (Langan *et al.*, 2001). This may be due to impaired mucus production which has been documented as an important defensive mechanism against predation by *Carabidae* (Pakarinen, 1994). By covering the beetle mouthparts with sticky mucus, the beetle must take time to clean itself before it can attack again (Mair & Port, 2002). This preference for scavenging dead slugs brings into question the ability of this species to effectively control live slug populations in the field. The effects of methiocarb on mortality of *P. melanarius* are much greater than for metaldehyde to which it is relatively insensitive (Büchs *et al.*, 1989), but this difference in susceptibility is likely to have little effect in reality as *P. melanarius* feeds preferentially on slugs containing metaldehyde as opposed to methiocarb (Langan *et al.*, 2004).

Kendall *et al*., (1986) observed an increased incidence of Barley Yellow Dwarf Virus (BYDV) on plots treated with twice the normal rate of methiocarb pellets when compared with untreated plots. At the time they suggested this to be a possible result of a reduction in aphid predators, namely carabid beetles, but no significant differences in the numbers of *Carabidae* or *Staphylinidae* in treated and untreated plots were recorded in support of their argument. More direct evidence later showed that methiocarb pellet use does cause a drop in carabid activity (Kennedy, 1988; Purvis & Bannon, 1992; Purvis 1996). A four year trial was conducted by Purvis & Bannon (1992) using isolated plots, to assess the affect of methiocarb-based slug pellets on *Carabidae* populations. Pellets were either drilled into the

seedbed at the time of sowing, or broadcast on the soil surface after sowing. Pitfall traps were used to assess beetle and spider activity at seven-day intervals from the week prior to autumn sowing, until the following June. Twenty-seven carabid species were identified throughout the trial.

In a minority of species, broadcast application caused a greater reduction in abundance than when pellets were drilled into the soil. Perhaps unsurprisingly, the winter-active species that were most active at or close to the time of application, showed the greatest reduction in number following application (Purvis & Bannon, 1992). These species (*Bembidion obtusum, Nebria brevicollis and Trechus quadristriatus*) remained suppressed until their natural seasonal decline. Adult *B. obtusum* persist throughout the winter, breeding in spring and so are particularly vulnerable to methiocarb treatments of winter cereals which can have serious affects on breeding success and subsequent generations. This species generally has reduced, non-functional wings and so is very slow to repopulate an area after large-scale elimination. Mortality of adult *T. quadristriatus* is particularly high as a result of winter methiocarb use however, the long-term effect on the species is small (Purvis & Bannon, 1992). This can be attributed to the fact that this species breeds in the autumn and larvae are relatively unharmed by methiocarb (probably due to different feeding behaviour/diet) and persist through the winter so that the subsequent generation is unaffected.

*N. brevicollis* was almost completely eliminated by winter methiocarb application (Purvis & Bannon, 1992). Following autumn breeding, this beetle over-winters as a larval population which is active on the soil surface so it is of little surprise that it was worst affected on broadcast treated plots where the larvae would be more likely to come into contact with the pellets. The adults are most active after emergence from pupation in early summer and at the time of breeding, and it is thought that population recovery observed in this study was due to redistribution of the species during these periods of high activity. Few effects are described with respect to the effects of winter use of methiocarb on summer-active species.

In summary, although the abundance of most *Carabidae* species is reduced following contact with methiocarb-based slug pellets, the long term effects are dependent upon the life cycle, dispersive ability and the period of greatest activity of the species. It is however important to note that preservation of field margins is essential to maintain a habitat in which *Carabidae* and other beneficial invertebrates can over-winter and from which they can repopulate arable land following cultivation and pesticide applications.

## 1.4 Why Are New Controls Required?

There is a strong case for the innovation of new molluscicides and management strategies. Over recent decades, changes in the climate and to farming practice have favoured an increase in slug populations (Willis *et al.*, 2006). Increased areas of oilseed rape and breeding of 'double low' varieties, the ban on straw burning, reduced cultivation, non-cropped set aside and increased autumn sowing are all changes to farming practice that affect the control of slugs in agriculture whether their effects were previously known or not. Economic factors are partly to blame for such changes, where a reduction in required time and labour through fewer cultivations for example, could offer considerable savings for the farmer. Drilling fewer seeds can reduce seed costs but damage to seedlings becomes more important as there are no plants to take their place and areas of fields may need re-drilling as a result. Economic factors are an important consideration when developing a new control method, and more effective controls that afford greater control at a lower cost are highly desirable.

As detailed earlier, current chemical controls are not effective enough and are dependent upon the optimisation of many factors including the number and species of slugs present, the plant variety, weather and climate, the crop history of the field, cultural practices and the time of application with respect to the crop stage and the time of year. The formulation of chemical baits poses its own problems as they must attract the slug, encourage feeding and ensure that a lethal dose is consumed whilst at the same time being able to withstand adverse weather conditions and remain active and viable in the field for as long as possible. Im recent years the development of "wet process" slug pellets, where the protein and gluten content of wheat flour is used to bind the pellets, has improved integrity under wet conditions (De Sangosse Ltd, 2007). Application of molluscicides in bait form neglects those slugs that are not surface active, so large reservoirs of slugs may remain in the soil. The limited effectiveness of chemical molluscicides at the time of their introduction, was only a small problem because the way that the land was farmed lessened their importance (Hunter, 1967). But as farming practice changes such to increase the likelihood of slug damage, a greater proportion of our slug defence is dependent upon molluscicide baits which do not always offer sufficient control. The effects of molluscicides on non-target organisms such as earthworms and carabid beetles are clearly undesirable (Purvis & Bannon, 1992; Wellmann & Heimbach, 1996; Van Toor, 2006) and modern farming practice is under increasing pressure to re-examine its environmental impact of chemical usage. Furthermore, the action

of such beneficial organisms has a viable role to play in ICM where through a combination of cultural, chemical and biological control, pests can be controlled whilst maintaining biodiversity and reducing the amounts of pesticides used (Denyer, 2000; Leake, 2000). Specificity of pesticides is key to effective integrated pest management (IPM), where highly selective chemicals allow part control of pests whilst leaving biological control agents unharmed and able to assist to further reduce the pest population by natural means.

In recent years, all chemicals used in crop protection have been subject to increased scrutiny under the EU pesticide directive 91/414 implemented in the UK from 2005. As a result of this review process, thiodicarb was withdrawn in 2008, on grounds of its potential risk to human health and the environment. Under the same legislation, methiocarb which is considerably more toxic to animals than metaldehyde, is due for review by the end of September 2011 (European Commission, 2007). Combined with the current usage limits and risk of further regulatory action with regards to water contamination by metaldehyde residues, the future for chemical molluscicides is far from secure. With metaldehyde currently accounting for up to 90% of molluscicides applications in the UK, and the alternative chemicals costing at least twice as much per application, the increased cost of slug control should metaldehyde face a ban or restrictions could have severe consequences for farmers' profit margins. All of the above factors combine to present a strong case for the need for innovation in order to achieve significant advances in the control of gastropod molluscs.

## 1.5 Symbiosis

Symbiosis is the persistent close association between two organisms which may be beneficial (to one or both parties) (mutualistic), harmful (pathogenic) or commensal (Klepzig *et al.*, 2009). Symbioses have been recorded in a number of different organisms, particularly invertebrates. Some of the better described symbioses are in insects of importance in medicine and agriculture including aphids, weevils, tsetse fly, cockroaches (Munson *et al.*, 1991; Aksoy, 2000; Lefevre *et al.*, 2004; Akman Gunduz & Douglas, 2009). The status of a symbiont as beneficial, harmful or harmless is not always exclusive as it can depend on environmental factors or the physiological status of the host (McCreadie *et al.*, 2005). Many insects house symbiotic bacteria in specialist cells called mycetocytes (Moran & Telang, 1998). Molecular analysis of mycetocyte associated symbionts shows that they share a long

evolutionary history and are strictly vertically inherited by their hosts. These bacteria are usually obligate symbionts, and not able to survive outside of the host. Many symbionts have a nutritional role which allows the host organism to exploit an ecological niche by providing essential nutrients to a host feeding on a nutritionally imbalanced diet such as plant phloem sap (Akman Gunduz & Douglas, 2009) or vertebrate blood (Aksoy, 1995). However, not all insects with mycetocyte-associated symbionts consume such narrow diets, for example cockroaches (Bandi *et al.*, 1994), ants (Sauer *et al.*, 2000) and weevils (Lefevre *et al.*, 2004) are all generalist feeders. Many insects cannot grow or reproduce normally without the symbiont while others grow more slowly but survive and reproduce normally.

Symbionts especially those associated with mycetocytes, often cannot be cultured *in vitro*, and extracted mycetocyte bacteria only remain viable for a number of hours and do not replicate in the lab, making studies of their function difficult. This is because symbionts through co-evolution are selected for the conditions within the host (Moran & Telang, 1998). In the last decade, molecular techniques have allowed researchers to characterise these bacteria and their phylogenetic origins. The use of additional paleontological information estimates that these symbioses have co-evolved over hundreds of millions of years – 150 million years in the case of aphids and their symbionts *Buchnera aphidicola* (Moran & Telang, 1998; Clark *et al.*, 1999). Phylogenetic analyses show the virtual absence of horizontal transfer which would result in incongruent DNA sequences in the genome as symbionts underwent horizontal gene transfer with free-living bacteria. Most of the studied insect symbionts are identified as belonging to the phylum of *Proteobacteria* (de Vries *et al.*, 2001; Oliver *et al.*, 2010).

The second main class of symbionts are facultative symbionts which are not generally required for optimum host fitness and development. Facultative symbionts are usually horizontally transferred, with an aposymbiotic phase in the host life-cycle being followed by acquisition of the symbiont from a free-living reservoir. Transmission of symbionts by this means however prevents long-term co-speciation due to the risk of cross-infection. Studying these symbioses is difficult because it is not possible to produce aposymbiotic organisms (free from symbiont) by administering antibiotic as they are able to re-acquire the symbiont from the environment. As a result, little is understood about the function of these symbioses but they appear to have some mutualistic benefits to host survival and reproduction.

In contrast, vertically transmitted, obligate symbionts cannot re-colonise a host if removed by antibiotic treatment and so their study is simplified. Perhaps one of the best studied insect:symbiont associations is that between the aphid and its symbiotic bacteria *Buchnera aphidicola* (Munson *et al.*, 1991; Prosser & Douglas, 1991; Sasaki *et al.*, 1991; Douglas, 1998). *Buchnera* account for approximately 10% of aphid biomass which equates to $10^7$ cells per mg of aphid tissue and approximately 60% of the mycetocyte cytoplasmic volume (Douglas, 1998). *Buchnera* synthesise the amino acid tryptophan which is absent from the plant phloem sap that solely makes up the aphid diet. The biosynthetic enzyme in *Buchnera* is coded for by the trpEG gene (Lai *et al.*, 1996). The presence of this gene on a plasmid coincides with greater growth rates for the aphid host, compared to aphids with bacteria possessing only chromosomal copies of trpEG. It is these growth benefits for the host that results in the positive selection for this association in evolutionary history. Baumann and Baumann (1994) showed that *Buchnera* cell count and aphid growth are tightly coupled such that the symbionts do not proliferate liberally. Instead, *Buchnera* demonstrate a doubling time of around two days which is considerably longer than the approximately 20 minutes of many studied bacteria, and appears to be because *Buchnera* and indeed other symbiotic bacteria only have a single copy of the rRNA genes (Baumann & Baumann, 1994). Ribosomes are the machinery of protein synthesis and essential for replication, with more copies of the gene permitting a greater rate of protein synthesis and replication.

A number of authors have written about the potential exploitation of symbioses in insect pest control (Douglas, 2007; Darby, 2009). There are two ways in which this may be achieved. Firstly, symbionts upon which the host insect depends for normal survival and function could be selectively removed to elicit control, in this case, obligate vertically transmitted symbionts would appear to be the most viable targets for pest control, because once removed from the host they cannot be re-acquired from the environment. Secondly, some symbionts not required by a host have the ability to alter the host traits that are connected to their pest status (Douglas, 2007; Darby, 2009). An example of this approach encompasses what is termed paratransgenesis in which symbiotic bacteria are genetically modified to confer traits that modify the insect phenotype. This has been demonstrated successfully in *Rhodinus prolixus*, a blood-feeding insect and vector of the parasite *Trypanosoma cruzi* which causes Chaga disesase, prevalent in South America. Researchers have been able to transform gut bacteria of *R. prolixus* with a gene that encodes a protein which lyses *T. cruzi* resulting in a over 1000-fold reduction in *T. cruzi* transmission (Durvasula *et al.*, 1997; Beard *et al.*, 2002). The group produced a formulation called CRUZIGARD which exploits the insects' habit of

consuming faecal pellets in a delivery method that combines transformed bacteria with artificial faeces. However, the fundamental principal behind the success of this biocontrol is the fact that *R. prolixus* are dependent on the gut bacterium *Rhodococcis rhodii* for normal function which ensures that the transformed bacteria are maintained within the insect gut.

## 1.6 Summary and Aims of the Study

Despite many decades under the spotlight, and innovations in chemical and biological control, slugs continue to pose a significant threat to a very broad range of agricultural and horticultural produce. Aspects of slug behaviour and physiology make them a particularly persistent and challenging crop pest. Chemical molluscicides represent the most reliable method of slug control currently, but effective control can be subject to the optimisation of climatic and agronomic factors and non target effects may be detrimental to beneficial organisms. Recent pesticide legislation and the discovery of untreatable metaldehyde residues in water courses puts the future of slug control at great uncertainty. The loss of such chemicals without effective alternatives to replace them could prove disastrous to the sustainability of UK farming. Innovation is therefore urgently required to ensure that slug control can be maintained in the future. This project aims to investigate whether symbiont-mediated biological control of slugs could contribute to future control, by asking whether *D. reticulatum* possesses beneficial symbiotic bacteria.

Following a short chapter detailing general methods (Chapter 2) used throughout the thesis, three experimental chapters (Chapters 3-5) will each focus on one of three main aspects of the study:

- Characterisation of slug gut bacteria to highlight potential symbionts
- Microbial ecology in the slug gut to establish whether any bacterial associations are geographically conserved and therefore more likely to be symbionts
- Bioassays to assess the importance of slug gut bacteria for the normal survival and fitness of *D. reticulatum*.

Each chapter will outline specific methods and contain an independent introduction and discussion. A general discussion (Chapter 6) will summarise findings in the context of the project hypothesis, as well outlining scope for future research.

# Chapter 2

# General Methods

## 2.1 Experimental Slugs

### *2.1.1 Collection and maintenance in the laboratory*

For all experiments *Deroceras reticulatum* (Müller) were field-collected from various locations throughout Midlothian, Northumberland and further afield in the UK. Each of the following chapters detail the origin of the slugs used in specific experiments. Slugs were collected from either winter wheat *(Triticum aestivum)* or oilseed rape *(Brassica napus)* fields throughout the year as required. Slugs were most numerous in the autumn months of late September and October and the spring months of May and June however *D. reticulatum* can be collected year-round if conditions permit. On mild autumn and winter days, fields sown with winter wheat in a rotation following oilseed rape (OSR) yielded good numbers of slugs providing the soil was not frozen or water-logged. At this time of year slugs were always small (usually not greater than 200 mg) and were most successfully found under large clods of earth and within oilseed rape stalks remaining from the previous crop.

As soil and air temperatures increased through spring and towards summer, wheat fields were found to be too dry and slugs were difficult to find. In these months, collection was most successful in OSR fields particularly following or during wet weather. The dense canopy of OSR shelters the soil and creates an ideal microclimate keeping the soil moist and, slugs are readily observed on both the surface of the soil and the underside of lower leaves of the OSR plants. The tramlines in the crop provide small tunnels through which the collector can crawl to gain access. Slugs collected from OSR fields in late spring were regularly larger than those collected from wheat fields on the same day (often more than 0.5 g heavier).

If conditions were less than favourable and slugs were not visible in suitable numbers on the surface, refuge traps were successful in increasing yield in some instances. Traps comprised 15 x 15 cm squares of 0.9 cm thick plywood which were placed in sheltered positions within the crop, baited with Alpen™ muesli, and left overnight (Figure 2.1). Traps were found to often yield up to ten slugs per trap. Traps were checked early the following morning before the sun warms the trap and slugs retreat into the soil to cooler shelter.

Slugs were transported in plastic containers lined with several layers of moistened laboratory tissue and air holes punched in the lid. Leaves from the field were added as food and for shelter for the slugs and the containers were kept cool and out of the sun until arrival at the laboratory. In the laboratory, slugs were stored in an incubator at 15 °C, 95% relative

humidity (RH) and with a 12 hour:12 hour light:dark period. When not part of an experiment or being starved in preparation for an experiment, slugs were fed *ad libitum* on shop bought carrot and Chinese cabbage which were thoroughly washed in distilled water. Slugs were transferred to clean containers every three to four days.

**Figure 2.1.** Plywood boards (15 x 15 cm) can be used as refuge traps, baited with muesli and left overnight. Photo P. Wilkinson

### *2.1.2 Artificial diet*

During experiments slugs were fed an artificial diet based on a method by Walker (1997). The diet was prepared by homogenising 60 g fresh weight of Chinese cabbage with 40 ml distilled water in a mortar and pestle until a smooth paste was achieved. To this, 2 g of wheat bran was added. Meanwhile 3 g of nutrient agar (Oxoid) was added to 50 ml of distilled water and heated whilst stirring until boiling and all agar was dissolved. The cabbage and bran mix was slowly added to the agar, continually stirred to ensure even incorporation, whilst heating to return the mixture to the boil. The diet was autoclaved at 121 °C for 15 minutes, dispensed into 9 cm Petri dishes and allowed to set. The diet was then sealed with Parafilm (Pechiney Plastic Packaging Company, Neenah, WI, USA) and stored at 4 °C. When required, pieces were cut using an alcohol/flame sterilised 8 mm cork borer and

offered to the slug. For carrot and muesli based diets (Chapter 4), 60 g of Chinese cabbage was replaced with 100 g of grated carrots or 40 g of ground muesli respectively.

To incorporate antibiotics into the diet for bioassays (Chapter 5), firstly, three solutions of chloramphenicol and tetracycline in equal quantities were prepared in sterile distilled water to final concentrations of 16, 80 and 160 mg per ml and 0.2 μm filter-sterilised. When the diet had cooled to approximately 40 °C, 10 ml of the appropriate antibiotic solution was added to yield a diet with a final antibiotic content of either 1, 5 or 10 mg.g diet$^{-1}$. The still molten diet was dispensed into 90 ml Petri dishes and allowed to set, sealed with Parafilm (Pechiney Plastic Packaging Company, Neenah, WI, USA) and stored at 4 °C. When required, pieces were cut using an alcohol/flame sterilised 8 mm cork borer and offered to the slug.

### *2.1.3 Dissection of slugs*

Slugs to be dissected were weighed and placed individually into clean plastic containers (9 cm diameter, 7 cm deep). The slugs were then anaesthetised and killed by gently covering with supermarket-bought carbonated water (Lincoln & Sheals, 1979). The carbonated water was from a newly opened bottle as reduced effervescence lowers the efficiency of the anaesthetic process- 'flat' water is ineffective. The carbon dioxide in the water acts to anaesthetise the slug which relaxes, fully stretched out, and is immobilised in around one minute. Prior to dissection, the slug was removed from the water and surface-sterilised by immersing in 70% ethanol and agitating for one minute.

A dissection dish was prepared by lining the base of a square Petri dish (10 x 10 x 2 cm) with a piece of corrugated polypropylene sheet cut to a tight fit, to which the slug could be secured using insect pins. After swabbing the base of the dish with alcohol to sterilise, the slug was positioned on its right-hand side and held with two pins, one through the tail (taking care not to push the pin through the internal visceral mass) and one through the buccal mass. The dissection was carried out under sterile distilled water (SDW) to support and prevent desiccation of the tissue and to improve visibility.

All dissection instruments were first alcohol- and flame-sterilised and dissection was carried out under a dissection microscope at 20-times magnification. A longitudinal incision was made in the body wall just dorsal to the foot, from the buccal cavity to the tail, using a

scalpel (No. 11 blade, Swann-Morton Ltd, Sheffield. UK) and fine forceps. The body wall was carefully lifted free of the internal organs and the blood vessels and rectum were cut to free the visceral mass from the mantle. The large orange/pink digestive gland fills most of the internal volume (Figure 2.2), and posterior and dorsal to this is the dark ovo-testis or hermaphrodite gland. Using forceps and fine scissors the ovo-testis was carefully removed. The hermaphrodite duct running anteriorly from the ovo-testis was located and teased out and the remainder of the reproductive system was separated from the digestive system and moved aside. The nerve ring, surrounding the oesophagus, posterior to the buccal mass, was cut with scissors. The digestive system was carefully moved aside, cutting connective tissue and finally the oesophagus to complete the dissection.

The digestive tract was transferred to a pre-weighed 1.5 ml microcentrifuge tube and weighed again to determine the tissue mass. Gut samples were either used immediately for microbiological study, or stored at -80 °C until required for molecular work.

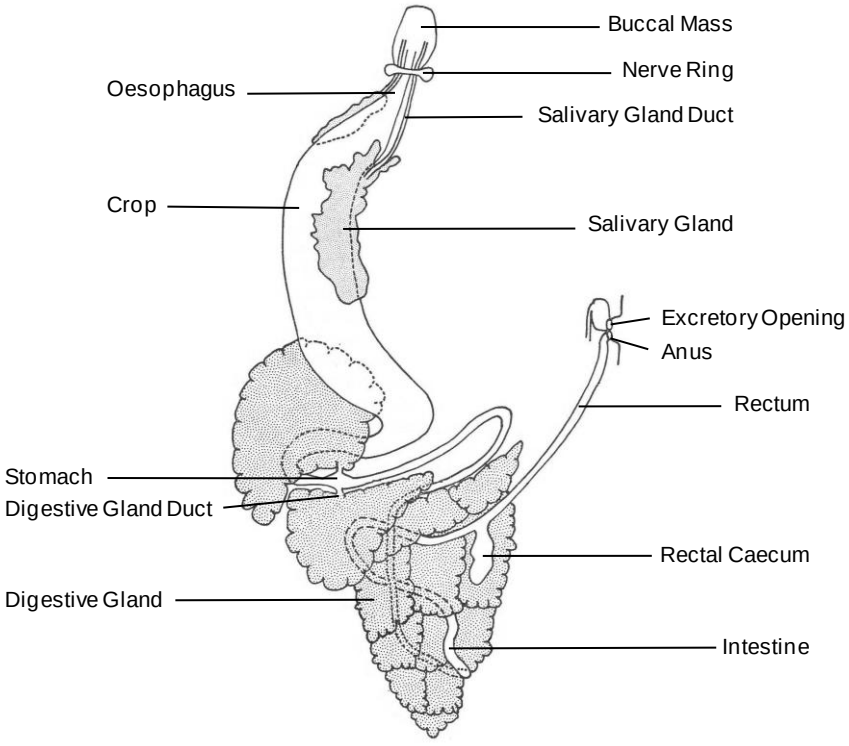



**Figure 2.2.** *D. reticulatum* digestive tract. From Runham & Hunter (1970), from work by Walker (1969)

## 2.2 Molecular Protocols

### *2.2.1 Purification of DNA from slug gut*

DNA purified from the slug gut was studied using molecular techniques to quantify, identify and assess the diversity of bacterial populations present within the slug digestive system. The method of DNA extraction was modified from Henckel *et al*. (1999). The dissected slug gut tissue was first ground to a fine powder under liquid nitrogen using a pestle and mortar. The ground tissue was then transferred to a sterile 2 ml screw-cap centrifuge tube containing approximately 1.0 g of sterilized 0.1 mm diameter glass beads (BioSpec Products Inc. Oklahoma. USA). To the tubes, 800 µl of sodium phosphate buffer (120 mM, pH 8.0) and 260 µl of a sodium dodecyl sulphate (SDS) solution (10% SDS; 0.5 M Tris-HCl, pH 8.0; 0.1 M NaCl) was added before briefly vortexing to suspend the contents. A Fastprep instrument (Model FP120, Qbiogene Inc., Carlsbad, CA. USA) was used to lyse the cells by shaking the tubes for 45 seconds at a speed of 6.5 $m.s^{-1}$. After centrifuging for 3 minutes at 12,000 *g*, 700 µl of the supernatant was removed to a clean 1.5 ml microcentrifuge tube. The tissue-bead mixture was extracted a second time by resuspending in 700 µl of sodium phosphate buffer, repeating the centrifugation step and removing a further 700 µl of supernatant to the 1.5 ml tube. The pooled supernatant was then split, transferring 700 µl to a clean 2 ml microcentrifuge tube which would be taken forwards to nucleic acid purification whilst the remaining portion was stored at -80 °C in case the extraction should need to be repeated. Proteins were precipitated from the working sample by adding 300 µl of 7.5 M ammonium acetate and incubating on ice for 5 minutes. In a further purification step, 1ml of phenol:chloroform:isoamyl alcohol mix (25:24:1) was added to the tube which was inverted several times and then centrifuged for 3 minutes at 12,000 *g*. The nucleic acids were pelleted from 850 µl of the upper aqueous layer by adding 600 µl of ice cold isopropanol in a clean 1.5 ml tube and centrifuging for 45 minutes at 12,000 *g* and a temperature of 4 °C. The resulting DNA pellet was washed twice with ice cold 70% ethanol and then dried in a vacuum centrifuge for 15 min. The DNA was resuspended in 400 µl of Tris-EDTA buffer (10 mM Tris base; 1 mM EDTA; pH 8.0), quantified using a Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA. USA), diluted to a concentration of 20 $ng.\mu l^{-1}$ with sterile distilled water and then stored at -20 °C until required.

### *2.2.2 Enumeration of bacterial DNA with real-time quantitative PCR*

In addition to plate counts of viable bacteria in crude slug gut extracts, a TaqMan probe-based real-time quantitative PCR assay was used to quantify bacterial DNA present in the slug digestive tract. The assay used broad-specificity primers that target a highly conserved region within the bacterial 16S rRNA gene across all bacteria, without targeting slug DNA, giving a sensitive assay that is capable of quantifying even bacteria that cannot be cultured and therefore would be excluded from a plate count.

#### *Q-PCR primers and probes*

The assay used primers and a probe published by Bach *et al.*(2002) and synthesised by Eurogentec S.A. (Liege, Belgium). Table 2.1 shows the sequences of each oligonucleotide.

**Table 2.1.** Sequences of oligonucleotides used in real-time quantitative PCR of total bacterial DNA. *Position refers to the nucleotide position of the primer binding site in the E. coli genome. From Bach et al. (2002).

| Oligonucleotide | Sequence | Position (nt)* |
|---|---|---|
| Forward Primer | 5'-GGTAGTCYAYGCMSTAAAG-3' | 799-818 |
| Probe | 5'-FAM-TKCGCGTTGCDTCGAATTAAWCCAC-TAMRA-3' | 951-975 |
| Reverse Primer | 5'-GACARCCATGCASCACCTG-3' | 1044-1063 |

#### *Bacterial DNA standards for absolute quantification*

Standards were prepared using genomic DNA extracted from the bacterium *Acinetobacter psychrotolerans* (NCIMB 14062) using the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany). Briefly, a single colony of the bacterium was picked from an overnight culture on nutrient agar and used to inoculate a test tube containing 5 ml of nutrient broth, and incubated for 48 hours at 20 °C. The cells were harvested from 4 ml of the broth culture by centrifuging for 10 minutes at 5,000 *g* and discarding the supernatant. The DNA was extracted as per the manufacturer's instructions and quantified using a Nanodrop 1000 spectrophotometer. A five-fold dilution series was prepared and eight standards ranging from $9.13 \times 10^{-1}$ to $7.13 \times 10^{4}$ picograms of DNA per reaction were used to generate standard curves for the PCR.

*Quantitative PCR Reaction Conditions*

PCR was performed with a Sratagene MX3000P (Agilent Technologies, La Jolla, CA, USA) system using polypropylene 96-well plates. Each reaction contained 12.5 µl of 2x Platinum® Quantitative PCR SuperMix-UDG (Invitrogen, Carlsbad, CA, USA), 5 pmol of each primer, 2.5 pmol of the probe, 2.5 pmol of ROX reference dye (Invitrogen), 2.5 µl of mixed template DNA (from slug gut, 20 $ng.\mu l^{-1}$) and PCR grade water (Roche, Mannheim, Germany) to a total reaction volume of 25 µl. All reactions were run in triplicate on the same plate. The amplification reaction conditions comprised 50 °C for 2 minutes, 94 °C for 2 minutes, followed by 40 cycles of 94 °C for 15 seconds and 60 °C for 1 minute.

## 2.3 Data Analysis

Data analysis methods are explained in detail in each chapter however a brief summary is included below.

Analysis of variance (ANOVA) was used to compare treatment effects in experimental data. ANOVA compares the variance of experimental factors with the natural (residual) variance within two datasets. Where experimental variance is considerably greater than residual variance it is deemed that the treatment has a large effect on the results. A resultant F-probability describes the significance of this effect.

Bacterial 16S rDNA and intergenic spacer region DNA sequences were compared to the Genbank database using the BLASTN search algorithm (Altschul *et al.*, 1990). Multiple sequence alignments were generated with CLUSTALX (Thompson *et al.*, 1997) and phylogenetic anaylsis by the maximum likelihood method was conducted in PHYLIP (Felsenstein, 1989).

Analysis of molecular fingerprints obtained from ribosomal intergenic spacer analysis (RISA, Chapter 4) of slug-associated bacterial communities was conducted using macros in Microsoft Excel. A series of indices describing the species diversity and abundance within bacterial communities were calculated from fingerprint data and compared with ANOVA in Genstat (Payne *et al.*, 2009).

For analysis of slug mass and food intake during bioassays (Chapter 5), the data for each individual slug were summarised using two response features which described an aspect of the subject's response curve. In this case the mean mass over the duration of the bioassay and the rate of change of mass were used as summary measures which were themselves compared using analysis of variance (ANOVA).

Slug survival data in bioassays were analysed using the Cox proportional hazard model. The basis for this analysis is the assumption of a baseline hazard function which describes the risk of a terminating event (in this case death of the slug) occurring to individuals of the control group at any point in time (Collett, 2003). Taking into account the rate of death at all timepoints at which death has occurred, the hazard function of a treatment group relative to the control is expressed as proportion of the baseline hazard function.

# Chapter 3

# Characterisation of Slug Gut Bacteria

## 3.1 Introduction

Bacteria associated with some invertebrates have been well-studied with regards to their specific roles in nutrient metabolism (Douglas, 1998; Dugas *et al.*, 2001; Visotto *et al.*, 2009). Little previous work has been carried out to identify the bacteria associated with the slug, with existing studies concentrating mostly on the clinical significance of associated bacterial species (Shrewsbury & Barson, 1947; Elliott, 1970). In more recent years, Walker (1997) studied the role of proteinase secreting bacteria in slug digestion and the potential for their exploitation in slug biocontrol, but no formal identification of the bacterial flora was undertaken.

Before the advent of molecular biology, microbiology was wholly dependent upon culture-based techniques in bacterial identification and classification. Biochemical tests were used to study the phenotypical reactions of an organism and when combined with observations of morphological characteristics, identification could be achieved. These traditional techniques are labour intensive and time consuming, and rely upon the ability to culture the organism of interest. In the clinical laboratory in particular, where in many cases a quick diagnosis of often fastidious or opportunistic bacteria is required, these are distinct disadvantages. In addition to this, reliable interpretation of phenotypic test results requires experience and can be very subjective (Tang *et al.*, 1998). Differentiation of closely related bacteria sharing very similar metabolic activities can be very difficult using phenotypical methods (Balcazar *et al.*, 2007). The discriminating power of some tests can be adversely affected by the variable expression of metabolic factors amongst members of the same phylotype (Bannerman *et al.*, 1993; Heikens *et al.*, 2005). Furthermore, from a phylogenetic perspective, little information about the evolutionary relationships can be inferred between groups of organisms sharing similar phenotypes. Various kits are available on the market that standardise the testing process and help to remove some of the subjectivity associated with phenotypical identification. These include carbon source utilisation with the Biolog System (Biolog Inc. Haywood, CA, USA ), gas chromatographic analysis of cellular fatty acids using the Sherlock system developed by MIDI Inc (Newark, DE, USA) and metabolic profiling with the analytical profile index system (API, BioMérieux, La Rochelle, France). These methods still however rely upon the ability to produce a pure culture of the query organism, and there is a time factor associated with this.

It is now possible and quickly becoming the norm, to group bacteria by similarities in their genetic makeup which better reflects their evolutionary relationships (Woese, 1987; Hugenholtz *et al.*, 1998; Balcazar *et al.*, 2007). As DNA sequencing became possible, work by Woese and others in the early 1980s highlighted the significant part that comparative studies of the bacterial genetic code could play in bacterial phylogeny (Woese *et al.*, 1985; Woese, 1987; Olsen & Woese, 1993). In the time since then, bacterial identification by molecular methods has become more widespread and is generally regarded to be quicker, easier and less subjective than more traditional phenotypic identification. The 16S ribosomal RNA (rRNA) gene is one of the most common targets for molecular taxonomy owing to its high degree of conservation and universal presence in bacteria. The 16S rRNA gene can be compared throughout bacteria, archaea and with the 18S rRNA gene in eukaryotes (Clarridge, 2004). The high degree of conservation in this gene is probably due to the critical functional role that it has, meaning that mutations are much less tolerated than in a gene coding for an enzyme (Woese *et al.*, 1975). Mutations in the rRNA genes occur with a clock-like frequency, that is, changes occur at genotype level at a relatively constant rate and regardless of phenotypic evolution or selection pressures (Woese, 1987). This property makes rRNA genes particularly useful for inferring evolutionary relatedness and allows differentiation at the genus or even species level across all bacteria. The gene is around 1500 bp long and comprises conserved regions at the beginning, at around 500 bp and at the end which flank highly variable regions. For sequencing, the heterogeneous regions are amplified using PCR primers that are designed to be complementary to the conserved regions of the gene. Time and resources may be saved by sequencing only the first 500 bp of the 16S rRNA gene which still allows reliable inference of relationships and it is suggested that this portion can exhibit greater diversity per length than the full gene sequence (Kattar *et al.*, 2000).

In this chapter, bacterial isolates were cultured from the gut of four field-collected slugs from two different locations. These bacteria were identified using both phenotypical and molecular approaches. A greater understanding of the types of bacteria commonly associated with the slug is important in helping to initially highlight bacteria that may be symbiotic in the slug. It will also assist in the selection of appropriate antibiotics for bioassays which aim to test the potential for symbiotic bacteria to exist in the slug. A comparison will also be drawn as to the relevant merits of phenotypical identification versus a molecular approach.

## 3.2 Materials and Methods

### *3.2.1 Experimental Samples*

For the isolation and identification of culturable bacteria, slugs were collected by hand from a winter wheat field at Milrig Farm, Newbridge, West Lothian (NT101732GB) (Figure 3.1A). Estimates of bacterial populations were performed also on slugs from Jerusalem Farm, Pencaitland, East Lothian (NT647700GB) (Figure 3.1B), and slugs purchased from Blades Biologicals Ltd., Edenbridge, Kent. Slugs were collected as described in section 2.1.1.

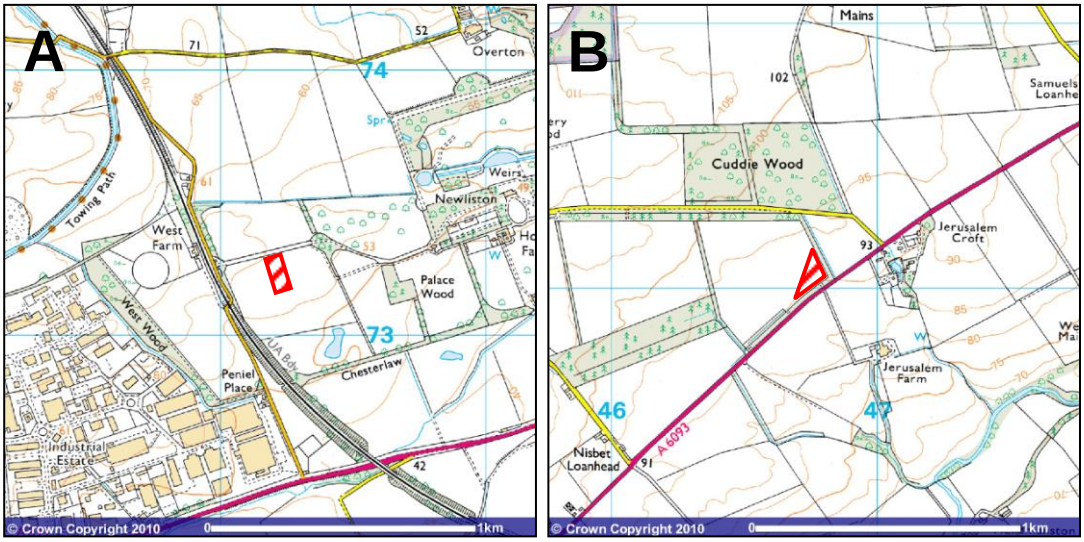



**Figure 3.1.** Location of sampling sites at A) "West Lodge", Milrig Farm, Newbridge, West Lothian; and B) Jerusalem Farm, Pencaitland, East Lothian.

### *3.2.2 Isolation of culturable bacteria from the slug digestive tract*

To culture bacteria from the slug gut, live slugs were first purged by starving for three days to reduce populations of transient bacteria then anaesthetised and dissected as per section 2.1.2. The masses of the excised whole digestive tracts were recorded then the tissue homogenised to a smooth paste with 100 µl of sterile quarter strength Ringer's solution (Oxoid, UK) using a sterile polypropylene pestle. Sterile Ringer's solution was added to make a total volume of 1 ml and a 10-fold dilution series prepared by transferring 100 µl of crude gut homogenate to 900 µl dilution blanks of Ringer's solution. Duplicate cultures were prepared on plate count agar (Oxoid) and MacConkey agar (Oxoid) by spreading 200 µl of dilutions from $10^{-2}$ to $10^{-6}$ onto the plates using a sterilised glass spreader rod.

Plates were incubated at 20 °C for two days after which colonies were counted on plates containing around 50 to 300 colonies. The colony counts were converted to colony forming units per mg tissue (cfu.mg tissue$^{-1}$) and a mean of ten counts recorded. Morphologically different colonies from four slugs were labelled and recorded in correspondence with the morphology descriptions in figure 3.2, and these colonies were picked with a sterile inoculating loop and sub-cultured until pure by streaking onto fresh plates of either plate count agar or MacConkey agar. Pure cultures were preserved by inoculating a tube of cryo-preservation beads (Protect™ storage system, Technical Service Consultants (TSC) Ltd, Lancashire, UK) and incubating whilst shaking for one hour at 30 °C during which time the bacteria adhere to the beads. After incubating the cryopreservation fluid was removed and the tube containing the beads was sealed and stored at -80 °C. To recover bacteria from the cryopreservation beads, the vial was removed from the freezer and using a sterile dissecting needle, a single bead was removed and used to inoculate a suitable culture medium.

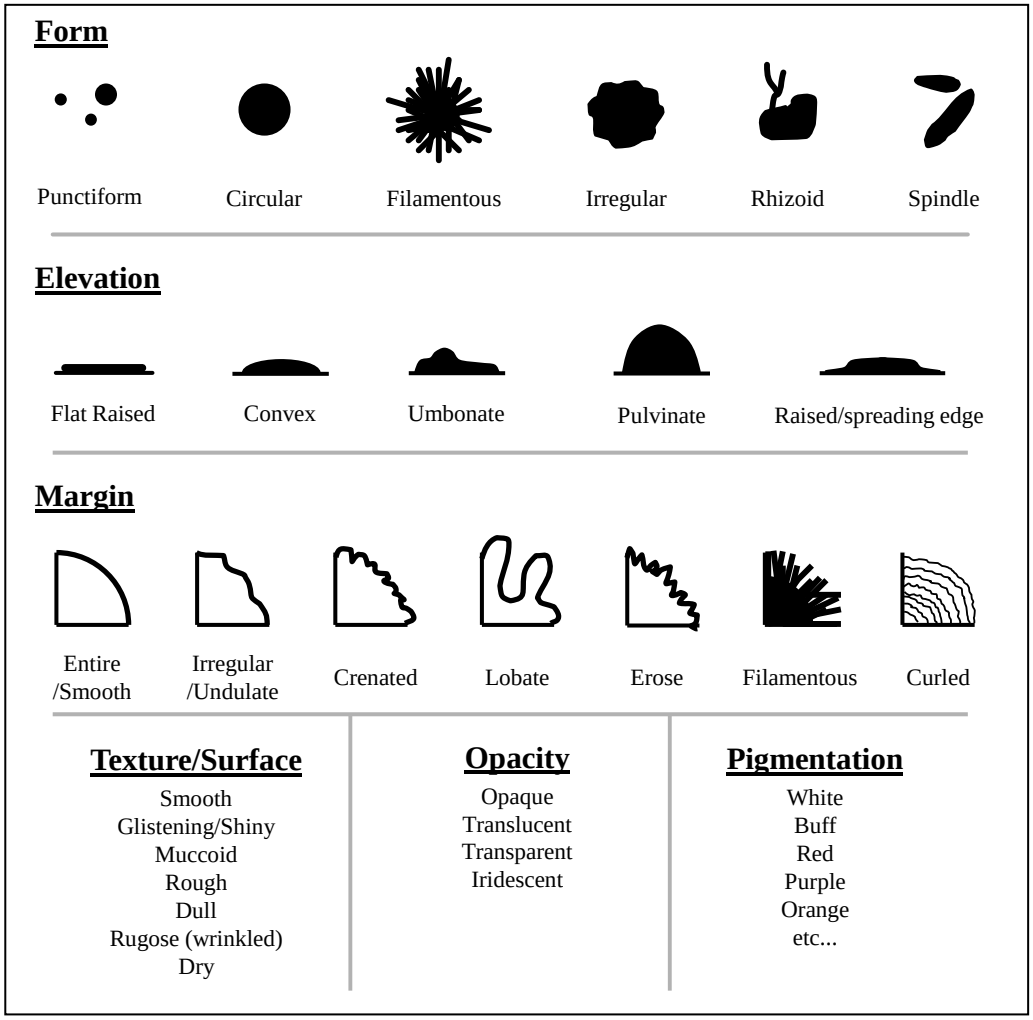



**Figure 3.2.** Bacterial colony morphology. Based on Prescott *et al.* (1990)

### *3.2.3 Phenotypic identification of bacterial isolates*

Gram determination of bacteria isolated from the slug digestive tracts was carried out using the potassium hydroxide (KOH) string test (Arthi *et al.*, 2003). Briefly, colonies that produce viscous "strings" when mixed with a drop of 3% KOH on a glass slide are recorded as Gram negative. Bacteria were identified using the API-20E identification system (BioMérieux) which comprises a strip of 20 biochemical tests (Figure 3.3, Table 3.1) each containing a different metabolite and an indicator which undergoes a colour change in response to bacterial utilization of the reagents.


**Figure 3.3.** API 20E test strip. A colour change in either of the 20 wells represents utilisation of the metabolite by the bacterium. The resultant profile is cross-referenced with the APILab database to return an identification.

**Table 3.1.** API 20E biochemical tests

| Test | Reagent | Reaction/Enzyme |
|---|---|---|
| ONPG | 2-nitrophenyl-βD-galactopyranoside | β-galactosidase |
| ADH | L-arginine | Arginine Dihydrolase |
| LDC | L-lysine | Lysine Decarboxylase |
| ODC | L-ortithine | Ornithine Decarboxylase |
| CIT | Trisodium citrate | Citrate utilisation |
| H2S | Sodium thiosulphate | $H_2S$ production |
| URE | Urea | Urease |
| TDA | L-tryptophane | Tryptophane Deaminase |
| IND | L-tryptophane | Indole production |
| VP | Sodium pyruvate | Voges-Proskauer – acetonin production |
| GEL | Gelatin | Gelatinase |
| GLU | D-glucose | Fermentation/Oxidation of Glucose |
| MAN | D-mannitol | Fermentation/Oxidation of Mannitol |
| INO | D-inositol | Fermentation/Oxidation of Inositol |
| SOR | D-sorbitol | Fermentation/Oxidation of Sorbitol |
| RHA | L-rhamnose | Fermentation/Oxidation of Rhamnose |
| SAC | D-sucrose | Fermentation/Oxidation of Saccharose |
| MEL | D-melibiose | Fermentation/Oxidation of Melibiose |
| AMY | Amygdalin | Fermentation/Oxidation of Amygdalin |
| ARA | L-arabinose | Fermentation/Oxidation of Arabinose |

A single well isolated colony was picked with a sterile inoculating loop and transferred to 5 ml of sterile distilled water in a 15 ml centrifuge tube and emulsified to achieve a homogeneous suspension. Each well in the strip was filled with the suspension and the ADH, LDC, ODC, $H_2S$ and URE tests were overlaid with mineral oil to create anaerobiosis. The strip was incubated at 30 °C for 24 hours before the colour changes for each test were interpreted and recorded as positive or negative reactions according to the test instructions. If fewer than three reactions were positive, the strip was incubated for a further 24 hours and checked again. A number of the tests must be revealed with the addition of separate reagents. The results profile from each test strip was entered into the ApiLab software which compares the pattern of results to a database and returns an interpretation together with possible identities for the test organism. Profiles are assigned an identification percentage (%ID, the degree to which the profile corresponds to the taxon in the database), T-index (how closely the profile corresponds to the most typical set of reactions for that taxon) and a comment based on the values of both the %ID and T-index (Table 3.2).

**Table 3.2.** Comments assigned to API profiles

| Comment | %ID | T-index |
|---|---|---|
| Excellent | ≥99.9 | ≥0.75 |
| Very Good | ≥99.0 | ≥0.5 |
| Good | ≥90.0 | ≥0.25 |
| Acceptable | ≥80.0 | ≥0 |

### *3.2.4 Molecular identification of bacterial isolates*

In addition to and as a comparison to phenotypic identification, some of the bacteria isolated from the slug gut in section 3.2.2 were also identified by sequencing of the bacterial 16S ribosomal RNA (rRNA) gene. The full gene length was sequenced where possible.

#### *Extraction of bacterial DNA from pure cultures*

Fresh bacterial cultures were prepared on nutrient agar and incubated for two days at 20 °C. A single well-isolate colony was picked with a sterile inoculating loop and suspended in 100µl of sterile distilled water (SDW) in a 1.5 ml microcentrifuge tube. To each cell suspension was added 367 µl sodium phosphate buffer (120 mM; pH 8.0), 30 µl SDS (10% SDS; 0.5 M Tris-HCl; pH 8.0; 0.1 M NaCl) and 3 µl Proteinase K (Qiagen). The tubes were inverted several times and incubated for 1 hour at 37 °C. The proteins were precipitated by

adding 200 µl of 7.5 M ammonium acetate, inverting the tube to mix and incubating on ice for 5 minutes. This was followed by adding 700 µl of phenol:chloroform:isoamyl alcohol mix (25:24:1) and inverting several times. After centrifugation at 12,000 g for 3 minutes the upper aqueous layer was transferred to a new tube and the DNA was precipitated by adding 300 µl of isopropanol and centrifuging at 12,000 g for 45 minutes at 4 °C. The isopropanol was poured from the tube and the remaining DNA pellet was washed twice in 70% ethanol before resuspending in 400 µl Tris-EDTA buffer (10 mM Tris base; 1 mM EDTA; pH 8.0). The DNA was quantified with a Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific).

*Sequencing of the 16S rRNA gene*

The complete 16S rRNA gene was amplified using a PCR protocol modified from (Egert *et al.*, 2003) utilising previously described primers 27f (5'-AGA-GTT-TGA-TCC-TGG-CTC-AG-3') (Edwards *et al.*, 1989), and 1492r (5'-TAC-GGY-TAC-CTT-GTT-ACG-ACT-T-3') (Lane, 1991). PCR was run with reaction volume of 50 µl which comprised 10 µl of 5x Colourless GoTaq® Flexi Buffer (Promega), 1.5 mM $MgCl_2$, 50 µM concentration of each dNTP, 1.25 U GoTaq® DNA Polymerase (Promega), 0.1 µM of each primer and as a template, 2 µl of a 1:100 dilution of bacterial genomic DNA. The amplification was performed on a GeneAmp 9700 PCR system (Applied Biosystems) and reaction conditions were 94 °C for 3 minute followed by 32 cycles of denaturation at 94 °C for 30 seconds, annealing at 57 °C for 30 seconds and extension at 72 °C for 1 minute, finishing with a final extension step 72 °C for 7 minutes. PCR products were run on an agarose gel (1.5% agarose; 0.1 $\mu l.ml^{-1}$ GelRed (Biotium Inc. CA, USA); 100 V; 1 hour) to check that a single product of around 1500 bp had been achieved, and that it was free from contaminating primer dimers. Products were deemed to be suitable for direct sequencing so the remaining product was purified using the High Pure PCR product purification kit (Roche) following the manufacturers' instructions. The purified 16S rDNA was sequenced from both directions using Big Dye terminator chemistry on an Applied Biosystems 3730 DNA Analyser at DBS Genomics, Durham University, UK. Forward and reverse sequences were trimmed and assembled into a contig covering the whole 16S rRNA gene (~1400 bp) using the sequence assembly program CAP3 (http://pbil.univ-lyon1.fr/cap3.php) (Huang & Madan, 1999). For identification, the assembled 16S rRNA sequences were compared to sequences in the GenBank database using the BLASTN search algorithm (http://www.ncbi.nlm.nih.gov/blast) (Altschul *et al.*, 1990). Query sequences were aligned with their closest matches and

reference sequences from type-cultures using CLUSTALX (Thompson *et al.*, 1997), and the resultant alignments used to generate phylogenetic trees by the maximum likelihood method in PHYLIP (Felsenstein, 1989) (http://evolution.genetics.washington.edu/phylip.html).

## 3.3 Results

### *3.3.1 Enumeration of bacteria in the slug*

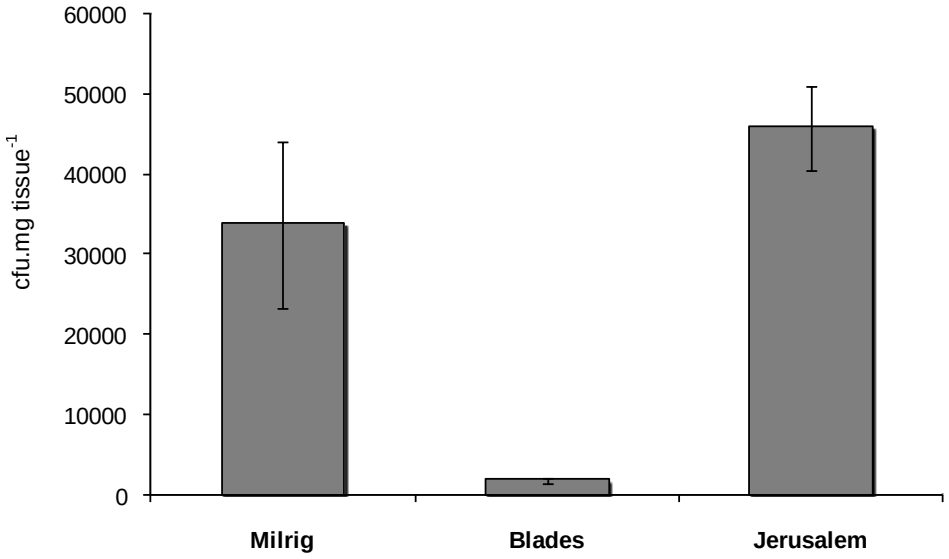



**Figure 3.4.** Estimates of slug gut bacterial load from three locations. Counts are expressed as mean colony forming units per mg of tissue (cfu.mg tissue$^{-1}$). Error bars represent SEM from ten samples per location.

Gut bacterial load was estimated for a total of 30 slugs, ten from each of three locations. Bacterial counts ranged between 496 and 100888 colony forming units per mg of tissue (cfu.mg tissue$^{-1}$) with slugs from Blades Biological exhibiting a 19-fold and 26-fold lower mean bacterial count than those collected from Milrig and Jerusalem farms respectively (Figure 3.4) (ANOVA $p<0.001$). Despite a 1.5-fold difference in mean bacterial load in slugs from Milrig Farm and Jerusalem Farm this was not statistically significant given the high degree of variation observed (Table 3.3). Standard deviation was similar in magnitude to the mean for both Milrig and Blades samples. The very large range exhibited by the Milrig data in particular reflects a high degree of variation which is not uncommon with bacterial colony counts which are notorious in their poor reproducibility (Collins *et al.*, 2004).

**Table 3.3**. Mean viable cell counts expressed as colony forming units per mg of tissue (cfu.mg tissue-1) from three locations. Standard deviation and range are quoted for ten repetitions per location. Different letters represent statistical significance in the means; ANOVA, $p<0.001$.

| Location | cfu.mg tissue $^{-1}$ | Standard deviation | Range |
|---|---|---|---|
| Milrig Farm | 33716 [a] | 32846 | 91707 |
| Blades Biological | 1777 [b] | 1066 | 3718 |
| Jerusalem Farm | 45762 [a] | 16530 | 48768 |
| All slugs | 27085 | 27862 | 100406 |

### *3.3.2 Phenotypical identification of slug-associated bacteria*

Between 11 and 14 morphologically different bacterial colonies were isolated from each of four different slugs making a total of 49 isolates for identification. Based on colony morphology alone, the bacteria inhabiting the slug gut appear to be rather diverse, exhibiting a range of different morphological traits (Table 3.4). The most abundant colonies on plate count agar were circular, raised or convex, with entire margins, muccoid texture, opaque and white in colour.

Of the 49 isolates cultured, 24 from two slugs were identified using the API 20E identification system. Thirteen of the 24 isolates tested were designated "unacceptable profiles" and no identification was given. Of the remaining cultures tested, the %ID varied from 39.7% to 99.8%, with only three organisms being assigned a %ID greater than 95% (Table 3.5). All isolates for which an identity was returned were Gram negative rods. Two organisms from slug MRW001 were identified as *Pseudomonas aeruginosa* and assigned a %ID of 99.8% which is regarded as "good" and "very good" identifications based upon their T indices of 0.27 and 0.5 respectively. Other notable identifications were 1P10 and 1P7, each identified to the genus level as *Pseudomonas fluorescens/putida* at 95.1% ID (T=0.19) and 82.6% ID (T=0.28) respectively and P6P5A assigned to the species *Rahnella aquatilis* at 80.7% ID (T=0.34). Two organisms, each from a different slug, had identical profiles and were identified as *Serratia liquefaciens* (isolates 1M1 and 6P5). Even though the %ID of these identifications is quite low (78.9%), the T-index is good at 0.31 and because these isolates have identical profiles, it is likely that these are indeed identical organisms.

**Table 3.4.** Colony morphology of bacterial isolates cultured from the digestive tract of four slugs. Morphology is recorded as described in figure 3.2. The media on which the colony was cultured is indicated by either 'M' (MacConkey agar) or 'P' (Plate count agar) in the isolate reference. The most abundant colony morphologies for each slug are indicated in bold type.

| | Colony Morphology | | | | | |
|---|---|---|---|---|---|---|
| **Ref** | **Form** | **Elevation** | **Margin** | **Texture** | **Opacity** | **Pigmentation** |
| *Slug MRW001* | | | | | | |
| 1M1 | Circular | Convex | Entire | Muccoid | Translucent | Pink |
| 1M2 | Circular | Raised | Entire | Shiny | Translucent | White |
| 1M3 | Circular | Convex | Crenated | Muccoid | Translucent | Deep Pink |
| **1M4** | **Circular** | **Flat** | **Entire** | **Dull** | **Translucent** | **Colourless** |
| **1M5** | **Circular** | **Raised** | **Entire** | **Muccoid** | **Opaque** | **White** |
| 1P1 | Circular | Flat | Crenated | Shiny | Translucent | Yellow |
| **1P2** | **Circular** | **Convex** | **Entire** | **Muccoid** | **Opaque** | **White** |
| 1P3 | Circular | Flat | Entire | Rough/Dry | Translucent | Cream |
| 1P6 | Irregular | Convex | Irregular | Muccoid | Opaque | White |
| 1P7 | Irregular | Raised | Irregular | Rough | Opaque | Yellow |
| 1P8 | Circular | Spreading | Entire | Muccoid | Opaque | Purple, white margin |
| 1P9 | Circular | Raised | Entire | Shiny | Translucent | White |
| 1P10 | Circular | Raised | Crenated | Muccoid | opaque | White |
| *Slug MRW003* | | | | | | |
| 3M1 | Circular | Raised | Irregular | Shiny | Opaque | Deep pink |
| 3M2 | Circular | Raised | Irregular | Shiny | Opaque | Pink |
| 3M3 | Circular | Convex | Entire | Muccoid | Opaque | Yellow, pink margin |
| 3M4 | Circular | Convex | Entire | Muccoid | Opaque | Yellow, pink margin |
| 3M5 | Circular | Raised | Entire | Muccoid | Translucent | Colourless |
| 3M6 | Circular | Flat | Entire | Dry | Translucent | Pale pink |
| **3M7** | **Circular** | **Flat** | **Irregular** | **Rough/dry** | **Translucent** | **Colourless** |
| 3M8 | Irregular | Flat | Irregular | Rough/dry | Translucent | Colourless |
| 3M9 | Circular | Flat | Crenated | Dry | Translucent | Deep pink |
| 3P1 | Circular | Spreading | Crenated | Dry | Translucent | Yellow |
| **3P2** | **Circular** | **Raised** | **Entire** | **Muccoid** | **Opaque** | **White** |
| 3P4 | Circular | Flat | Entire | Muccoid | Translucent | White, Colourlessmargin |
| 3P5 | Circular | Flat | Entire | Smooth | Opaque | Purple, white margin |
| 3P6 | Circular | Flat | Entire | Shiny | Translucent | Yellow |
| *Slug MRW005* | | | | | | |
| 5M1 | Circular | Raised | Entire | Dry | Opaque | Yellow, red margin |
| **5M2** | **Circular** | **Convex** | **Entire** | **Muccoid** | **Opaque** | **White** |
| 5M3 | Circular | Flat | Entire | Dry | Translucent | Colourless |
| 5M4 | Irregular | Convex | Irregular | Smooth | Translucent | Pink |
| 5M5 | Circular | Spreading | Entire | Muccoid | Opaque | Yellow, pink margin |
| 5P2 | Irregular | Umbonate | Irregular | Muccoid | Opaque | White |
| 5P3 | Irregular | Flat | Irregular | Dull | Translucent | Colourless |
| 5P4 | Circular | Raised | Entire | Rugose | Opaque | Purple, white margin |
| **5P5** | **Circular** | **Convex** | **Entire** | **Muccoid** | **Opaque** | **White** |
| 5P6 | Circular | Spreading | Entire | Muccoid | Translucent | Yellow tint |
| 5P7 | Circular | Convex | Entire | Dry | Translucent | Yellow |
| *Slug MRW006* | | | | | | |
| 6P1 | Circular | Raised | Crenated | Rough | Opaque | White |
| 6P2 | Circular | Convex | Entire | Muccoid | Opaque | White |
| **6P3** | **Circular** | **Raised** | **Entire** | **Dry** | **Translucent** | **Buff** |
| 6P4 | Circular | Spreading | Entire | Dry | Opaque | White |
| 6P5 | Circular | Flat | Entire | Dry | Translucent | Buff |
| **6P6** | **Circular** | Raised | **Entire** | **Shiny** | **Opaque** | **White** |
| 6P7 | Circular | Convex | Entire | Shiny | Opaque | Yellow |
| 6P8 | Circular | Spreading | Entire | Shiny | Opaque | White |
| 6P9 | Circular | Raised | Entire | Smooth | Translucent | Buff |
| 6P10 | Circular | Raised | Entire | Smooth | Translucent | Yellow |
| 6P11 | Circular | Raised | Entire | Smooth | Translucent | White |

Where the %ID is especially low, the ApiLab database may highlight the closest possible matches whilst suggesting complementary tests that can be performed to obtain more information for the identification. These clearly increase the workload and give no guarantee of significantly improving the conclusions that can be drawn. Although it is not clear why so many of the samples tested returned a result of "*unacceptable profile*", it is most likely that the profiles did not closely resemble any of the taxa in the database. API is primarily designed for clinical laboratory application, thus the database is orientated towards bacteria of clinical importance. Since the bacteria in the slug gut is most likely to reflect soil bacteria it is therefore perhaps unsurprising that API failed in its identification of many of the bacteria cultured from the slug. The API 20E strips used in this test are specific to the

**Table 3.5.** Identification results for 24 slug gut bacterial isolates identified using the API 20E identification system (BioMérieux), sorted by descending %ID.

| Isolate | Taxon | ID Comment | %ID | T index |
|---|---|---|---|---|
| 1M2 | *P. aeruginosa* | "Very good ID" | 99.8 | 0.50 |
| 1P9 | *P. aeruginosa* | "Good ID" | 99.8 | 0.27 |
| 1P10 | *P. flourescens/putida* | "Acceptable ID to genus" | 95.1 | 0.19 |
| 1P7 | *P. flourescens/putida* | "Acceptable ID to genus" | 82.6 | 0.28 |
| 6P5 | *Rahnella aquatilis* | "Low Discrimination" | 80.7 | 0.34 |
| 1M1 | *S. liquefaciens* | "Good ID to genus" | 78.9 | 0.31 |
| 6P8 | *S. liquefaciens* | "Good ID to the genus" | 78.9 | 0.31 |
| 1M3 | *Pantoea spp. 4* | "Acceptable ID to species" | 62.2 | 0.26 |
| 1M5 | *P. flourescens/putida* | "Acceptable ID to genus" | 56.6 | 0.20 |
| 1P8 | *Aer. salm. salmonicida* | "ID not valid" | 40.4 | 0.22 |
| 1P6 | *Pantoea spp. 2* | "ID not valid" | 39.7 | 0.30 |
| 6P1 | *Chrom. violaceum* | "Unacceptable Profile" | N/A | N/A |
| 6P2 | *Pantoea spp. 2* | "Unacceptable Profile" | N/A | N/A |
| 6P4 | *P. flourescens/putida* | "Unacceptable Profile" | N/A | N/A |
| 6P6 | *Pantoea spp.1* | "Unacceptable Profile" | N/A | N/A |
| 6P7 | *Serratia spp.* | "Unacceptable Profile" | N/A | N/A |
| 6P9 | *Myroides/Chrys. indologenes* | "Unacceptable Profile" | N/A | N/A |
| 6P10 | *P. aeruginosa* | "Unacceptable Profile" | N/A | N/A |
| 6P11 | *Pantoea spp.1* | "Unacceptable Profile" | N/A | N/A |
| 1M4 | N/A | "Unacceptable Profile" | N/A | N/A |
| 1P1 | N/A | "Unacceptable Profile" | N/A | N/A |
| 1P2 | N/A | "Unacceptable Profile" | N/A | N/A |
| 1P3 | N/A | "Unacceptable Profile" | N/A | N/A |
| 6P3 | N/A | "Unacceptable Profile" | N/A | N/A |

identification of gram negative enteric bacilli which were considered most likely to be present in the slug gut based on previous studies (Elliott, 1970; Watkins & Simkiss, 1990; Walker, 1997; Walker *et al.*, 1999). Other API strips are available which allow for identification of different bacterial groups, and it may be the case that API 20E are not the most suitable, for example if there is a large Gram positive population in *D. reticulatum*, or indeed the only tests required for a broad and thorough survey of this particular bacterial population.

### *3.3.3 Molecular identification of slug-associated bacteria*

In total, the 16S rRNA genes of 49 bacterial isolates were amplified and sequenced in both the forward and reverse directions. In most instances, a sequence read length of between 700 and 1000 base pairs was achieved allowing a full length 16S rRNA gene sequence to be assembled, around 1400 base pairs. For a number of samples, reads of insufficient length prevented full sequences being assembled, in which case the forward and reverse sequences were both used in subsequent analysis and database searching. Genbank queries allowed all isolates studied to be assigned to one of 11 genera, with individual slugs yielding between four and seven genera of cultured organisms (Table 3.6).

**Table 3.6.** Frequency of occurrence of different bacterial genera based on identification by 16S rRNA gene sequencing of morphologically different colonies isolated from the digestive tracts of four slugs.

| Slug | *Pseudomonas* | *Rahnella* | *Serratia* | *Janthinobacterium* | *Sphingobacterium* | *Flavobacterium* | *Buttiauxella* | *Ewingella* | *Carnobacterium* | *Stenotrophomonas* | *Chryseobacterium* | Total |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| MRW001 | 7 | 2 | 1 | 1 | 1 | 1 | | | | | | 13 |
| MRW003 | 3 | 3 | | 2 | 1 | 2 | 2 | 1 | | | | 14 |
| MRW005 | 6 | 3 | 1 | | | | 1 | | | | | 11 |
| MRW006 | 4 | 1 | 1 | 1 | | | | | 2 | 1 | 1 | 11 |

The majority (84%) of isolates were gram negative rod-forming bacteria belonging to the phylum *Proteobacteria,* with the genera *Pseudomonadaceae* (41%) and *Enterobacteriaceae* (31%*)* dominating (Figures 3.5, 3.6). Other phyla identified were *Bacteroidetes* (12%) and *Firmicutes* (4%). Appendices 3.1 to 3.4 show phylogenetic trees of the partial 16S rRNA gene sequences obtained from bacteria isolated from the individual slugs used in the study. Here it is apparent that *Pseudomonas, Rahnella, Serratia* and *Janthinobacterium* comprised the majority of the identified bacteria in the slugs studied, whilst other genera were less common. Although only fours slugs were used as a source of bacteria for this study it could expected that a larger sample size would result in a similar composition of bacterial flora with a core of more abundant genera accompanied by other more incidental bacteria. Considering the slugs were collected from the same field, there is a large degree of diversity between the make-up of the gut flora of the individuals, with only four genera identified in three or more slugs (Table 3.6). It was only possible to identify 18 isolates to the species level out of the 49 studied because the closest nucleotide sequence matches in Genbank in these instances were from previously unidentified bacterial clones.

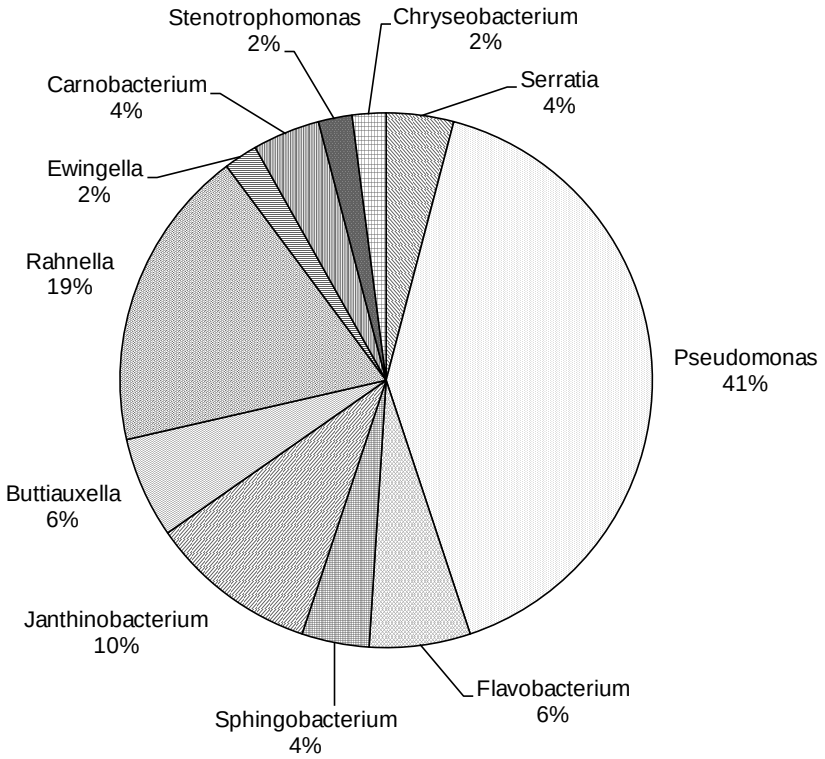



**Figure 3.5.** Bacterial isolate identities displayed by genus showing the percentage of 49 isolates assigned to each genus by 16S rRNA gene sequencing.

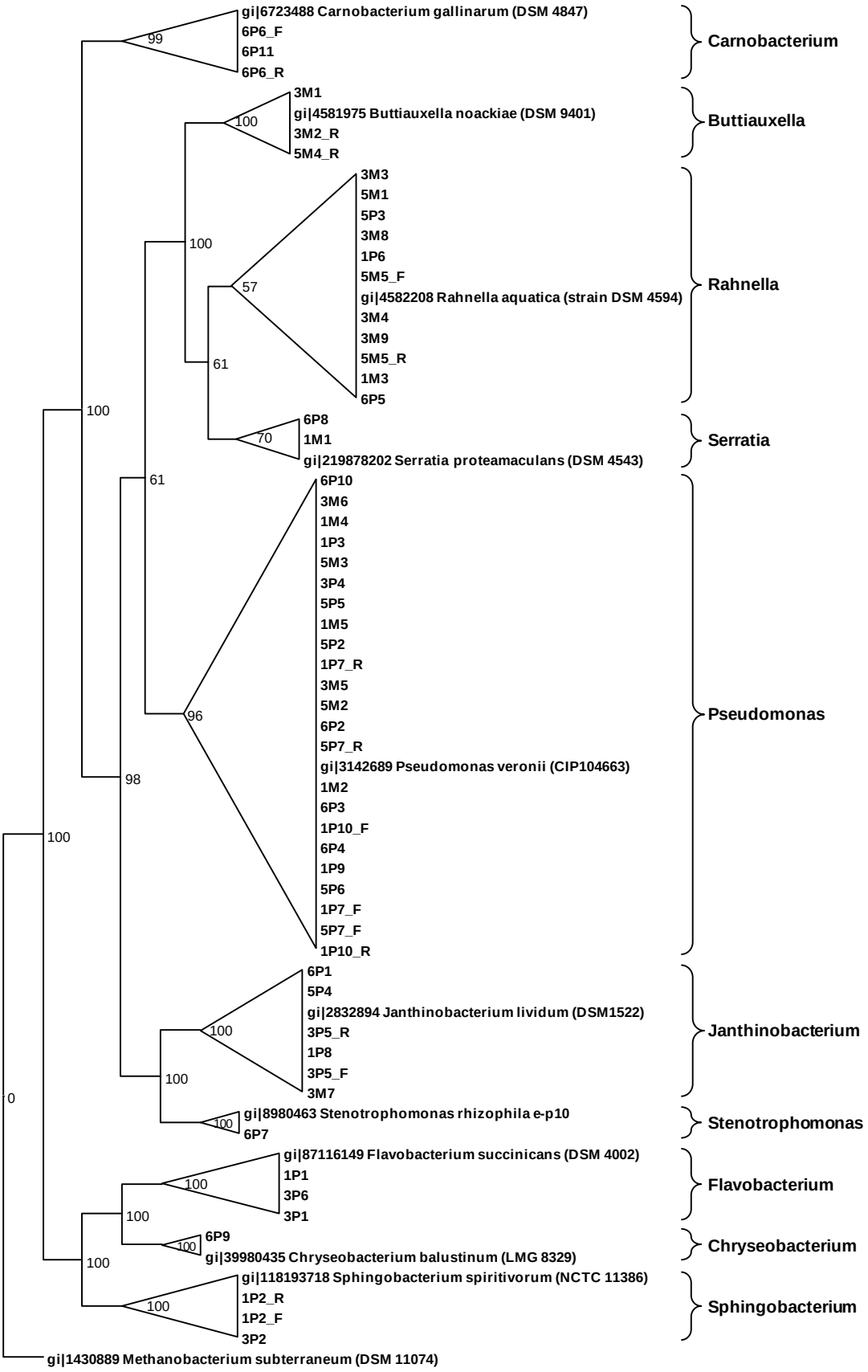



**Figure 3.6.** Maximum likelihood tree of 16S rRNA gene sequences from slug gut bacterial isolates. The values at the nodes represent the number of times that branch occurred in 100 boot strap replicate trees. The archaeal *Methanbacterium* is included as an outgroup root.

## 3.4 Discussion

Residing primarily in soil, slugs are in constant contact with soil-dwelling microorganisms so it is a simple hypothesis that their digestive tract could harbour bacterial species commonly found in the soil environment. Indeed in this study, bacteria from 11 different genera were identified in *D. retictulatum* all of which have previously been identified in soil, water and other environmental samples. Bacteria from the genus *Pseudomonas* are ubiquitous in soil and water samples and requiring no growth factors, are able to grow in the presence of a single organic compound so it is unsurprising that the majority of isolates in this study were assigned to this genus and that they were present in all of the slugs studied (Buchanan & Gibbons, 1975). *Pseudomonas* species identified included *P. aeruginosa*, *P. fluorescens, P. putida, P. reactans* and *P. trivialis.* Of these, *P. aeruginosa* is a serious opportunistic human and animal pathogen (Kerr & Snelling, 2009).

*Rhanella* species formed a second major genus, accounting for 19% of the isolates studied. *Rhanella aquatilis* has recently been found to express antibacterial factors that could prove important in the biological control of a number of bacterial pathogens of fruit trees, vines and tomatoes (Laux *et al.*, 2002; El-Hendawy *et al.*, 2005; Chen *et al.*, 2007). Although slugs are not major pests in these crops it is useful to know about the presence of such bacteria in *D. reticulatum* and to be aware that slugs may harbour other bacteria that elicit biocontrol in different crops. For example, *Pseudomonas* strains offer a possible application in biocontrol due to their production of the antibiotic 2,4-diacetyl-phloroglucinol (DAPG) that can help protect a plant from microbial pathogens (Keel *et al.*, 1992; Farzaneh *et al.*, 2007; Siasou *et al.*, 2009). It is possible to imagine that slugs and other soil dwelling invertebrates may play a role in the distribution and transmission of such biological control agents around the field and this potential contribution to crop protection should be born in mind when integrated systems for crop management are being developed. Furthermore, any slug control measure that is designed to target slug-associated bacteria must be careful not to disrupt bacteria that may at first seem superfluous to the slug, yet afford benefits to the crop in which the slug is living.

*Serratia* species are commonly isolated from insects where they may exist as pathogens (Grimont & Grimont, 1978) or as part of the natural commensal flora of some insects. Some species commonly cause hospital-acquired infections in humans (*S. marcescens* and *S. liquefaciens*) (Hejazi & Falkiner, 1997). Considering the likelihood of human contact with

*D. reticulatum*, especially through fresh produce, it may be important to consider the clinical significance of any slug-associated bacteria. Most of the bacteria identified in this study have been implicated in clinical cases at some point in time. Of the species identified in this study, *P. aeruginosa* is a serious opportunistic human and animal pathogen (Kerr & Snelling, 2009). The other genera identified are mainly opportunistic pathogens and transmission to the food chain by the slug is likely to be of little importance (Chang *et al.*, 1999; Kati *et al.*, 1999). It is however useful to be aware of potential environmental reservoirs of any human pathogens as such knowledge could be of epidemiological importance in the future.

Of the 24 isolates identified by both genotypical and phenotypical methods, ten were identified to the same genus or species by both methods (Table 3.7). Seven identities agreed only to class, phylum, order or family level which is unsuitable for identification purposes. The problem most probably lies in the application of the API system itself. Being targeted towards the clinical laboratory, many of the bacteria more commonly associated with environmental samples are simply not present in the database if they have no history as human pathogens. This is reflected in the fact that the API matches with the greatest %ID and T-index values were those of *Pseudomonas* species (Table 3.5) of which *P. aeruginosa* is a well-characterised opportunistic human pathogen (Kerr & Snelling, 2009). The API range comprises a number of different strips depending on the likely identity of the organism in question. In this case, the API 20E test strips used are specific to Gram negative rod-shaped bacteria to which most of these bacteria isolated from *D. reticulatum* in this study comply. The only exception are isolates 6P6 and 6P11 which through 16S rRNA gene sequencing were identified as belonging to the genus *Carnobacterium* which are Gram positive and rod-like in their cell morphology. Therefore, although in this instance the API 20E test was the most suitable, based on the likely dominance of the slug digestive tract by Gram negative bacteria (Elliott, 1970; Watkins & Simkiss, 1990), for identification of slug associated bacteria, it is an important criteria of the API system that a little is known about the query organism in advance of identification so that the most appropriate test may be selected. This makes the API system a rather inefficient choice for a full-scale study of the bacterial flora of a given environmental sample where no prior characterisation of the bacteria has been carried out. In most cases, API was only able to identify slug bacterial isolates to the genus level which suggests one of two things. Either the query species is not present in the database as mentioned above, or the profile obtained from the test strip was not accurate.

**Table 3.7.** Comparison of bacterial isolate identities assigned by molecular (16S rRNA gene sequencing) and pheonotypical (API 20E, BioMeriéux) methods showing to which level the two methods agree. The API% ID is shown in parenthesis next to the API identities –"N/A" represents incidences where the API profile was "unacceptable", the genera in these cases are closest matches only and not formal identifications. Shaded isolates show where identifications from both methods agree to the genus level.

| Isolate | 16S rDNA Sequencing | API 20E | Agree |
|---|---|---|---|
| 1M1 | *Serratia proteamaculans* | *Serratia liquefaciens (78.9%)* | Genus |
| 1M2 | *Pseudomonas sp.* | *Pseudomonas aeruginosa (99.8%)* | Genus |
| 1M3 | *Rahnella aquatilis* | *Pantoea spp. 4 (62.2%)* | Family |
| 1M4 | *Pseudomonas sp.* | N/A | N/A |
| 1M5 | *Pseudomonas sp.* | *Pseudomonas flourescens/putida (56.6%)* | Genus |
| 1P1 | *Flavobacterium frigidimaris* | N/A | N/A |
| 1P2 | *Sphingobacterium sp.* | N/A | N/A |
| 1P3 | *Pseudomonas sp.* | N/A | N/A |
| 1P6 | *Rahnella sp.* | *Pantoea spp. 2 (39.7%)* | Family |
| 1P7 | *Pseudomonas sp.* | *Pseudomonas flourescens/putida (82.6%)* | Genus |
| 1P8 | *Janthinobacterium sp.* | *Aeromonas salmonicida.salmonicida (40.4%)* | Phylum |
| 1P9 | *Pseudomonas sp.* | *Pseudomonas aeruginosa (95.1%)* | Genus |
| 1P10 | *Pseudomonas sp.* | *Pseudomonas flourescens/putida (95.1%)* | Genus |
| 6P1 | *Janthinobacterium sp.* | *Chromobacterium/Ps. Aeruginosa (N/A)* | Phylum |
| 6P2 | *Pseudomonas sp.* | *Pantoea spp./Citro freundii/Klebsiella (N/A)* | Class |
| 6P3 | *Pseudomonas reactans* | N/A | N/A |
| 6P4 | *Pseudomonas putida* | *Ps. fluor/putida/aeruginosa (N/A)* | Genus |
| 6P5 | *Rahnella aquatilis* | *Rahnella aquatilis (80.7%)* | Species |
| 6P6 | *Carnobacterium sp.* | *Pantoea spp.1 (N/A)* | N/A |
| 6P7 | *Stenotrophomonas rhizophila* | *Serratia spp. (N/A)* | Class |
| 6P8 | *Serratia proteamaculans* | *Serratia liquefaciens (78.9%)* | Genus |
| 6P9 | *Chryseobacterium vrystaatense* | *Myroides/Chryseobacterium (N/A)* | Order |
| 6P10 | *Pseudomonas trivialis* | *Ps. aeruginosa/fluor/putida (N/A)* | Genus |
| 6P11 | *Carnobacterium sp.* | *Pantoea spp.1 (N/A)* | N/A |

The API tests rely upon colour changes which in reality can be ambiguous and difficult to assign a positive or negative result by eye, introducing a degree of subjectivity. There is no procedure to regulate the number of bacterial cells used when inoculating the strip and although a single colony is used each time, the size and presumably number of cells can vary from one colony to the next. The number of cells may therefore vary from one test to another and it is not known if this has an effect upon results and to what extent if it does. Indeed, Devonish and Barnum (1982) reported that increasing the inoculum size improved identification with API 20E. A variation in cell numbers introduces a degree of uncertainty regarding the optimal incubation time and could result in a very weak reaction being recorded as a negative reaction where the bacterium is assumed to have little or no activity

with respect to utilisation of the specific reagent. For example, an organism that would normally show a strong positive reaction may only show a weak reaction if insufficient cells were present, and there may be a risk of this being recorded as negative. This problem could be relatively easily overcome by duplicating the tests, providing that a fresh bacterial suspension was prepared using a separate colony although this would increase both the time and resources required in what is already a fairly labour-intensive method.

An attempt was made to estimate the size of the bacterial population living within the slugs studied. Bacterial counts in slugs from Milrig Farm and Jerusalem Farm were comparable to those recorded in *D. reticulatum* by Walker (1997; 1999) but around 50% lower than those quoted by Watkins & Simkiss (1990) in the snail *Helix aspersa*. The slugs ordered from Blades Biological showed a 20-25-fold lower mean bacterial load than those collected from Milrig Farm and Jerusalem Farm. As it is largely unknown how much time had elapsed between field collection and delivery of Blades slugs, it is possible that these slugs had been out of the field and therefore purged longer than those collected locally which would explain the considerable difference in bacterial count. Also, because the Milrig and Jerusalem Farm locations are geographically very close to one another than they are to Blades, there may be differences in the species composition of the slug gut microflora in different geographical locations such that fewer bacteria in the Blades slugs were culturable in the culture conditions used for plate counting. This highlights a requirement to measure the extent of geographical variation in slug gut microflora composition as part of this study such that any novel slug control utilises a target that is found universally in *D. reticulatum* to avoid regional specificity and therefore reduced potential market size for a future biocontrol product.

When enumerating bacterial populations using culture-dependent techniques it is necessary to understand that the results obtained from plate counts are likely to be large underestimations of counts that can be obtained through direct visual counting of cells using a microscope (Staley & Konopka, 1985; Torsvik *et al.*, 1990). There are several reasons for this. Firstly not all bacteria present in a sample may be able to grow on the media or in the culture conditions used, leaving uncultured or non-viable cells uncounted. Slow-growing organisms may not form distinguishable colonies in the incubation time or their colonies may be ‘engulfed’ by faster growing bacteria. When preparing spread plates, effort is taken to ensure the cells are well dispersed within the inocula, and spread evenly on the plate, however clumping of cells can still occur and as a result, colonies may originate from more

than one cell. It is for this reason that plate counts are reported as colony forming units rather than number of bacterial cells.

Other alternatives for bacterial identification are the automated systems developed by Biolog Inc. (Hayward, CA, USA) which comprises 96 carbon source utilisation reactions that are automatically analysed and cross-referenced with a database of over 1,900 species of bacteria, yeast, and fungi. This system is much broader than API in the bacteria that it can identify, and is suitable for wide uses including clinical and environmental applications. As with the API system, it is still required that a little is known about the query organism in advance so that the correct plate may be selected for inoculation, but in contrast to API, each plate type covers a broader group of microorganisms, so a quick Gram determination would be sufficient for plate selection. The use of an automatic plate reader to analyse the colour changes across the plate removes the subjectivity associated with the API system.

Although such tests are designed to remove a large amount of the work and resources required from a traditional culture-dependent phenotypic approach to microbial identification, there is still considerable work involved in obtaining pure cultures from a mixed bacterial sample. Perhaps the greatest drawback of such an approach is that only a relatively small proportion of bacteria in a given sample can be easily cultured, and the remaining non-cultured organisms which may be of considerable importance can be overlooked. It is estimated that less than 0.5% of soil bacteria can be cultured in the laboratory (Torsvik et al., 1990). In this study, an average of 12 morphologically different bacterial colonies could be identified by eye from each extracted slug gut. Given that a single incubation temperature and a maximum of two different culture media were used, it is a fair assumption that a significant number of bacteria may have been overlooked as the culture conditions did not suit their requirements. An exhaustive characterisation of slug gut microflora would require a broad range of culture media and incubation conditions to ensure as large a portion as possible of the bacteria present were cultured however, the material and time constraints would certainly be limiting factors in this case. There is significant subjectivity involved in first identifying morphologically ‘different’ colonies, where tiny slow growing colonies may be very difficult to identify as different or otherwise to larger, well-defined colonies in the culture. This problem may be alleviated by taking a random sample of the colonies on the plate for further culturing and identification, however this will not only greatly increase the workload and the potential to duplicate identification work on some organisms, but there still remains the possibility of not selecting all of the different

colonies present. It is very important that the uncultured bacteria associated with the slug are not overlooked. Many of the characterised insect symbionts have never been successfully cultured outside of their host, a probable outcome of their very niche habitats. If therefore *D. reticulatum* possesses a bacterial symbiont it is likely that it may not be discovered through culturing alone and a means of studying the uncultured bacteria is virtually a necessity.

There is perhaps no ideal way in which every species present in a bacterial population can be identified without omission but modern molecular approaches offer a significant improvement upon culture-dependent methods. Constructing a clone library of, for example, the 16S rRNA gene allows for the identification of non-cultured organisms as it requires only the presence of bacterial DNA in the sample. It is important that primers are well designed and the PCR conditions thoroughly optimised to ensure that a set of products representative of the whole population is amplified. Once the clone library has been prepared, clones must be sampled and the 16S rRNA gene inserts sequenced and compared with the Genbank database to elucidate identification and, depending on the number of clones sequenced, this may be a costly process. Modern high throughput sequencing and metagenomic analysis of the derived sequence data is another approach to the characterisation of microbial assemblages (Schloss & Handelsman, 2005; Hall, 2007; Rothberg & Leamon, 2008). Essentially a mixed DNA sample is sheared into hundreds of thousands of fragments which are each separately and simultaneously amplified and sequenced to yield upwards of 100 megabases of sequence data at a time (Rothberg & Leamon, 2008). Complex metagenomic analysis is then applied to assemble contiguous sequence runs from the output fragment sequences which are representative of the bacterial community being studied (Schloss & Handelsman, 2005; Field et al., 2006; Pachter, 2007).

In conclusion, the work in this study has highlighted the major culturable bacteria in the gut of *D. reticulatum*. Future work should focus on characterisation of the noncultured bacteria associated with *D. reticulatum* which are likely to be a significant population and a more probable source of potential symbionts. In the absence of a thorough molecular study, the range of culture media and conditions should be expanded in an attempt to culture as many bacteria as possible from the slug gut. It is important to take into account geographical variations in gut microflora. Slug populations in different parts of the country are effectively isolated from one another and there are few mechanisms for the mixing of geographically separate populations. For this reason, it is reasonable to expect that the commensal gut flora of *D. reticulatum* may have evolved separately in slugs from different locations. So although

this study identified bacteria living in slugs from a single location, it is important that any novel slug control that targets potential symbionts is effective in slugs from numerous locations. This issue is addressed in chapter 4.

# Chapter 4

# Microbial Ecology in the Slug Gut

## 4.1 Introduction

The microbial ecosystem is very diverse. Estimates made by DNA:DNA re-annealing experiments (Torsvik *et al.*, 1990; Torsvik *et al.*, 1998) indicate many times greater microbial genomes in the soil than can be isolated by traditional culture techniques. This is partly due to difficulties in preparing the optimum growth medium and conditions, and because fast-growing organisms out-compete those that are slow-growing so that they are not able to grow to detectable levels, resulting in a skewed representation of the true microbial community composition. Furthermore, it is unlikely that the less than 0.5% of soil bacteria that Torsvik *et al*. (1990) estimated to be culturable will be representative of the total microbial community in the soil (Ikeda *et al.*, 2006). By employing molecular techniques, the modern microbial ecologist can not only circumvent some of the problems associated with culture-dependent methods but also conduct phylogenetic analyses of the DNA sequences obtained from an environmental sample. A microbial community can be studied using a clone library approach which can yield high resolution phylogenetic data (Chandler *et al.*, 1997; Singleton *et al.*, 2001; Li *et al.*, 2007) however this method is labour-intensive and high-cost and considering the complexity of microbial communities and the number of clones that would need to be sequenced in order to obtain a representative sample of the population, it is not ideal for studying whole communities. Instead, molecular fingerprinting techniques are increasingly being used in the study of microbial communities in terrestrial (Gleeson *et al.*, 2006; Cherif *et al.*, 2008) and aquatic settings (Fisher & Triplett, 1999; Arias *et al.*, 2006; Danovaro *et al.*, 2006) as well as the microflora associated with animals as widespread as insects (Shi *et al.*, 2010), marine invertebrates (WardRainey *et al.*, 1996; Li *et al.*, 2007), fish (Pond *et al.*, 2006), humans (Jernberg *et al.*, 2007; Jakobsson *et al.*, 2010) and domestic animals (Guan *et al.*, 2008; Torok *et al.*, 2008). Knowledge and understanding of such microbial populations has far-reaching applications in environmental monitoring and bioremediation of chemically polluted environments (Hernandez-Raquet *et al.*, 2006; Lear *et al.*, 2009), biological pest control (Douglas, 2007), and in biofilters (Steele *et al.*, 2004).

A number of molecular fingerprinting techniques are possible, all varying in their degree of technical difficulty, reproducibility, and phylogenetic resolution, and each with inherent limitations that must be taken into account when planning a study (Nocker *et al.*, 2007). All methods share a basic structure which begins with the extraction and purification of the entire DNA in a sample. This is followed by polymerase chain reaction (PCR) using

universal primers to amplify a gene that is capable of highlighting phylogenetic differences between individual species in the community. Target genes are most commonly the 16S ribosomal RNA (rRNA) gene, the internal transcribed spacer (ITS) region between the 16S and 23S rRNA genes or functional genes. The PCR products are then separated by either length (Ribosomal intergenic spacer analysis (RISA), restriction fragment length polymorphism (RFLP)), sequence (Denaturing or temperature gradient gel electrophoresis (DGGE, TGGE)) or single stranded conformation (single stranded conformation polymorphism (SSCP)) to produce a fingerprint banding pattern characteristic of the particular bacterial community of interest. The resultant bands or peaks may be referred to as operational taxonomic units (OTU) or ribotypes and together they make up a molecular fingerprint which is considered to be unique to the microbial community of interest (Fisher & Triplett, 1999). Within a microbial community fingerprint, the number of OTUs is representative of the species richness as each OTU may be assumed to be contributed by a single species or strain of bacteria as dictated by the phylogenetic resolution of the target gene. The relative fluorescence of each OTU reflects the number of copies of that particular DNA fragment in the PCR product and is analogous to but not always correlated to species abundance, as the signal intensity may be influenced by ribosomal operon copy number or PCR biases (Suzuki & Giovannoni, 1996; Crosby & Criddle, 2003). Molecular fingerprints can be compared to elucidate information about a community's species diversity or as a means of monitoring the changes in community composition in response to external pressures.

In DGGE, fragments are separated according to sequence differences as they travel along a polyacrylamide gel with an increasing gradient of the denaturants urea and formamide (Muyzer & Smalla, 1998). Alternatively a temperature gradient can be used to denature the DNA fragments in TGGE. This denaturing method is able to resolve small differences in melting behaviour brought about by as little as a single base pair difference (Fischer & Lerman, 1983). The benefit of a gel-based separation is that bands of interest may be excised from a gel and either cloned and sequenced, or screened with molecular probes to elucidate further information (Theron & Cloete, 2000). A maximum fragment size of a few hundred base pairs can be analysed by DGGE making primer design more difficult than in other methods where a whole 16S rRNA gene can be analysed. It is possible for different sequences to possess similar mobility and so migrate together in a gel and therefore, as with other fingerprinting techniques, it is possible for a band to represent more than one species. In addition to these limitations, preparation of DGGE gels is technically laborious and

difficult to achieve reproducibility meaning that results from different laboratories can often not be standardised and compared.

SSCP analysis separates fragments according to sequence differences and relies upon the differential mobility of the secondary structure of single-stranded fragments under denaturing conditions caused by point mutations and sequence polymorphisms (Scheinert *et al.*, 1996). SSCP is a simpler analysis than DGGE with more straight forward apparatus and primer design however it shares similar limitations in its interpretation.

Alternatively, fragments may be separated according to length in one of a number of methods. Ribosomal gene amplification products may be digested with enzymes and the resulting fragments separated by size. This method known as RFLP produces more than one band per PCR product, depending on the number of times a restriction enzyme cuts the DNA, resulting in a complex profile that comprises several overlapping profiles from individual species and is inherently complicated to analyse. Terminally labelled restriction fragment length polymorphism (T-RFLP) which utilises a 5'-fluorescently labelled forward primer so that only the 5'-terminal restriction fragment is detected using a fluorescence detection system simplifies sizing of the fragments and interpretation of the resultant profiles as each PCR product produces a single detectable band (Osborn *et al.*, 2000). In T-RFLP, each band can be considered to be a single species in the microbial population and so this technique can be used to measure species richness and evenness and to compare the similarity of different profiles. T-RFLP has the advantage of being highly reproducible both within and between samples and the ability to theoretically predict restriction fragments allows inference of the bacterial composition of a sample (Dunbar et al., 2001). Phylogenetic resolution may be increased by digesting the PCR products with more than one restriction enzyme either at the same time or in different runs and comparing the resultant profiles. Care however must be taken to optimise conditions for restriction digestion as incomplete digestion may cause an overestimation of diversity (Osborn et al., 2000). Similarly, the formation of pseudo-terminal restriction fragments as PCR artefacts in T-RFLP can also lead to overestimation of diversity (Egert & Friedrich, 2003). In contrast to DGGE, due to the automated detection of fluorescently labelled terminal restriction fragments (TRFs) it is not possible to select and clone bands of interest from a profile for further study by sequencing or molecular probe hybridisation. The high running costs may be inhibitory factors in the wide-spread use of T-RFLP in microbial ecology where large sample sets are often required,

whereas the high cost of fluorescence detection systems may be disadvantageous to smaller scale studies where the frequency of use may not justify the capital outlay.

RISA provides a simple alternative method of detecting length heterogeneities in ribosomal gene amplicons with low running costs and simple setup. In contrast to the previously described microbial fingerprinting methods, RISA determines microbial diversity based on length heterogeneity of the internal transcribed spacer (ITS) region between the 16S and 23S rRNA genes (Fisher & Triplett, 1999). This region encodes several transfer RNA (tRNA) genes and contains highly variable regions which can be used for phylogenetic analysis of bacterial communities (Loughney *et al.*, 1982; Garcia-Martinez *et al.*, 1999). A simple PCR reaction using a forward primer complimentary to the 3' end of the 16S rRNA gene and a reverse primer to target the 5' end of the 23S rRNA gene, and universal to bacteria, produces a series of bands of different lengths which when separated electrophoretically, produce a profile where each band may be representative of a single species (Borneman & Triplett, 1997; Fisher & Triplett, 1999). The use of polyacrylamide sequencing gels affords a high degree of resolution and allows the direct cloning of RISA amplicons from excised bands. Alternatively, the incorporation of a fluorescently labelled forward primer in the automated version of RISA (ARISA) allows for the automated detection of RISA fragments (Fisher & Triplett, 1999) and offers time savings over the running of sequencing gels, as well as seamless computer-based quantification and data analysis. RISA is highly reproducible and allows for the direct cloning of bands of interest, by excising from a gel, as with DGGE, but with resolution similar to T-RFLP (Ikeda et al., 2004). Amongst the limitations of RISA is the bias caused by multiple copies of the ribosomal operon within many bacterial genomes resulting in an overestimation of abundance of certain RISA bands (Suzuki & Giovannoni, 1996; Crosby & Criddle, 2003). Furthermore, there is potential for interoperonic heterogeneity in the ITS regions to cause a single bacterium to contribute more than one band to a RISA profile again resulting in a misrepresentation of the species richness of the sample. (A)RISA has been applied to the study of microorganisms in marine and freshwater (Fisher & Triplett, 1999; Perez-Luz *et al.*, 2004; Hewson & Fuhrman, 2006), soil (de Oliveira *et al.*, 2006), food products (Ikeda *et al.*, 2005), biofilters (Steele *et al.*, 2004) and compost (Cherif *et al.*, 2008), as well as changes in microbial communities in response to pollutants (Ranjard *et al.*, 2000).

This experiment is primarily concerned with the question of whether there is a population of bacteria that is common to all or a majority of *D. reticulatum* from geographically different

locations, based on the assumption that one might expect a beneficial symbiont to be conserved within a species. Furthermore, if biological control of slugs is to be effective and commercially viable, the target must be one that is universally found otherwise the product would be limited geographically in its application. Ribosomal intergenic spacer analysis (RISA) was utilised to generate molecular fingerprints of the microbial populations associated with the gut of *D. reticulatum* which were then used to compare the diversity of samples from different locations and to look for conserved ribotypes that may indicate a beneficial bacterial symbiont. Subtracting the molecular profiles of soil and plant-associated bacteria from the slug profile allowed RISA fragments found only in the slug to be selected, cloned and sequenced for identification. This study also demonstrates the novel application of the Agilent 2100 Bioanalyzer for detection and quantification of RISA fragments.

## 4.2 Materials and Methods

### *4.2.1 Sample preparation*

Newly hatched slugs, plant and soil samples used in RISA profiling were collected on the same day from a winter wheat field at Embleton Mill Farm, Embleton, Northumberland (NU220212), figure 4.1. Juvenile slugs were used for rearing in sterile conditions in an attempt to reduce bacteria content of the slug gut to background levels free from transient associations. Samples of the weeds, crop and soil were also collected from Embleton Mill Farm. The major weeds present were Couch (*Elytrigia repens*) and Groundsel (*Senecio vulgaris*) whilst the crop was winter wheat (*Triticum aestivum).* Ten samples of soil and each plant were taken at locations throughout the area of slug collection and pooled to create a single sample of each. Plants were stored at -20 °C and soil at 4 °C until required for DNA extraction.


**Figure 4.1.** Location of the sampling site for slugs, soil and plants at Embleton Mill Farm, Embleton, Northumberland.

For comparison of the geographical variation in slug gut microbial ecology, slugs were collected from 13 locations throughout Northumberland and the Lothians from wheat or oilseed rape crops and pasture, gardens or fallow land (Figure 4.2, Table 4.1). All slugs were collected as described in section 2.1.1 and purged by starving for three days to reduce populations of transient bacteria prior to dissection as per section 2.1.3.

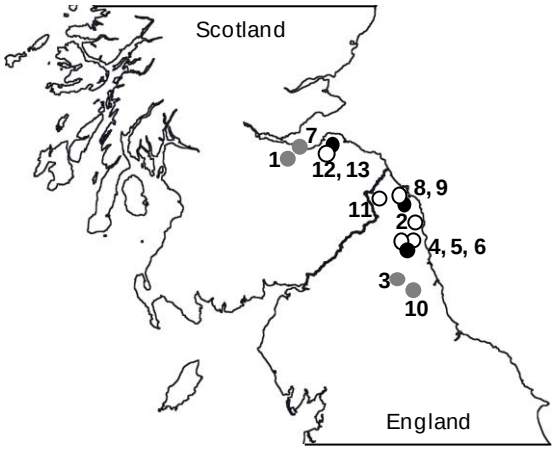



**Figure 4.2.** Map showing the origins of slugs used in this experiment. Marker colour represents the crop type at time of collection; (○) oilseed rape (OSR); (●) winter wheat; (● ) fallow/pasture/garden, numbers refer to individual sites as detailed in table 4.1.

**Table 4.1.** Origins of *D. reticulatum* used in microbial community analysis. Abbreviations will be used throughout this chapter. Map numbers correspond to the map of sites in figure 4.2.

| Map | Abrv. | Site | Crop | Grid Ref. |
|---|---|---|---|---|
| 1 | BN3 | Bush No.3, Penicuik, Midlothian | Fallow | NT241639 |
| 2 | BUR | Low Buston, Alnmouth, Northumberland | OSR | NU236080 |
| 3 | CLH | Close House, Newcastle-upon-Tyne | Garden | NZ127658 |
| 4 | EDR | Easrdon West Farm, Morpeth, N'Land | OSR | NZ195940 |
| 5 | EDV | Easrdon West Farm, Morpeth, N'Land | OSR | NZ191936 |
| 6 | EDW | Easrdon West Farm, Morpeth, N'Land | Wheat | NZ202941 |
| 7 | EFD | Eastfield, Edinburgh | Garden | NT325732 |
| 8 | EMR | Embleton Mill Farm, Alnwick, N'land | OSR | NU225213 |
| 9 | EMW | Embleton Mill Farm, Alnwick, N'land | Wheat | NU225211 |
| 10 | TAP | Tanfield, Marley Hill, Tyne & Wear | Pasture | NZ208571 |
| 11 | TLR | Turvelaws Farm, Wooler, N'land | OSR | NT992378 |
| 12 | WHR | Woodhead Farm, Gifford, East Lothian | OSR | NT515664 |
| 13 | WHW | Woodhead Farm, Gifford, East Lothian | Wheat | NT520667 |

### *4.2.2 Rearing slugs in sterile conditions*

Juvenile slugs (0.0075-0.0590 g) collected from Embleton Mill Farm (Figure 4.1) were reared under as close to sterile conditions as could be achieved with the available facilities in an attempt to purge the slugs of as much transient bacteria as possible. It was hypothesised that any potential symbiotic bacteria would remain within the slug gut whilst populations of transient bacteria would be significantly reduced through a combination of passage in the faeces and a sterile diet preventing ingestion of bacteria. Slugs were housed individually in sterile 15 ml screw top centrifuge tubes to which 2 ml of sterile distilled water and a loose plug of autoclaved cotton wool had been added. Tubes were replaced every two days. On a daily basis the lids of each tube were removed inside a class II microbiological safety cabinet to allow gaseous exchange. Slugs were fed three alternating sterile diets based on muesli, carrot and Chinese cabbage to ensure a balanced diet. The diet, based on a method by Walker (1997) is described in section 2.1.2. A total of 20 slugs were reared under these conditions for 28 days and a further ten were frozen prior to the experiment for later use to measure the starting bacterial populations. After 28 days, DNA was extracted from the slugs and the bacterial populations present were assessed using real-time quantitative PCR with universal bacterial primers as per sections 2.2.1 and 2.2.2. Molecular fingerprinting of the bacterial communities present in each slug was carried out (Section 2.2.3) and profiles used to select ribosomal intergenic spacer analysis (RISA) amplicon sizes that were common in the majority of slug samples to be cloned and sequenced to identify the bacteria they represent.

### *4.2.3 Extraction of DNA from plant and soil samples*

In order to compare the bacterial communities in slugs with those present in the soil and vegetation in the immediate locality, DNA was extracted from plant and soil samples. Microbial DNA was extracted from plant samples using the DNeasy Plant Mini kit (Qiagen). One hundred milligrams of plant material was ground under liquid nitrogen in a mortar and pestle and the extraction performed as per the kit instructions. DNA was extracted from soil using a CTAB/Chloroform method (Standard operating procedure No. LAB/MOL/022, SAC, Edinburgh, UK). Soil samples were first dried overnight at room temperature then passed through a 2 mm sieve to remove stones and plant debris. The sample was thoroughly mixed and 60 g subsample removed to the cup of a PM400 planetary ball mill (Retsch, Düsseldorf, Germany) together with 120 ml of sterile CTAB-EDTA buffer (50 mM CTAB; 100 mM Tris-HCl; 20 mM EDTA; 1.4 mM NaCl; 750 μM PVP-40; 6.4 mM dithiothreitol).

The samples were milled at 300 rpm for 5 minutes after which four 1.5 ml aliquots were taken from each sample and centrifuged at 6,000 g for 5 minutes. The supernatant was removed to a fresh tube and 0.9 ml of chloroform added before inverting the tube several times and centrifuging at 3000 g for 4 minutes. 0.9 ml of the upper aqueous layer was transferred to a new tube where 90 µl of 3 M sodium acetate and 900 µl isopropanol were added. Tubes were inverted to mix and incubated for 1 hour at room temperature. DNA was pelleted by centrifuging at 13,000 g for 4 minutes and the supernatant was discarded. After washing the pellet by centrifuging in 150 µl 70% ethanol at 13,000 g for 2 minutes, the pellet was dried in a vacuum centrifuge then resuspended in 100 µl sterile distilled water and stored overnight at 4 °C. DNA was purified by passing through micro spin columns (Bio-Rad Laboratories, Hercules, CA, USA) loaded with polyvinylpyrrolidone (PVPP) and quantified using a Nanodrop 1000 spectrophotometer.

### *4.2.4 Extraction of DNA from slugs and slug eggs*

The method of DNA extraction from slug gut tissue is described in section 2.2.1. DNA was extracted from slug eggs to establish whether there were bacteria associated with the egg that could potentially be passed down maternally to the offspring as is common in some insect symbioses. Eggs laid by slugs kept in the laboratory were collected and stored in Petri dishes lined with autoclaved filter paper and moistened with sterile distilled water. The Petri dishes were sealed with laboratory film and stored at 4 °C for a maximum of two months until required. Individual eggs were surface sterilised prior to DNA extraction (Figure 4.3) by washing single eggs with 200 µl of 70% ethanol in sterile 1.5 ml centrifuge tubes. The eggs were briefly vortexed in the alcohol and then left to stand for 5 minutes after which the ethanol was removed and the eggs washed twice with 200 µl of sterile distilled water for 2 minutes. The sterile water was removed and the eggs crushed with a sterile polypropylene pestle and 200 µl sodium phosphate buffer (120 mM; pH 8.0). A further 200 µl sodium phosphate buffer and 130 µl 10% SDS was added before vortexing and incubating in a water bath set at 65 °C for 30 minutes to lyse the cells. Proteins and cell debris was precipitated with 800 µl phenol:chlorofom:isoamyl alcohol mix (25:24:1) followed by centrifugation for 10 minutes at 13,000 g. The supernatant containing the nucleic acids was transferred to a clean tube and 600 µl of isopropanol and 40 µl of 7.5 M ammonium acetate was added to precipitate the nucleic acids. After storage at -20 °C overnight, the samples were thawed and centrifuged for 20 minutes at 13,000 g and the resultant pellet washed with ethanol and dried in a vacuum centrifuge. The DNA was resuspended in 200 µl Tris-EDTA buffer (10 mM Tris; 1 mM EDTA; pH 8.0) and quantified using a Nanodrop spectrophotometer.

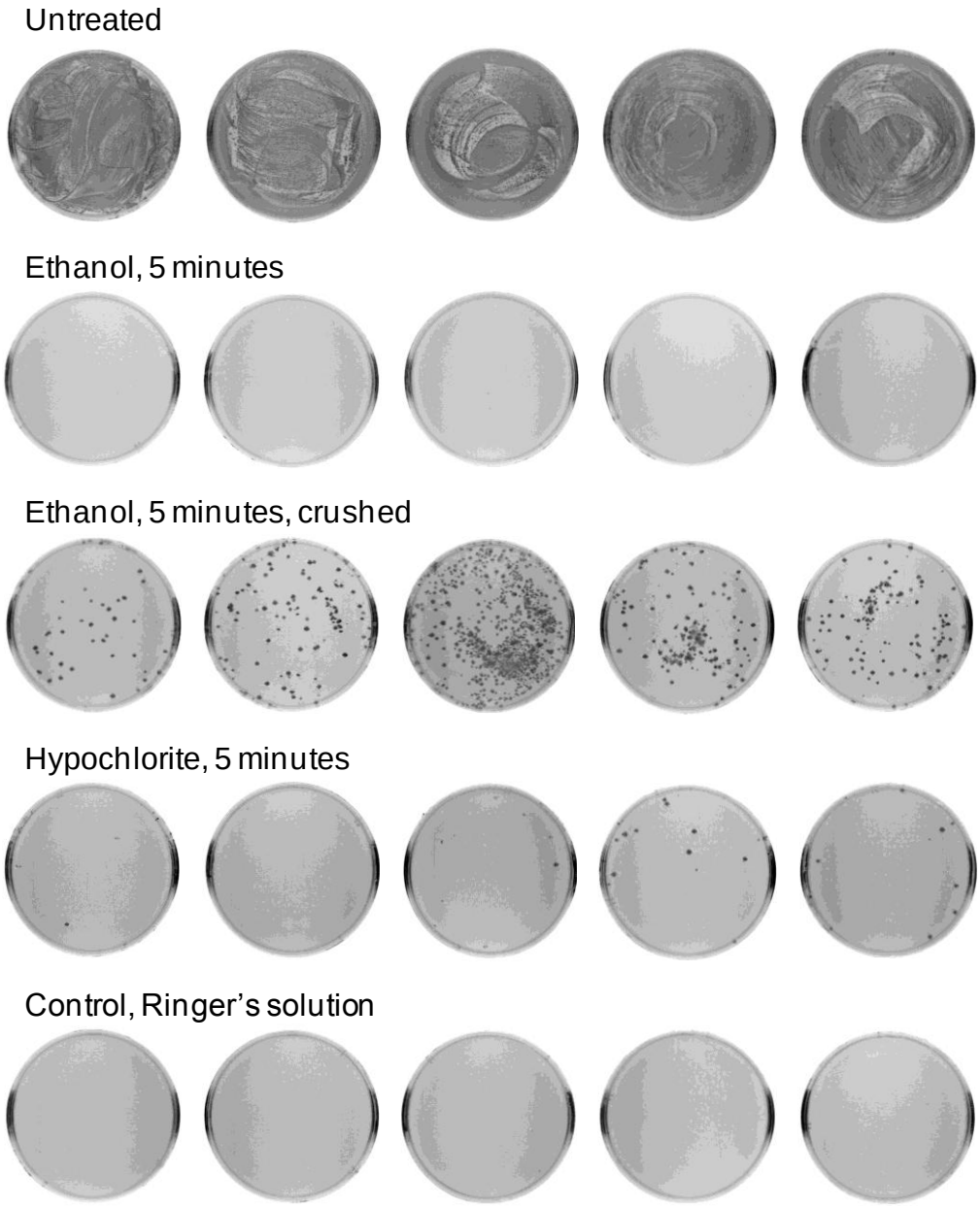



**Figure 4.3.** DNA was extracted from slug eggs for microbial community analysis by ribosomal intergenic spacer analysis (RISA) (Section 4.2.4). Slug eggs were surface sterilised prior to DNA extraction. To test surface sterilization methods, groups of ten eggs were subjected to various treatments in five replicates. After treatment eggs were washed in 200 µl sterile insect ringer's solution and rolled over the surface of a nutrient agar plate which was incubated at 20 °C for 48 hours. This figure shows the resultant bacterial growth for eggs that were untreated, sterilised in 70% ethanol for 5 minutes, sterilised in 70% ethanol for 5 minutes then crushed with a sterile PTFE pestle before plating, sterilised in 1% sodium hypochlorite solution for 5 minutes, and for the control which comprised 200 µl insect ringer's solution.

### *4.2.5 Ribosomal intergenic spacer analysis (RISA) of microbial communities*

Ribosomal interspacer analysis (RISA) is a PCR-based molecular profiling technique which studies species diversity in bacterial communities. This is achieved by analysing the PCR products generated when universal primers are used to amplify a heterogeneous spacer region between the 16S and 23S rRNA genes in the bacterial genome. The theory and analysis of the technique is described elsewhere in section 4.1, but below is a description of the method.

#### *PCR*

Sample slugs were dissected and DNA was extracted from the slug digestive tracts and diluted to a concentration of 20 ng.µl$^{-1}$, as described in sections 2.1.3 and 2.2.1. Due to their small size, juvenile slugs were not dissected and instead DNA was purified from whole slugs after surface sterilisation in 70% ethanol for 30 seconds. The RISA PCR protocol is based on Cardinale *et al.*(2004) with each reaction comprising 5 µl Go Taq Flexi buffer (Promega, Madison, WI. USA), 2 mM $MgCl_2$, 0.2 mM of each deoxynucleoside triphosphate (dNTP), 0.25 µM each primer (Table 4.2), 0.625 units of Go Taq DNA Polymerase (Promega), 40 ng of template DNA, and PCR grade water (Roche) to a total reaction volume of 25 µl. The PCR was performed on a GeneAmp 9700 PCR System (Applied Biosystems, Foster City, CA. USA) with the following thermal profile setup; 94 °C for 3 minutes followed by 30 cycles of 94 °C for 45 seconds, 55 °C for 1 minute and 72 °C for 2 minutes, with a final elongation step at 72 °C for 7 minutes.

**Table 4.2.** Sequence of primers for RISA-PCR. *Nucleotide position of each primer annealing site calculated from the E. coli O157:H7 ribosomal genes (Cardinale et al., 2004).

| Primer | Sequence | Position (nt)* |
|---|---|---|
| ITSF (Forward) | 5'-GTCGTAACAAGGTAGCCGTA-3' | 16S, 1403-1443 |
| ITSReub (Reverse) | 5'-GCCAAGGCATCCACC-3' | 23S, 23-38 |

The resulting PCR products were separated according to size by either electrophoresing on an agarose gel (3% agarose, 0.1 µl.ml$^{-1}$ GelRed (Biotium Inc., Haywood, CA.), 100 volts, 3 hours), or automatically using an Agilent 2100 Bioanalyzer and DNA 7500 kit (Agilent Technologies, Santa Clara, CA. USA) according to the manufacturer's instructions. The Bioanalyzer utilises micro-fluidics in the sizing and quantification of a range of biological entities including DNA. Briefly, the sample is loaded along with internal markers and a

fluorescent dye, onto a DNA chip primed with a gel matrix. When a current is applied in the instrument, the sample moves through microchannels on the chip base where the DNA amplicons are separated electrophoretically before their fluorescence is detected with a laser. The data is displayed as an electronic gel image and electropherogram which allows for accurate sizing and greater resolution than can be achieved with an agarose gel and with more simplicity than polyacrylamide gels. The bioanalyzer is also considerably quicker than running a gel, requiring around 30 minutes to run a chip containing twelve samples.

*Analysis*

The parameters of the Agilent 2100 Expert software were adjusted to ignore amplified fragments shorter than 250bp and peaks whose fluorescence was less than five times background signal were also excluded from molecular fingerprints as these peaks were not distinguishable from instrument noise. The area under each peak expressed as the percentage of the total area under all peaks is representative of the relative abundance of the operational taxonomic unit (OTU) within the community fingerprint. Profile data were exported for further analysis in Microsoft Excel.

| Size (bp) | BN3 220 | BN3 221 | BN3 222 | BN3 223 | BN3 224 | BN3 225 | BN3 226 | BN3 227 | BN3 228 | BN3 229 |
|---|---|---|---|---|---|---|---|---|---|---|
| 660 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 661 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 662 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 663 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 664 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 665 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1.85 |
| 666 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 667 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 668 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 669 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 670 | 0 | 0 | 0 | 9.54 | 0 | 0 | 0 | 0 | 0 | 0 |
| 671 | 0 | 27.31 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 672 | 0 | 0 | 0 | 0 | 0 | 0 | 12.53 | 0 | 0 | 0 |
| 673 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 674 | 0 | 0 | 0 | 0 | 0 | 11.86 | 0 | 0 | 0 | 2.57 |
| 675 | 0 | 0 | 14.24 | 0 | 87.83 | 0 | 0 | 0 | 0 | 0 |
| 676 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 677 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 678 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 679 | 29.18 | 0 | 0 | 0 | 0 | 0 | 0 | 5.71 | 24.04 | 0 |
| 680 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

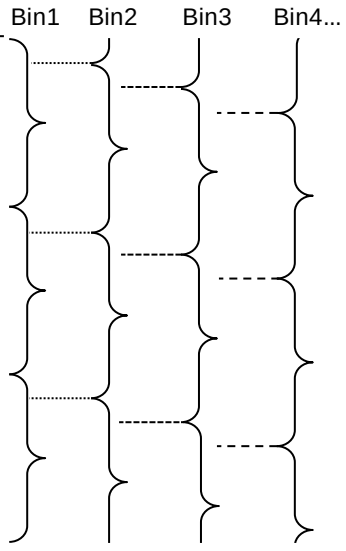
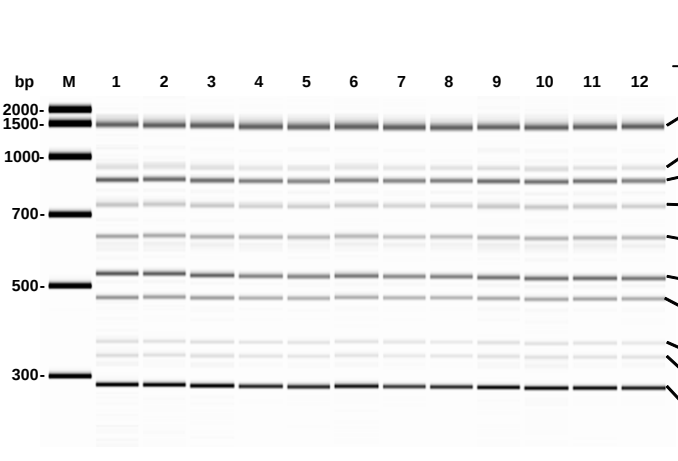



**Figure 4.4.** Example of the binning strategy for RISA fingerprints. Shown here are partial fingerprints from slugs from the location BN3 (Table 4.1). The figures represent the percentage contribution that each fragment size contributes to the total RISA DNA fluorescence and is analogous to species abundance. Assigning profiles to bins attempts to overcome the errors in the calculated similarity between fingerprints that may be introduced by instrument sizing imprecision. Bins windows are incrementally staggered so that all possible frames can be considered. Similarity indices are then calculated for each set of bins, taking the maximum value for each pairwise comparison. Diagram modified from Hewson & Fuhrman .

In order to adequately compare different communities with molecular profiles, it is necessary to be able to precisely assign a size to each OTU and match OTUs within the profiles to be compared. This task is made difficult due to the inherent imprecision in fragment analyzers which will be defined here as the ability of the instrument software to precisely determine the size of a single fragment between runs or of the same fragment loaded several times in one run. Because samples and size markers may move through a gel at different rates, variation is introduced into fragment sizing which can affect comparisons and cause errors in calculated values of similarity indices (Swerdlow & Gesteland, 1990; Kaplan & Kitts, 2003). Assigning the OTUs to bins with a window width comparable to sizing imprecision is one way to overcome this problem (Hewson & Fuhrman, 2004; Brown *et al.*, 2005; Hewson & Fuhrman, 2006) but this itself risks the introduction of artefacts for example where OTUs may be split between two bins, resulting in an underestimation of similarity. To overcome some of these issues, Hewson (2005) suggested taking into account all possible binning window frames and expressing similarity indices as the maximum of all window frames (Figure 4.4).

In this study, bin widths were set in relation to the measured sizing precision of the Agilent 2100 Bioanalyzer determined by running a RISA-PCR product containing a number of bands evenly distributed between 280 bp and 1450 bp 12 times on one bioanalyzer chip (Figure 4.5). Using the sizes assigned by the bioanalyzer to each band in this run, bins were set at 7




| Fragment size (bp) | | | |
|---|---|---|---|
| Mean | Min | Max | Range |
| 1455 | 1442 | 1484 | 42 |
| 942 | 936 | 949 | 13 |
| 876 | 870 | 882 | 12 |
| 745 | 739 | 754 | 15 |
| 637 | 634 | 641 | 7 |
| 527 | 521 | 534 | 13 |
| 473 | 470 | 476 | 6 |
| 375 | 372 | 378 | 6 |
| 345 | 342 | 348 | 6 |
| 282 | 279 | 285 | 6 |

**Figure 4.5.** Assessing sizing imprecision in the Agilent 2100 Bioanalyzer. A single sample was run in 12 wells of a bioanalyzer chip (DNA 7500 assay) and the assigned sizes compared. The gel image output from the 2100 Expert software is an electronic representation of what the sample would look like on an agarose or polyacrylamide gel. The table shows the mean, maximum and minimum size values assigned to the 12 replicates of each OTU and the range indicates the maximum discrepancy between sizings.

bp wide from 250 bp to 700 bp, 15 bp wide from 701 bp to 1109 bp and 40 bp wide from 1110 bp to 1560 bp. To achieve the best estimate of similarity between fingerprints, all possible window frames must be taken into account, and as the largest bin window is 40 bp it is possible that this window can have 40 possible starting values and thus all 40 possible window frames must be considered. An Excel macro was created that took raw fingerprint data and aligned peak sizes with the corresponding bin frames and repeated the process for all 40 possible bin windows (Appendix 4.1).

### *4.2.6 Diversity indices*

A series of diversity indices were calculated to allow comparison of the abundance and distribution of OTUs in the RISA fingerprints of slugs from the different locations studied (Legendre & Legendre, 1998). Simpson's diversity index (*1-D*) describes the probability that two specimens picked at random from a community will belong to different species or in this case OTUs. The Simpson index may have a value between 0 and 1 where 1 represents the maximum sample diversity. The Shannon index (*H*) is a measure of the amount of uncertainty or disorder in a species distribution (Krebs, 1999). This index is somewhat unusual in that its maximum value varies depending on the number of species present such that $H_{max}$ (when all species are equally abundant) is equal to the log of the number of species. It is important to be aware that the choice of logarithm base will affect the calculated value of the Shannon index and consistency should be maintained when making comparisons between Shannon index values. In this study logarithm base ten was used in all calculations. Dividing the Shannon index by the log of the number of species in the sample provides an evenness index (*E*) which is more easily interpretable and is independent of logarithm base. The evenness of a sample population can range between 0 and 1, with an evenness of 1 representing all species in equal abundance. Simpson's index and Shannon index were calculated in Microsoft Excel using the following equations where $p_i$ is the proportion that the *i*th peak contributes to the total fluorescence signal (the area under the peak as a percentage of the total area) and *S* is the sample richness (number of peaks in the fingerprint).

$$\text{Simpson's Index } (1\text{-}D) = 1 - \sum_{i=1}^{n} p_i^2$$

$$\text{Shannon Index } (H) = -\sum_{i=1}^{n} p_i \log_{10} p_i$$

$$\text{Evenness } (E) = \frac{H}{\log_{10} S}$$

As a measure of similarity between communities, the Jaccard ($S_j$) and Whittaker ($S_w$) similarity indices were calculated, also in Excel. The Jaccard index (Jaccard, 1912) compares the presence-absence of each OTU in pairs of fingerprints whereas the Whittaker index (Whittaker, 1952) additionally takes into account the relative fluorescence of each OTU. These indices were calculated using the equations below where *W* is the number of shared OTUs between communities 1 and 2, and $a_1$ and $a_2$ are the total numbers of OTUs in each respective community; and where $b_1$ and $b_2$ represent the percentage of the total amplified DNA (total area under all peaks) that the *i*th peaks in communities 1 and 2 contribute.

$$\text{Jaccard Index, } S_j = \frac{W}{(a_1 + a_2 - W)}$$

$$\text{Whittaker Index, } S_w = 1 - \sum_{i=1}^{n} \frac{|b_1 - b_2|}{2}$$

Maximum values for the Jaccard and Whittaker indices were subjected to hierarchical cluster analysis in Genstat using the unweighted pair group mean average (UPGMA) method, and the data output in dendrograms.

### *4.2.7 Cloning and sequencing of RISA bands*

A total of ten bands from the RISA profiles of slugs and eggs were chosen for sequencing according to their high incidence in the bacterial community fingerprints from the gut of slug profiles (both sterile reared and field collected) and absence from the bacterial communities of the surrounding soil and plant matter. RISA-PCR products containing strong examples of

the desired bands were chosen from the previously described RISA data and run on an agarose gel (3% agarose; 0.1 µl.ml$^{-1}$ GelRed; 125 V; 3.5 hours) with a 100 bp size marker. The bands of interest were located and excised using GelX pipette tips (Cleaver Scientific Ltd, Rugby, UK) before purification of the DNA using the MinElute Gel Extraction Kit (Qiagen) as per the manufacturer's instructions.

Purified RISA band DNA was cloned using the pGEM-T Easy Vector system (Promega) with minor modifications. Ligation reactions were set up as per the manufacturer's instructions and incubated overnight at 4 °C. For transformation, ligation tubes were spun down and 2 µl of each ligation reaction was transferred to a clean tube and put on ice. Meanwhile JM109 high efficiency competent cells (Promega) were thawed in an ice bath and 50 µl of cells were transferred to each transformation tube, mixed by flicking and incubated on ice for 20 minutes. Cells were heat shocked for 45 seconds in at water bath set at 42 °C and immediately returned to ice for 5 minutes. Cells were gently suspended in 450 µl room temperature SOC medium (Invitrogen) and incubated with shaking for 1 hour 20 minutes at 37 °C. The transformations were next centrifuged at 5000 rpm for 5 minutes and 400 µl of the supernatant discarded. The cells were resuspended in the remaining 100 µl of medium and all 100 µl spread on an LB agar plate containing ampicillin (100 µg/ml) and previously spread with IPTG (100 µl; 100 mM) and X-Gal (22 µl; 50 mg/ml), and incubated at 37 °C for 16 hours.

Ten single, well isolated transformed colonies per cloned RISA band were selected and enriched on a fresh LB agar plate overnight. A colony PCR was conducted to check for the presence of the insert in each clone (Corporation, 2005). A single well isolated colony was picked, half of which was used to inoculate 5 ml LB broth plus ampicillin (100 µg/ml) for overnight enrichment, while the other half was suspended in 50 µl of sterile distilled water. The cell suspensions were boiled on a heating block for 10 minutes, centrifuged at 16,000 g for 5 minutes, and 2.5 µl of the supernatant was used as template DNA for the colony PCR. The PCR was run using the two plasmid specific primers T7 (5'-TAA-TAC-GAC-TCA-CTA-TAG-GG-3') and SP6 (5'-TAT-TTA-GGT-GAC-ACT-ATA-G-3') (Corporation, 2005). The 25 µl reactions comprised 12.5 µl GoTaq® Colourless Mastermix (Promega), 25 pmol of each primer, 2.5 µl template DNA and 5 µl sterile distilled water. Reaction conditions comprised an initial denaturation step of 2 minutes at 94 °C followed by 35 cycles of 94 °C for 30 seconds, 55 °C for 1 minute and 72 °C for 2.5 minutes, and a final step at 72

°C for 10 minutes. PCR products were run on an agarose get (1.5%; 0.1 μl.ml$^{-1}$ GelRed; 100 V; 1 hour) to check the presence and size of the cloned inserts.

Plasmids were purified using the Hurricane Plasmid DNA MiniPrep kit (Gerard Biotech, Oxford, OH, USA). Two millilitres of liquid culture was transferred to a microcentrifuge tube and spun at 12,000 rpm for 1 minute. The supernatant was discarded and plasmid DNA extracted from the remaining cells as instructed in the kit manual. Plasmid DNA was quantified using a Nanodrop 1000 spectrophotometer and sent for sequencing in both directions by DBS Genomics, Durham University, UK. Forward and reverse sequences were trimmed and assembled into a contig covering the whole 16S-23S ITS region (Approximately 470 - 1400 bp) using the sequence assembly program CAP3 (http://pbil.univ-lyon1.fr/cap3.php) (Huang & Madan, 1999). For identification, the assembled ITS region sequences were compared to sequences in the GenBank database using the BLASTN search algorithm (http://www.ncbi.nlm.nih.gov/blast/) (Altschul *et al.*, 1990). Query sequences were aligned with their closest matches and reference sequences from type-cultures using CLUSTALX, and the resultant alignments used to generate phylogenetic trees by the maximum likelihood method in PHYLIP (http://evolution.genetics.washington.edu/phylip.html) (Felsenstein, 1989).

## 4.3 Results

### *4.3.1 Data binning strategy*

To test the intra-run sizing precision of the Agilent 2100 Bioanalyzer a single sample was run 12 times in a single chip (Figure 4.5). The data from these runs were used to set the window widths for binning of the fingerprint data for similarity comparisons (Section 4.2.5). Because a single sample was analysed 12 times, one would expect that the profiles should all be identical, with all pairwise comparisons yielding Jaccard index values of 1. Without binning however, the mean Jaccard index for all pairwise associations was 0.20, with no two repeat profiles being identical (Figure 4.6A). When bin widths used in previous studies (3 bp wide from 400 to 700 bp, 5 bp wide from 701 to 1000 bp and 10 bp wide from 1001 to 1200 bp (Hewson & Fuhrman, 2004; Brown *et al.*, 2005; Hewson & Fuhrman, 2006) were applied to the profiles, taking the maximum Jaccard index values of the ten possible bin frames, the mean Jaccard index rose to 0.47 but only three pairs of profiles were identical (Figure 4.6B, $S_j = 1$; Pairs 5 and 9, 7 and 8, 11 and 12.). This highlighted the necessity to further refine the bin widths to suit the fragment analyzer used in this study. By widening the bins to 7 bp wide from 250 bp to 700 bp, 15 bp wide from 701 bp to 1109 bp and 40 bp wide from 1110 bp to 1560 bp in line with the range of assigned fragment sizes (Figure 4.5), this time taking maximum Jaccard values from 40 possible bin frames, a mean Jaccard index of 0.80 was achieved, where a number of associations shared a Jaccard value equal to 1 (Figure 4.6C).

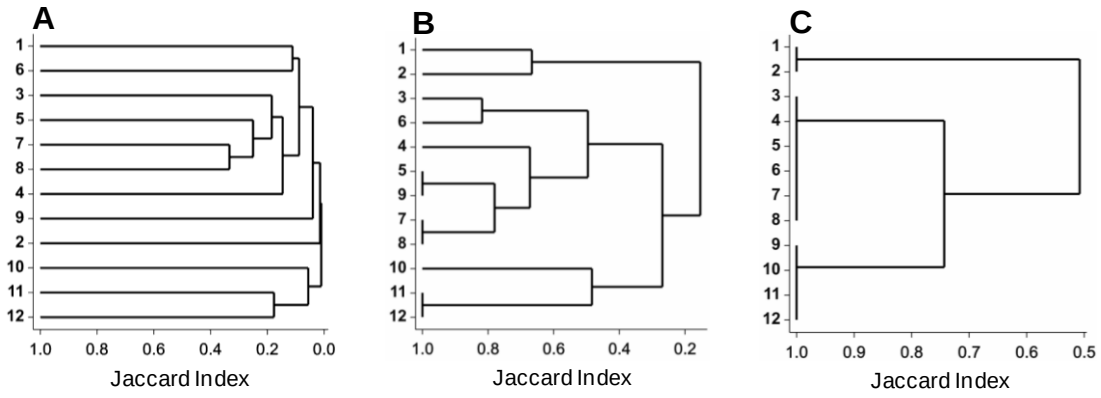



**Figure 4.6.** Jaccard index UPGMA clustered dendrograms for twelve repeats of a single RISA fingerprint sample, to assess sizing imprecision. A) unbinned data, B) maximum Jaccard index values from 10 bin frames with widths from the literature (Hewson & Fuhrman, 2004; Hewson & Fuhrman, 2006) and C) maximum Jaccard values for 40 window frames of adjusted widths. See main text for bin widths.

These results highlight the difference in precision between DNA sequencing machines as are more commonly used, and the Agilent 2100 Bioanalyzer for fragment electrophoresis, detection and sizing, and the importance of assessing the sizing precision and capability of individual machines to be used in a molecular community analysis protocol. Although the Agilent 2100 Bioanalyzer cannot size fragments with the precision commonly obtained with DNA sequencers, it remains an affordable option when access to sequencing machines is not possible as it still offers an improvement in speed, simplicity and resolution over traditional polyacrylamide or agarose gel based techniques.

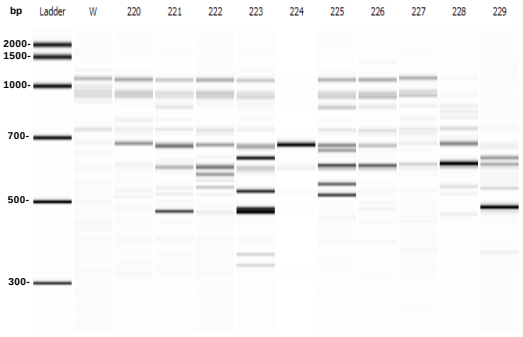
The same effects of binning were reflected in analysis of fingerprint data from a total of 129 slugs from 13 locations (Figure 4.7). The mean richness of the microbial community profiles of individual slugs was between 9 and 14 (Table 4.3) but total richness per location was much greater than this. When comparing the ten individual fingerprints from each location without binning, total richness is around eight times greater than the individual richness which would suggest that approximately 80% of fragment lengths obtained from individual slugs are unique but in fact this is not the case. When the fragment sizes were assigned to bins as set out in the literature (Brown *et al.*, 2005; Hewson & Fuhrman, 2006; Hewson *et al.*, 2007), the total species richness per location was between 50 and 80, approximately five to six times individual richness. Similarly when fingerprints were binned to the revised bin

**Table 4.3.** Mean richness of individual samples compared to the total richness for each location based on 10 samples when not binned, binned to original bins as described in Hewson *et al.* (2006), and when binned with revised bin widths based on the Agilent 2100 Bioanalyser sizing precision. Refer to table 4.1 for location descriptions.

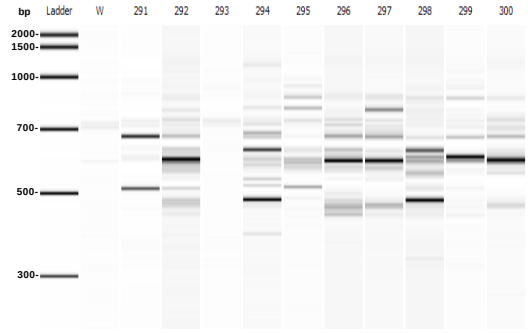
| Location | Mean Sample Richness | Total Species Richness | | |
|---|---|---|---|---|
| | | Not Binned | Original bins | Revised bins |
| BN3 | 10 | 82 | 57 | 38 |
| BUR | 10 | 80 | 56 | 38 |
| CLH | 14 | 113 | 80 | 54 |
| EDR | 10 | 83 | 59 | 40 |
| EDV | 14 | 108 | 70 | 43 |
| EDW | 9 | 74 | 50 | 34 |
| EFD | 13 | 106 | 73 | 47 |
| EMR | 13 | 108 | 67 | 42 |
| EMW | 10 | 83 | 57 | 37 |
| TAP | 13 | 90 | 57 | 38 |
| TLR | 11 | 86 | 61 | 41 |
| WHR | 13 | 106 | 74 | 47 |
| WHW | 12 | 106 | 75 | 48 |

**Figure 4.7.** Ribosomal intergenic spacer analysis (RISA) profiles of the microbial communities in the gut of *D. reticulatum* collected from 13 locations in Northumberland, Tyne and Wear and the Lothians, as shown in figure 4.2. RISA-PCR products were analysed with the Agilent 2100 Bioanalyzer and gel-like images produced.

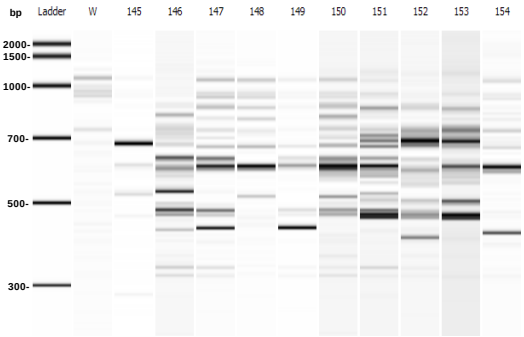
BN3 - Bush No.3



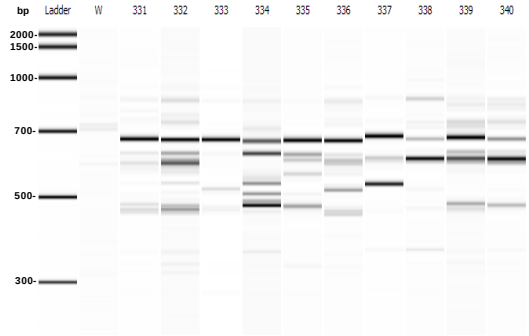

BUR – Low Buston OSR



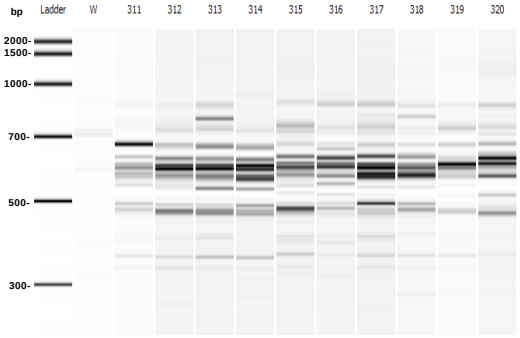

CLH – Close House



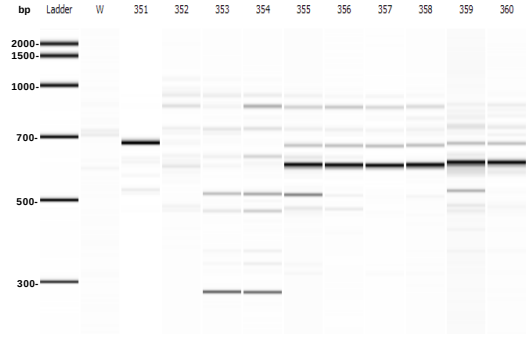

EDR – Earsdon, OSR




EDV- Earsdon OSR




EDW- Earsdon Wheat




EFD - Eastfield

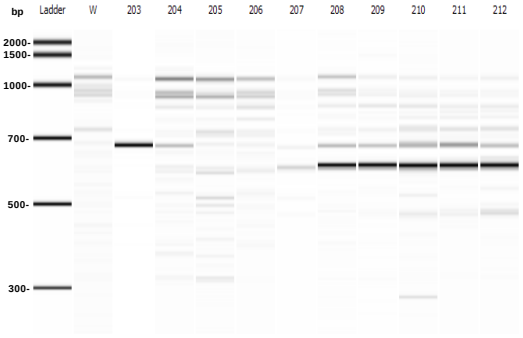
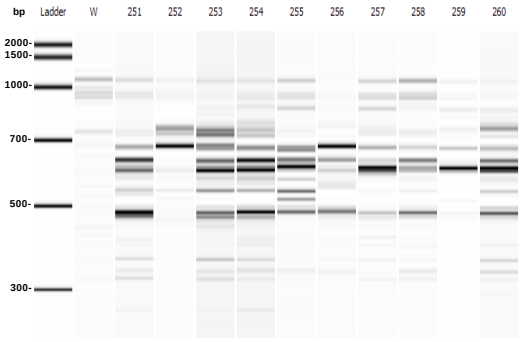
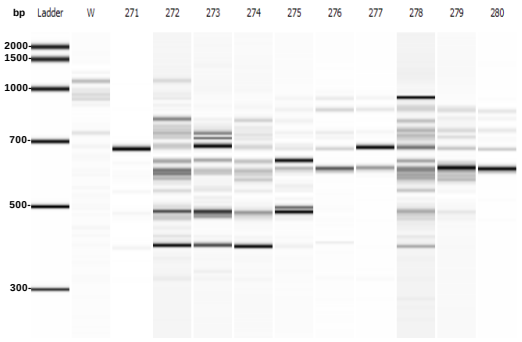


**Figure 4.7** Continued

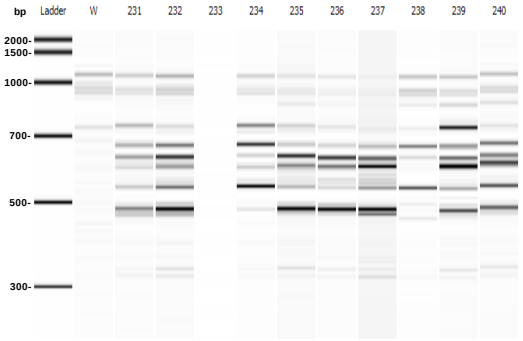
EMR - Embleton Mill, OSR



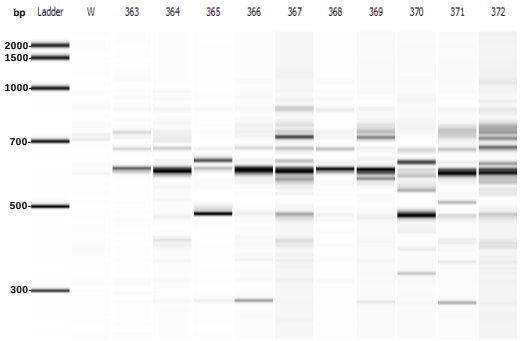

EMW- Embleton Mill, Wheat



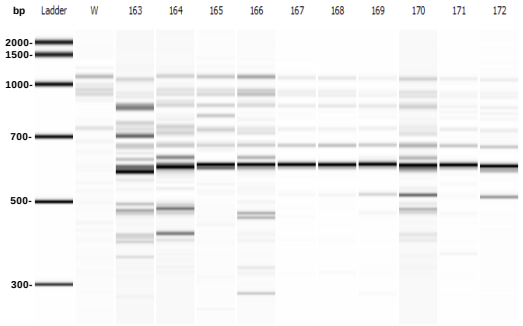

TAP - Tanfield



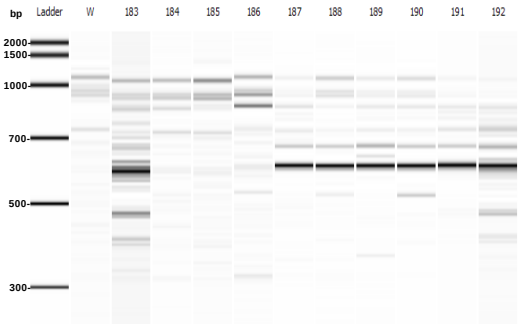

TLR – Turvelaws, OSR




WHR – Woodhead, OSR




WHW – Woodhead, Wheat



widths, the total richness was further reduced to between 34 and 54. This reduction in total richness indicates that as bin width is increased, the chance of two fragments being matched also increases such that fragments that were originally separated by a small number of base pairs will now be grouped as one fragment size for analysis purposes. When comparing microbial fingerprints from environmental samples, it is difficult to say whether small size differences between fragments is due to sizing imprecision or natural fragment length heterogeneity and indeed it is not possible or at least very difficult to be certain of the actual richness of a microbial community without large-scale sequencing of fragments.

### *4.3.2 Analysis of microbial community fingerprints*

Figure 4.7 shows the RISA fingerprints of the microbial communities in the digestive tracts of slugs from all the locations in the study. Pair-wise comparisons of intra- and inter-site similarity by the Jaccard index revealed that slugs within a single location shared greater gut microbial community similarity than slugs from different locations (Figure 4.8). This gives evidence of a transient bacterial population whereby the gut microbes presumably resemble the environmental bacteria which may differ from one location to another. This suggests little evidence of a highly conserved bacterial association with the slug, however the Jaccard index alone is perhaps not an ideal method of highlighting symbioses. It is not unreasonable to imagine that a beneficial association with the slug could comprise a single species or strain of bacteria which when investigated on a community level using similarity indices, may be lost in the background. For example, it only requires a single shared OTU to give a Jaccard index of greater than zero and as all Jaccard index values for intra- and inter-site

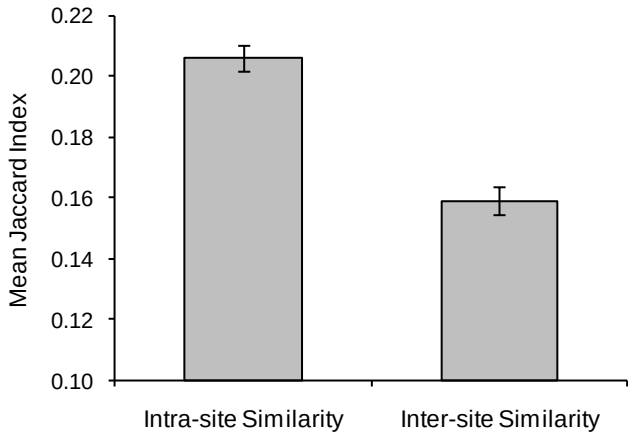



**Figure 4.8.** Mean intra- and inter-site similarity by Jaccard index of gut microbial communities in slugs from 13 locations. Error bars show SED, $p<0.001$, ANOVA.

comparisons were greater than zero this indicates that at least one OTU was identical in each pair-wise comparison. Therefore even low Jaccard indices could indicate a common bacterial species and possible symbiont and so similarity indices alone are not suitable indicators of symbiont presence.

To overcome the limitations of similarity indices, it was necessary to look more closely at individual fragment sizes. Figure 4.9 shows the frequency of occurrence of RISA fragment sizes in a total of 129 molecular fingerprints of bacterial communities in the gut of slugs from 13 locations, aligned to the revised bin widths. Only three fragment sizes, 675 bp, 740 bp and 880 bp, were present in more than 50% of communities, 64%, 59% and 60% respectively. These fragments theoretically could have the greatest chance of being potential symbionts and would be a good starting point for closer investigation, however these are low percentage abundances and one would expect an obligate symbiont to be present in considerably greater than 60% of individuals.

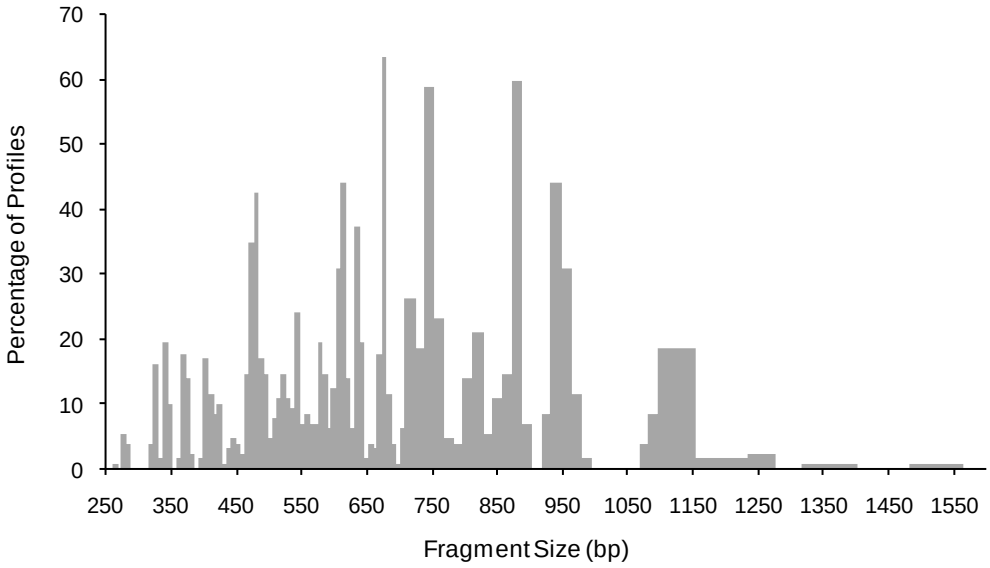



**Figure 4.9**. Percentage presence of fragment lengths in RISA profiles of bacterial communities in the gut of 129 slugs from 13 locations. Profiles were aligned to revised bins and he maximum of 40 window frames displayed.

Cluster analysis of Whittaker and Jaccard indices does not suggest particularly high similarity of microflora in slugs from different locations (Mean $S_w$ = 0.59, $S_j$ = 0.62). The low similarity is likely to be due to differences in the soil types and the composition of the

soil and environmental microflora at a particular location. Reflecting the differences in intra and inter-site similarity (Figure 4.8), there is some clustering of samples from the same farm, with Earsdon samples (EDV, EDR, EDW) clustering together by the Jaccard index, and those from Woodhead Farm (WHR, WHW) clustering with both Whittaker and Jaccard indices (Figure 4.10). Appendix 4.2 shows dendrograms for individual locations with the ten microbial fingerprints from each location clustered by both Whittaker and Jaccard indices of similarity.

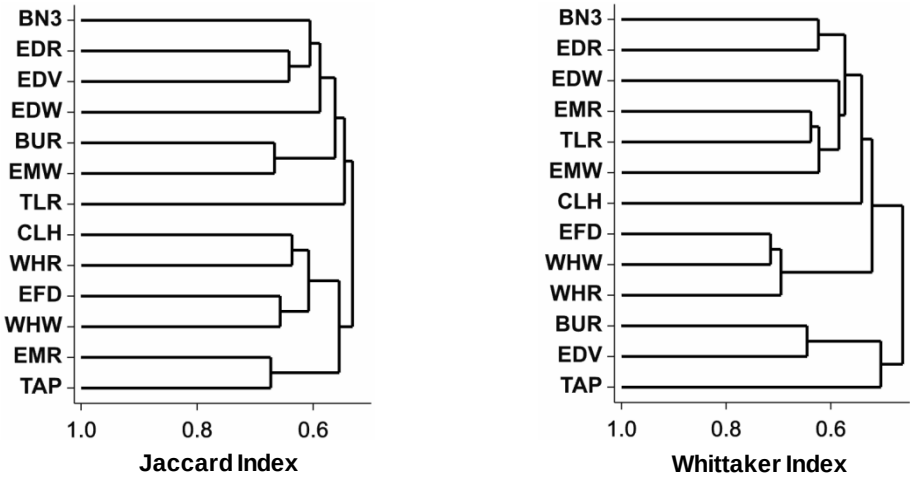



**Figure 4.10.** Dendrograms showing the similarity of slug gut microbial community RISA fingerprints calculated using the Jaccard and Whittaker indices of similarity. Dendrograms were produced by hierarchical cluster analysis, by the UPGMA method in Genstat. Values used were the maximum values from 40 bin window frames.

The richness of slug gut bacterial communities was variable, with location mean values between 8.8 and 14 (Figure 4.11A) for individuals. Differences between locations were significant (ANOVA, $p<0.001$). Evenness values were between 0.63 and 0.82 (Figure 4.11D) suggesting a slight skew in the abundance of some components of the bacterial community. Differences in mean evenness between locations showed slight significance at the 5% level (ANOVA, $p=0.044$). The evenness values are echoed by the Shannon index which yields similar mean values of between 0.61 and 0.90 (Figure 4.11C) with significance at the 1% level ($p=0.003$). Finally, the Simpson index also displays similar variability (0.61 - 0.84) indicating a fairly diverse microbial population which most likely reflects the bacterial community in the soil (Figure 4.11 B). Despite differences in these indices between locations, no consistent patterns with regards to crop type or locality were evident.

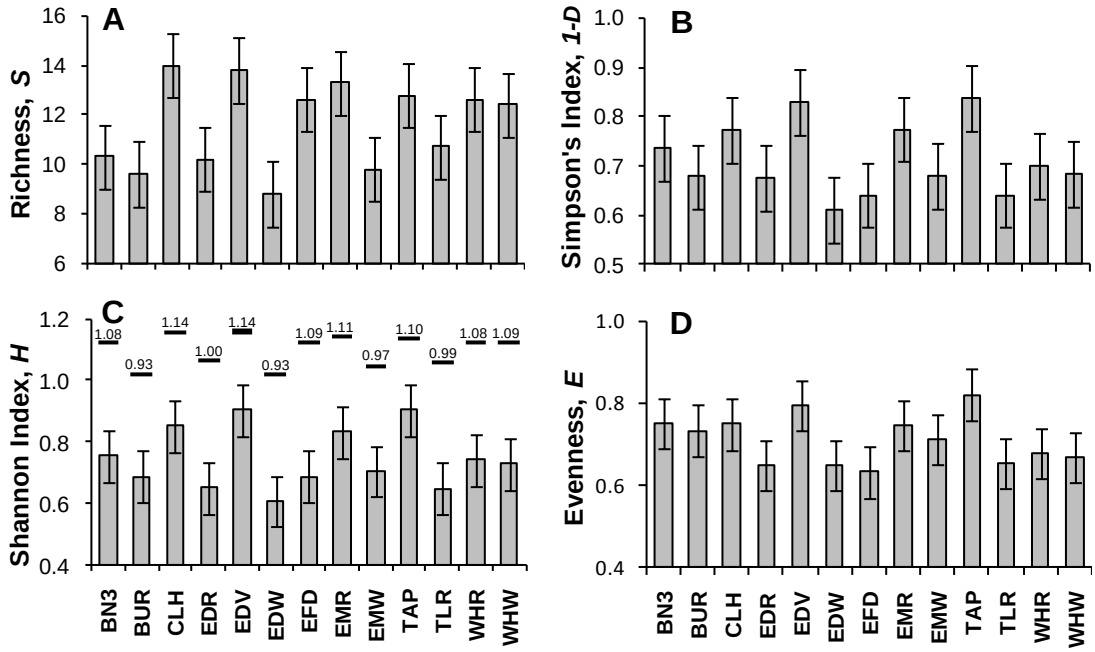



**Figure 4.11.** Mean richness (A), Simpson's index (B), Shannon index (C) and Evenness (D) of microbial communities from the gut of *D. reticulatum* studied by RISA. Because the maximum Shannon index varies depending on the number of species in a sample, maximum values ($H_{max}$) for each dataset are indicated with bars on graph C. The values show the mean of 10 samples per each of 13 locations (9 from TAP). Error bars show SED from ANOVA in Genstat (p values <0.001, 0.024, 0.003, 0.004 respectively).

### *4.3.3 Comparison of microbial communities in slugs and environmental samples*

Rearing slugs in sterile conditions did result in a decrease in the mean quantity of bacterial 16S rDNA detectable in the slugs by real-time quantitative PCR (Figure 4.12) however analysis of variance showed this difference was not significant (p=0.123). These values were however similar to those observed in slugs in the bioassays described in chapter 5 so it could show that bacterial numbers were at a level approaching background. The mean richness was between 7 and 13 for field-collected slugs, sterile-reared slugs, slug eggs, soil and plant matter (Figure 4.13). The field-collected and sterile-reared slugs did not exhibit significantly different richness values (ANOVA, p<0.001) which again supports the theory that rearing of slugs under sterile conditions does not further reduce the bacterial content of the gut compared to purging by starvation for three days post-collection. Mean evenness for the same samples was between 0.73 and 0.78 which suggests a slightly skewed distribution of OTUs in the fingerprints. Differences between samples were not significant (ANOVA, p=0.918).

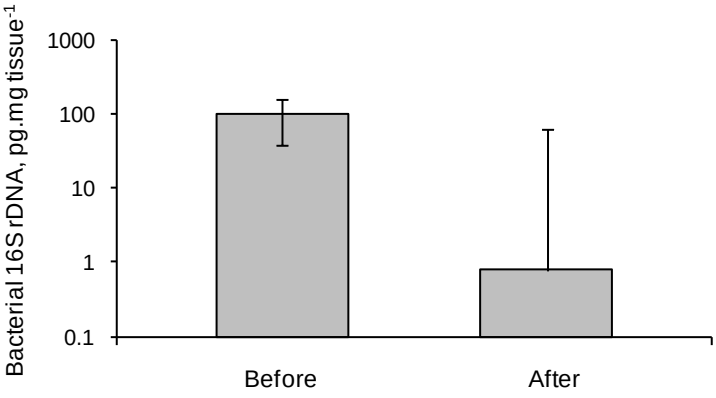



**Figure 4.12.** Bacterial 16S rDNA present in slugs reared in sterile conditions for 28 days, estimated by real-time qPCR. Error bars show s.e.d (ANOVA, p=0.123). Data plotted on a log scale.

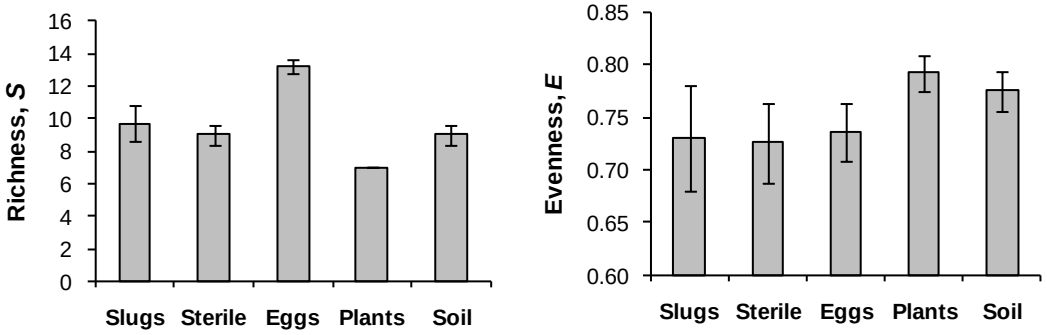



**Figure 4.13.** Mean richness and evenness of microbial community RISA fingerprints obtained from field-collected slugs, slugs reared in sterile conditions for 28 days, surface-sterilized slug eggs, and plants and soil from the place of slug collection. Error bars show SEM

There was a marked difference in the appearance of RISA fingerprints from slugs that were reared under sterile conditions compared to those only starved for 3 days after collection (Figure 4.14A & B). Although both sets of profiles share similar bands in places such as at around 615 bp and 930 bp, the patterns are markedly different. It could be argued that maintaining slugs in the laboratory for long periods of time may be disruptive to the natural balance of microflora in the slug gut and so the profiles from the sterile reared slugs may be misleading or unrepresentative of the true bacterial flora present. However it may be reasonable to assume that important associations will still remain and even if the profile becomes unsuitable for meaningful studies of the slug gut microflora in its natural configuration, it may still be useful for identification of more persistent associations between slug and bacteria.

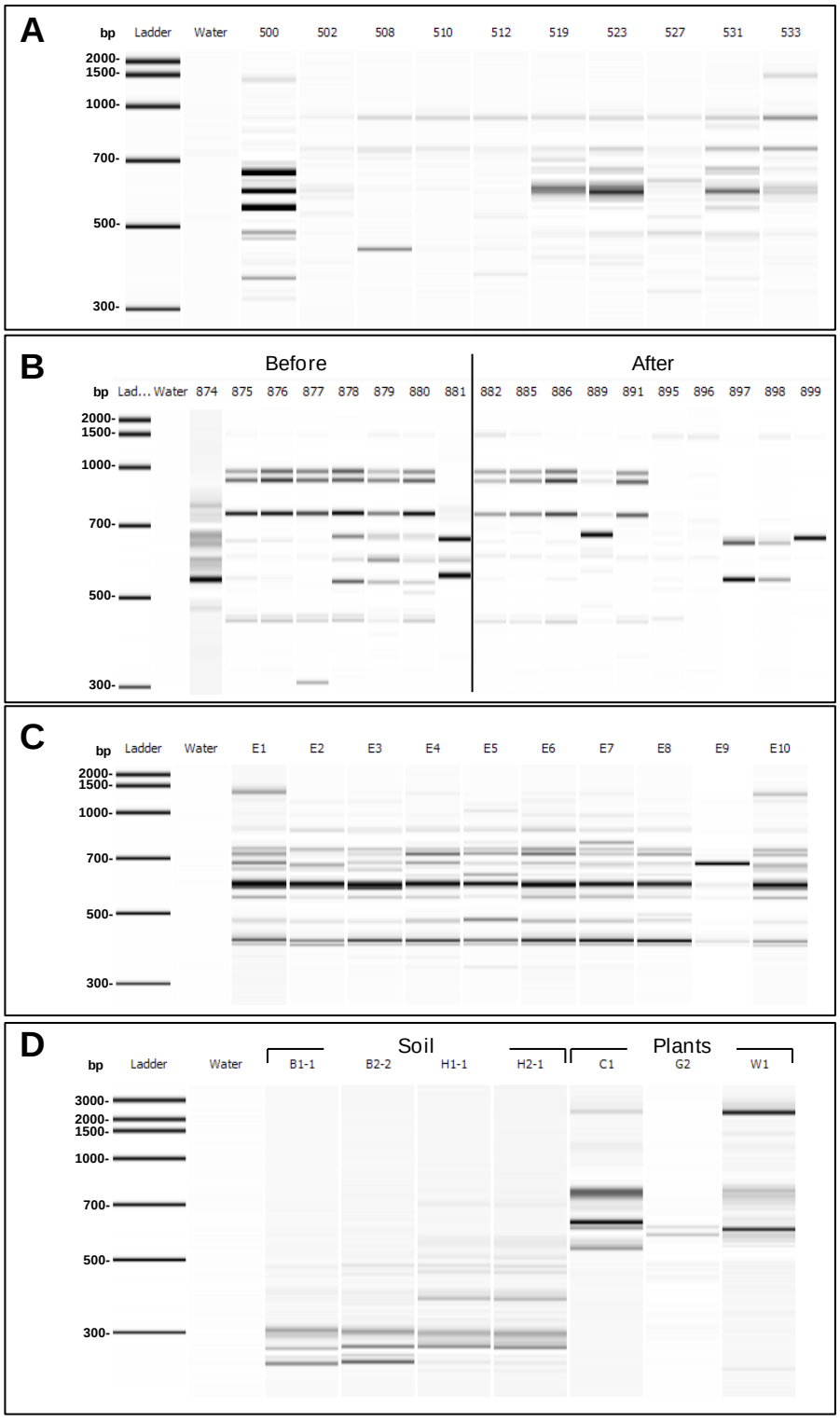
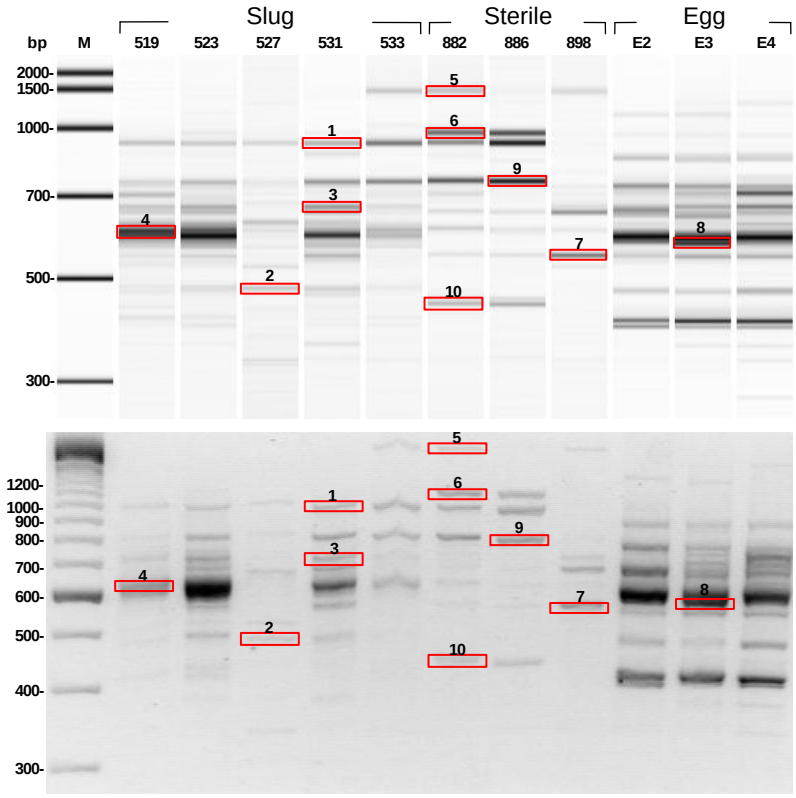



**Figure 4.14.** RISA-PCR molecular fingerprints of microbial communities present in **A**) field-collected slugs purged for three days, **B**) juvenile slugs reared in sterile conditions for 28 days after collection, **C**) surface sterilised slug eggs and **D**) soil and plant samples from the site of slug collection. Soil samples were from the same field as the slugs (B1-1, B2-2) and from an adjacent field (H1-1, H2-1) also under wheat crop. Plant samples represent the crop, wheat (W1) and major weeds, Couch (C1) and Groundsel (G2).

The high richness value of 14 shown in figure 4.13 is evident in the RISA profiles from surface sterilized slug eggs (Figure 4.14C). There is also a high degree of homogeneity between these samples with only one sample out of ten (E9) differing significantly from the others. If these profiles are indeed from slug symbionts that are transmitted to offspring either in or on the eggs, then the consistency in banding pattern reflects the fact that they have not been subjected to external influences on the molecular communities which might serve to generate more heterogeneous profiles. The profiles for soil and plants (Figure 4.14D) are very different from those observed for slugs and slug eggs. It is interesting to note that soil samples taken within a single field share very similar profiles while others taken from an adjacent field are markedly different.

Ten bands which were present in more than 50% of slug and slug egg RISA fingerprints but absent from soil and plants were selected and cloned (Figure 4.15). Ten clones were randomly picked from each transformation for sequencing. Some transformations were unsuccessful, resulting in a total of 78 16S-23S ribosomal internal transcribed spacer (ITS) sequences for bands one to nine but none for band ten. Sequences were cross-referenced with the GenBank database and best matching sequences were used to compile a phylogenetic tree (Figure 4.16). Most of the clones exhibited similarity to the genera of soil and environmental bacteria, *Pseudomonas*, *Erwinia*, *Serratia* and *Janthinobacterium* that were isolated from slugs previously in chapter 3. Consistent with previous findings, *Gammaproteobacteria*, in particular *Pseudomonas*, were dominant. Band five clones and some from band six matched most closely with the 5.8S rDNA sequence of *D. reticulatum* and other gastropod molluscs indicating that the primer pair used is not entirely unique to bacteria and highlighting the importance of primer selection according to the sample origin. For a large number of bands, high quality matches were not found in the GenBank nucleotide database and all clones from band two with the exception of clone 2-5 failed to match adequately to any sequences and so could not be identified. Based on the clustering of band 2 clones it is possible to conclude that they are likely to be members of the class *Mollicutes*.




| Band | Size (bp) | Band Abundance | | |
|---|---|---|---|---|
| | | Slugs | Sterile | Eggs |
| 1 | 935 | 100% | - | - |
| 2 | 482 | 60% | - | 80% |
| 3 | 673 | 50% | - | - |
| 4 | 614 | 50% | 90% | - |
| 5 | 1487 | - | 50% | - |
| 6 | 982 | - | - | 80% |
| 7 | 558 | - | 50% | 100% |
| 8 | 602 | - | - | 90% |
| 9 | 766 | 70% | 60% | 80% |
| 10 | 451 | 50% | 50% | - |

**Figure 4.15.** A gel image produced by the Agilent 2100 Bioanalyzer (Top) is compared with an image of an agarose gel (middle) both showing the positions of the bands selected for cloning. Samples represent microbial communities from field collected slugs, sterile-reared slugs and surface sterilised slug eggs. The table shows the approximate band sizes and abundance of each band within samples.

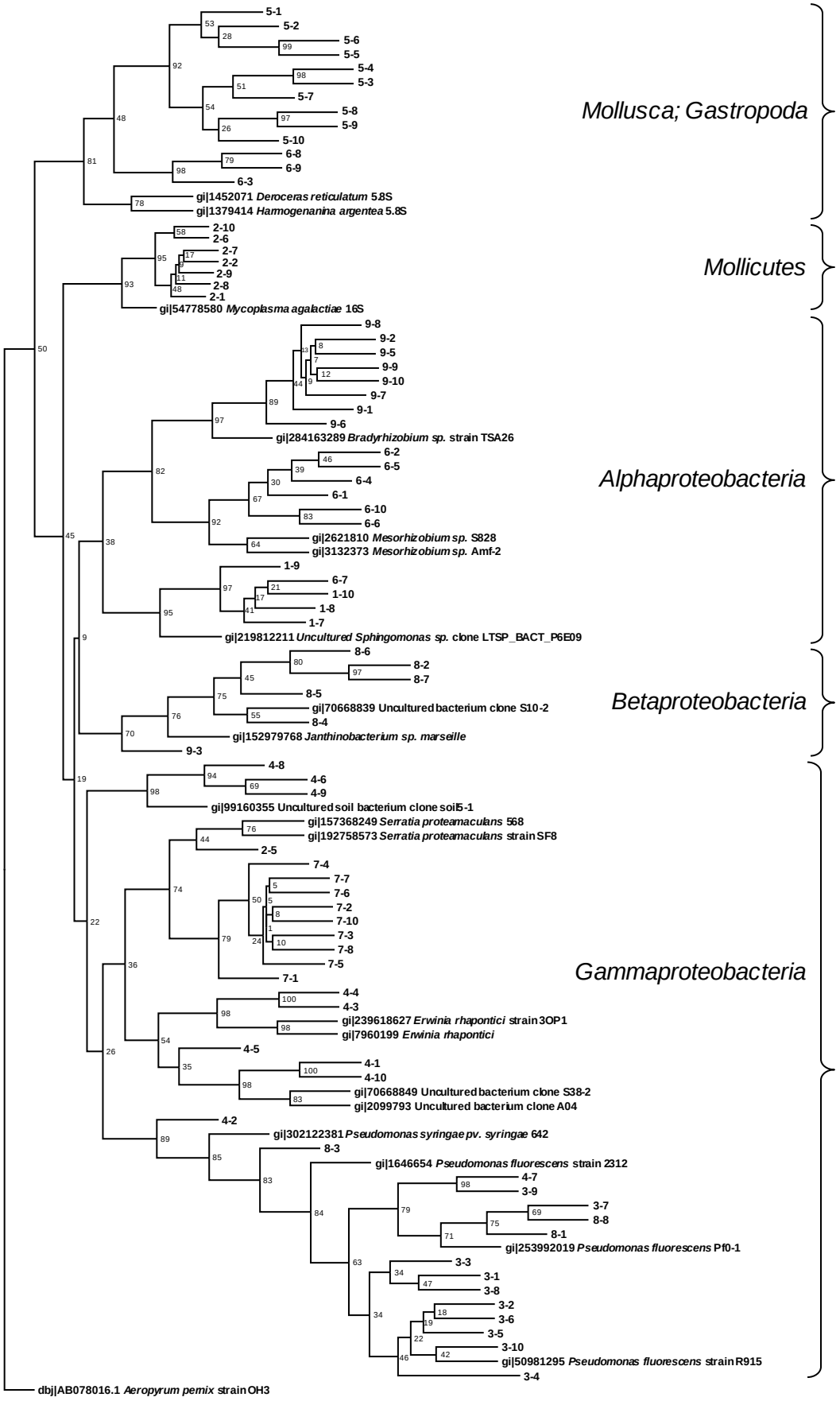



**Figure 4.16.** Maximum likelihood tree of RISA sequences obtained from slugs and slug eggs. Numbers at the nodes represent occurrence in 100 bootstrap replicate trees. The archaeal *Aeropyrum pemix* sequence is included as an outgroup root.

## 4.4 Discussion

Microbial populations in the digestive tracts of slugs from different locations exhibited low similarity, returning Jaccard indices below 0.20 (Figure 4.8) which suggests that the majority of slug gut bacteria undergo only transient associations with D. reticulatum. Differences in microbial communities in slugs from each location are likely to reflect differences in environmental microbial populations in the soil or on plants at the locations studied. Microbial populations in the soil are complex and dynamic and influenced by a number of external factors including plants, nutrient availability, chemical disturbances and physical properties (Torsvik et al., 1998; Wall & Virginia, 1999; Girvan et al., 2003). By comparing the abundance of different RISA fragment sizes, it was shown that very few OTUs were detected in more than 50% of the slug digestive tracts studied (Figure 4.9) suggesting there are no highly conserved bacteria that may be indicative of a beneficial symbiont.

Slug gut bacterial communities are diverse with up to 14 different OTUs detectable by RISA. Sequence analysis of cloned RISA bands from the slug gut identified primarily soil bacteria of the phylum Proteobacteria and supports findings in chapter 3. It is not surprising that such bacteria are found in the slug gut given they are soil-dwelling invertebrates and the likelihood of co-ingestion of soil with food material. When slugs are starved or kept in sterile conditions and fed sterile diets, bacteria remain within the slug gut, albeit at low levels, and are not removed altogether. This persistence of a small background population suggests possible commensalism which warrants further investigation to establish whether these have any benefits to slug fitness and survival.

Surface-sterilised slug eggs were used in conjunction with RISA to assess the possibility of obligate symbiotic bacteria being vertically transmitted to juvenile slugs as is common with many insect bacterial symbioses. RISA profiles generated from eggs had high richness which seems unlikely to be contributed by a maternally transmitted symbiont which one would expect to contribute much fewer bands. The lumbricid earthworms (Oligochaeta: Lumbricidae) possess symbiotic bacteria that are vertically transmitted (Schramm et al., 2003). Egg capsules released in reproduction contain albumin and symbionts and are also infected with environmental bacteria due to their external fertilization (Zachmann & Molina, 1993; Davidson & Stahl, 2006). During embryonic development, symbiotic bacteria, Verminephrobacter eiseniae are recruited into an embryonic duct before migration to the symbiotic organ, the nephridia, which have an excretory function (Davidson & Stahl, 2008).

At hatching, the gut lumen of the worm is colonized by the exogenous bacterial species. It is not known what role these symbionts play or what benefit they bring (if any) to the earthworm despite evidence of cospeciation (Schramm et al., 2003). This example demonstrates that it is possible for environmental bacteria to be present from fertilization onwards in invertebrates and that mechanisms of transmission of symbionts can be varied and complex. Although slug eggs are fertilized internally unlike the earthworm, and therefore less likely to be infected by exogenous environmental bacteria, research into potential slug symbionts would benefit from further investigation of the bacteria associated with the slug egg as evidence of vertical transmission. In this study, slug eggs were surface sterilized and bacteria inside these eggs were detected using RISA-PCR. The sterilisation method (70% ethanol) and subsequent washing in sterile distilled water was sufficient to kill viable bacteria on the surface which were subsequently not detected on nutrient agar however it is not certain whether this was sufficient to remove all dead bacteria and contaminating DNA from the surface which may have caused false positives in RISA-PCR. Despite this, the resultant RISA profiles suggested a diverse population of bacteria was present in or on the eggs and warrants further investigation into the source and function of these bacteria.

For many of the ITS sequences obtained from cloned RISA bands, high quality matches could not be retrieved from GenBank. This issue highlights one of the main drawbacks of using the 16S-23S ITS region in analyses of microbial ecology. Due to the relatively small number of ITS sequences in the GenBank database compared to 16S rDNA sequences, there is a much greater chance of a query sequence not matching any sequence in the database, or as is also often the case, the query may return a match to an uncultured and unidentified organism which can prevent definitive identification of the query organism. Indeed this was observed in this study, where a number of uncultured bacterial sequences were returned. It is for this reason that currently, until databases become populated by a greater number of 16S-23S ITS sequences, RISA may not be the best technique for studying microbial communities where high resolution identification of clones is a requirement. From this study the effect of sequence heterogeneity between RISA fragments of the same length becomes apparent. For example, clones from band eight cluster within the Gammaproteobacteria and Betaproteobacteria groups while those from band six cluster with both Alphaproteobacteria and Mollusca (Figure 4.16).

This experiment demonstrated the novel use of the Agilent 2100 Bioanalyzer for the fragment detection and sizing of RISA fingerprints so it would be appropriate to give a short summary of the suitability of the instrument for this application. This machine presents a quicker and technically simpler alternative to separating RISA-PCR products on polyacrylamide sequencing gels and allows a RISA profile to be generated from a sample, including DNA purification, in less than 24 hours. The Agilent Bioanalyzer is an affordable alternative to DNA sequencing machines in both unit and running costs. Several attempts were made at separating RISA products on polyacrylamide sequencing gels in this study, but difficulty in standardising the procedure and obtaining reproducible and reliable results was encountered and a simpler method was sought. In using automated detection of RISA products as demonstrated here and as in ARISA, the researcher is unable to directly excise and clone RISA bands for downstream use in sequencing or probe hybridisation studies as is possible with polyacrylamide gels. Conducting such further analyses is beneficial to the molecular microbial ecologist, allowing the elucidation of further structural or functional information about the microbial community being studied. In this study, bands were excised, cloned and sequenced by using the bioanalyzer and agarose gels side-by-side. The bioanalyzer provided the resolution and automation required to precisely detect and assign sizes to the bands and a print-out of a "virtual" gel image could be used to identify the correct bands for excision from the agarose gel. This however is not an ideal solution, and excision of RISA bands, although improved, is still limited by the resolution of the gel used, regardless of the resolution of fragment detection. This introduces difficulty in selecting the exact clone size and a possibility of selecting a number of clones that may be for example ± 10 bp. This is further complicated because broad peaks may represent very closely sized bands of different sequences, so selecting individual band sizes may be difficult.

On reflection, the precision of the bioanalyzer was insufficient to allow meaningful and reliable comparisons between complex microbial community fingerprints. Because of the variability experienced in sizing identical fragments in different wells of the same chip (Figure 4.5) a degree of uncertainty is introduced into similarity indices. As is the case with all molecular fingerprinting techniques, a major question arises – How do we know whether variation in the size of two fragments is due to instrument imprecision or natural length heterogeneity? This is a question that is rarely discussed in the literature but has important implications for the analysis of molecular microbial ecology data. This becomes a particular problem as instrument imprecision increases whereby greater uncertainty is introduced and so it is important to ensure the fragment analysis method employed is as precise as possible

to ensure uncertainty is minimised. This study demonstrated that the Agilent Bioanalyzer achieves sizing precision a number of times lower than the DNA sequencing machines more normally used in molecular fingerprinting and although it offers high resolution sizing and is suitable for estimations of individual sample richness and evenness, data should be treated with caution when profile similarity is to be calculated as objectivity is difficult to achieve. Similar conclusions have been drawn by Nachamkin et al. (2001) who assessed the application of the Agilent 2100 Bioanalyzer for the molecular typing by T-RFLP of the flagellin gene of clinical isolates of Campylobacter jejuni to bypass difficulties in standardising gel electrophoresis between laboratories. Comparing agarose gels with the bioanalyzer they reported problems in resolving multiple fragments within 8-20 bp of each other. They concluded that the machine did represent improvements over agarose gel electrophoresis, and that highly reproducible results could be obtained making it suitable for use in other applications where closely sized DNA fragments do not need to be resolved. It is not clear whether any modifications to the chip and instrumentation are planned by Agilent. The instrument remains an excellent alternative to conventional analysis of PCR products and also for quantifying and sizing biological entities from nucleic acids, to proteins and whole cells.

It is difficult to conceive that any treatment that involves housing a slug in conditions that are different to their natural habitat and feeding a diet that is different to their natural diet would not result in a change in the composition and dynamics of the bacterial flora associated with the slug. This highlights the importance of sample collection and preparation in microbial ecology studies, so as to preserve the natural composition of microbial communities without external influences.

Microbial fingerprinting techniques are subject to errors at almost every stage, from DNA extraction and purification to primer design and PCR reaction conditions. Apart from experimental errors that can be minimised by careful optimisation, inherent variations in genome size and number, and copy number of the ribosomal operon introduce uncertainty that is more difficult to account for. Of the different fingerprinting methods, RISA is considered to yield the highest phylogenetic resolution due to the high degree of heterogeneity within the 16S-23S ribosomal spacer region but it is not without its problems. One important issue relates to the discovery that some organisms may produce more than one fragment length from a RISA-PCR. Multiple peaks arise from differences in operon copy number and the number of peaks produced usually closely resembles the copy number

of the rRNA operon. The multiple peaks often differ by only 1-2 bp indicative of a minor deletion or insertion event and these cases, depending on the sensitivity of the separation technique, may not be detected as separate peaks, but will affect the signal intensity of the band. Alternatively additional RISA products may differ by tens or hundreds of base pairs, suggesting the inclusion of tRNA sequences and in this case would be visible as a clearly defined separate band on the fingerprint. Either way, these extra bands can lead to an overestimation of both the species richness and abundance in the microbial community. Underestimation of these values is also a risk and can occur when two different organisms yield RISA fragments that are the same length but differ in sequence. In this instance, the two organisms would be counted as one and the signal intensities combined resulting in misleading measures of diversity. It is possible for an organism not to produce a fragment in microbial fingerprinting methods due to universal primers not being compatible with sequence heterogeneities at the priming site and this is particularly a problem in organisms with a low copy number of the ribosomal operons. This should be minimised when designing primers and optimising the PCR reaction conditions.

Farelly et al. (1995) demonstrated that the amount of product obtained from PCR amplifications of the 16S rRNA gene was dependent on genome size and the number of 16S genes present in the bacterial genome. Using known information about these parameters they were able to predict the amount of product in 16S PCR amplifications of known mixtures of bacterial DNA. They concluded that unless equipped with prior knowledge of genome number and 16S copy number, it is not possible to accurately quantify bacterial species in mixed samples using 16S rDNA clone libraries. These findings do not affect the study of pure cultures for identification purposes, but are of greatest importance in quantitative and ecological studies of bacterial communities. Genome size can further complicate estimations of abundance in bacterial community analyses. Bacterial genomes can vary from 600 to 13,000kb in length (Cole & Saintgirons, 1994) which raises the issue of the ratio of non-ribosomal DNA to ribosomal DNA. Typically a known quantity of template DNA is used in microbial studies, in which the 16S rDNA template is assumed to be present in equimolar quantities for each of the bacterial constituents. However it is easy to imagine that, for example, 40 ng of template DNA comprising mostly bacteria with large genomes could contain a lower molar concentration of 16S rDNA template than 40 ng of template made up of small genomes, where the 16S rRNA gene accounts for a greater percentage of the total genome length.

It is unclear how molecular ecologists can ever be certain that a true and accurate representation of the microbial community has been obtained from a molecular fingerprinting study, given that culturing and 16S rDNA clone library sequencing each share biases that lead to over estimation of species richness and abundance. To achieve such certainty would require an accurate identification of every bacterial species present, together with detailed information regarding the genome number, genome size and ribosomal copy number such that molecular fingerprints could be reliably interpreted and estimates of diversity adjusted accordingly. Despite the fact that currently no such database holds the required genomic data for all known bacteria, the cost, time and complexity of analysis involved in such a study would be prohibitive to its routine use in molecular ecology.

Organisms with higher copy numbers of the ribosomal genes display greater adaptability to perturbations in environmental conditions such as caused by pollutants or nutrient influx (Klappenbach et al., 2000). Such organisms are able to respond quickly as they can produce ribosomes and synthesise the proteins necessary to metabolise different substances more rapidly than organisms with only a single copy of the rRNA genes. Crosby & Criddle (2003) suggest that these "fast-responding" organisms are the same ones that are over-estimated in the culture-based techniques and as a result of increased copy numbers they may also be overestimated in 16S rRNA-based molecular microbial ecology protocols. Despite inherent biases in molecular fingerprinting techniques described above, these methods continue to be used by the microbial ecologist. At this stage, no acceptable solution for these biases appears imminent, and researchers usually advise "caution" when quoting values of microbial diversity. Girvan et al. (2003) suggests that in light of this problem, diversity and similarity indices generated in molecular microbial ecology studies should be used only for comparative purposes and not be regarded as absolute. It is generally agreed that these biases are acceptable given the magnitude of the biases in culture-based techniques that they replace.

In conclusion, the D. reticulatum digestive tract contains a diverse bacterial community. Some OTUs of potential importance due to their presence in more than 50% of slugs have been determined and are an obvious base of further investigation into potential slug symbionts. There do not however appear to be any universally conserved symbiotic associations between bacteria and slug which has particular implications for the viability of a biological slug control. Bacterial RISA bands considered to be unique to the slug gut and absent from surrounding plants and soil were identified as belonging to major classes of soil

and environmental bacteria, in agreement with the identification of bacteria isolated from D. reticulatum in chapter 3. A number of the bacteria identified belonged to the same classes as other well-studied bacterial invertebrate symbionts (Munson et al., 1991; Moran & Telang, 1998; de Vries et al., 2001; Haynes et al., 2003).

# Chapter 5

# Antibiotic Bioassays

## 5.1 Introduction

Biological pest control offers a means of controlling pest organisms of agricultural, medical and veterinary importance through the use of natural predators, parasites or other mechanisms of a natural origin (VanLenteren & Woets, 1988; Georgis *et al.*, 2006). Biological micro- or macro-organisms may be introduced, encouraged or their numbers expanded such that the pest population may be suppressed and their damage controlled. For example, a gardener may grow plants specifically chosen for their attractant properties to encourage beneficial insects into the garden (Roberts, 2009), or insects such as ladybirds (*Coleoptera: Coccinellidae*) may be introduced into a greenhouse to help control their natural prey, aphids (*Hemiptera: Aphidoidea*) (Kindlmann & Dixon, 1993). The only commercially available biological control for slugs is the parasitic nematode *Phasmarhabditis hermaphrodita* (sold as NemaSlug and NemaSlug Xtra by Becker Underwood) which kills slugs by proliferating inside the shell cavity (Wilson *et al.*, 1993). Refer to section 1.2.4 for more detail about the molluscicidal action of *P. hermaphrodita*. In gardens and small systems, encouraging frogs and hedgehogs, or keeping ducks can help control slugs (Speiser *et al.*, 2001).

Fundamental to biocontrol is the identification of a relationship between the pest and a predator, pathogen, parasite or symbiont that can be manipulated in order to effect control. The exploitation of symbioses in biological pest control is a field which is growing in interest and offers much potential for novel biocontrols (Douglas, 2007; Darby, 2009). Endosymbionts are known to be present in and necessary for the normal growth, health and reproduction of a number of insect pest species including aphids (Lai *et al.*, 1996; Akman Gunduz & Douglas, 2009), tsetse flies (*Dipthera: Glossina* spp.) (Nogge, 1976; Rio *et al.*, 2003) and cockroaches (Order *Blattaria*) (Bandi *et al.*, 1994; Lo *et al.*, 2007) amongst others. As yet symbiont-based biocontrol of terrestrial molluscs has not been studied.

For symbiont-dependent biocontrol to be effective, ideally symbionts should be obligately vertically transmitted and removal of the symbiont should be detrimental to the normal development, reproduction and fitness of the pest. In any other situation, it may not be possible to gain meaningful reduction in an organism's pest-status by this method of biocontrol. Alternatively, commensal symbionts that are not required by a host but are capable of altering a phenotype connected to host pest status offer another potential target for biocontrol (Douglas, 2007). In this case, the pest population would not be affected, but instead it would be rendered harmless. This is however not likely to be achievable in slug

control which requires rapid cessation of crop consumption, and is most applicable to insect vectors of animal and plant pathogens (Durvasula *et al.*, 1997; Beard *et al.*, 2001).

In the laboratory, aposymbiotic insects, those lacking their symbiotic bacteria, can be generated by either feeding or injecting broad-spectrum antibiotics such as tetracycline or rifampicin, or by heat treatment in order to study the effects upon the host of removing an obligate symbiont. Researchers have used this technique to demonstrate the importance of symbionts in aphids (Prosser & Douglas, 1991), tsetse fly (Nogge, 1976) and weevil (*Sitphilus oryzae*) (Heddi *et al.*, 1993) amongst others.

Currently, the future of chemical slug control is unclear. Methiocarb is under continued scrutiny in relation to the EU Pesticide Directive 91/414, whilst metaldehyde residues have recently been identified in water courses. In 2008 metaldehyde accounted for over 90% of the chemical molluscicides applied to arable crops (Garthwaite *et al.*, 2008). The future use of this molluscicide is dependent on achieving suitable reductions in contamination levels in water through stewardship by farmers. If metaldehyde was to be withdrawn, or have reduced application rates imposed, farmers would be forced to turn to methiocarb or ferric phosphate-based pellets which cost around three times greater than metaldehyde per hectare, based on optimum application rates (Dr. K. A. Evans, SAC, personal communications). This increased cost of control would impact upon profit margins and the long term sustainability of farming in the UK. Biocontrol of slugs is likely to be a valuable tool when used as part of an integrated pest management strategy. One biological control product is currently available in the form of the parasitic nematode *Phasmarhabditis hermaphrodita* (Section 1.2.4) but costing over 10-times that of chemical molluscicides, so there is scope for innovation in this field.

This experiment set out to investigate whether *D. reticulatum* may contain a bacterial symbiont crucial to its normal fitness and survival, as a potential target for biocontrol. Antibiotics were administered to *D. reticulatum* to disrupt slug-associated bacteria. The effect of this upon feeding status, fitness and survival of the slug was measured in an attempt to elucidate any potential symbiotic relationships between the slug-associated microflora and its host. First, antibiotic susceptibility tests were conducted on bacteria isolated from the slug gut to assess which antibiotics were most effective against the gut flora of the slug and most suitable for use in the bioassay. Two preliminary bioassays are described. Initially antibiotics were administered by feeding but it was necessary to modify the method of antibiotic administration and a second preliminary assay tested injection of the antibiotic.

## 5.2 Materials and Methods

### *5.2.1 Experimental slugs*

Slugs originated from one of two sites on Embleton Mill Farm, Northumberland, (Figure 5.1). Slugs used in preliminary assays were collected in February from Bob's Close, a winter wheat field following oilseed rape in rotation. For the main assays which were conducted during the months of July and August 2009, slugs were collected from North Emmington which was under oilseed rape after barley. Section 2.1.1 details procedures of slug collection and maintenance in the laboratory.

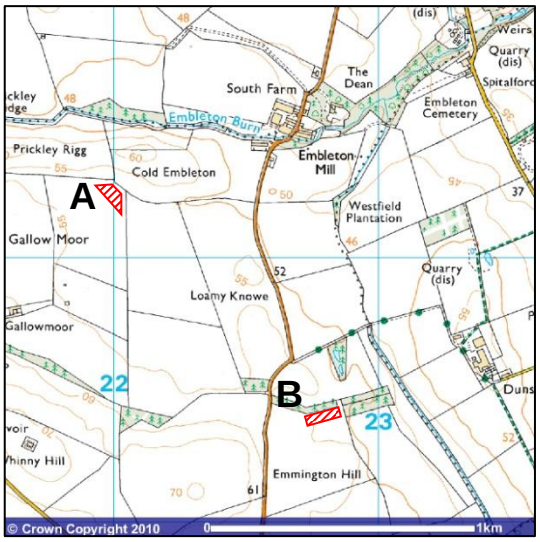



**Figure 5.1.** Location of sampling sites at Embleton Mill Farm, Northumberland. A) "Bob's Close" NU219212GB, winter wheat; B) "North Emmington" NU228204GB, oilseed rape.

### *5.2.2 Antibiotic susceptibility testing*

Crude gut extracts were prepared from ten slugs by first excising the digestive tract as described in section 2.1.2, and then homogenising in 200 µl of sterile quarter-strength Ringers solution (Oxoid, UK) with a sterile polypropylene pestle. Ten-fold dilutions were prepared and 200 µl of the $10^{-2}$ dilution from each slug was spread on each of 6 plates of nutrient agar. Eighteen antibiotic disks were prepared by laying sterile 6 mm Whatman antibiotic assay disks in a sterile petri dish and pipetting 25 µl of an appropriate antibiotic solution directly onto the disk. Disks were gently pressed onto the surface of the inoculated nutrient agar plates such that three plates per slug each contained 6 disks, and a further three plates were duplicates of these (Figure 5.2). The antibiotics were chosen to represent a broad

range of chemistries, target pathways and susceptibility. Antibiotics used and doses per disk were; amoxicillin (25 µg), ampicillin (50 µg), carbenicillin (100 µg), chloramphenicol (25 µl), chlortetracycline (10 µg), gentamicin (10 µg and 25 µg), erythromycin (25 µg), kanamycin sulphate (50 µg), nalidixic acid (15 µg), neomycin (25 µg), polymyxin B (100 U and 300 U), novobiocin (25 µg), penicillin G (20 U), rifampicin (25 µg), streptomycin sulphate (50 µg) and tetracycline (50 µg) (Sigma-Aldrich). Plates were incubated for 24 hours at 20 °C and the diameters of the zones of inhibition were measured in millimetres.

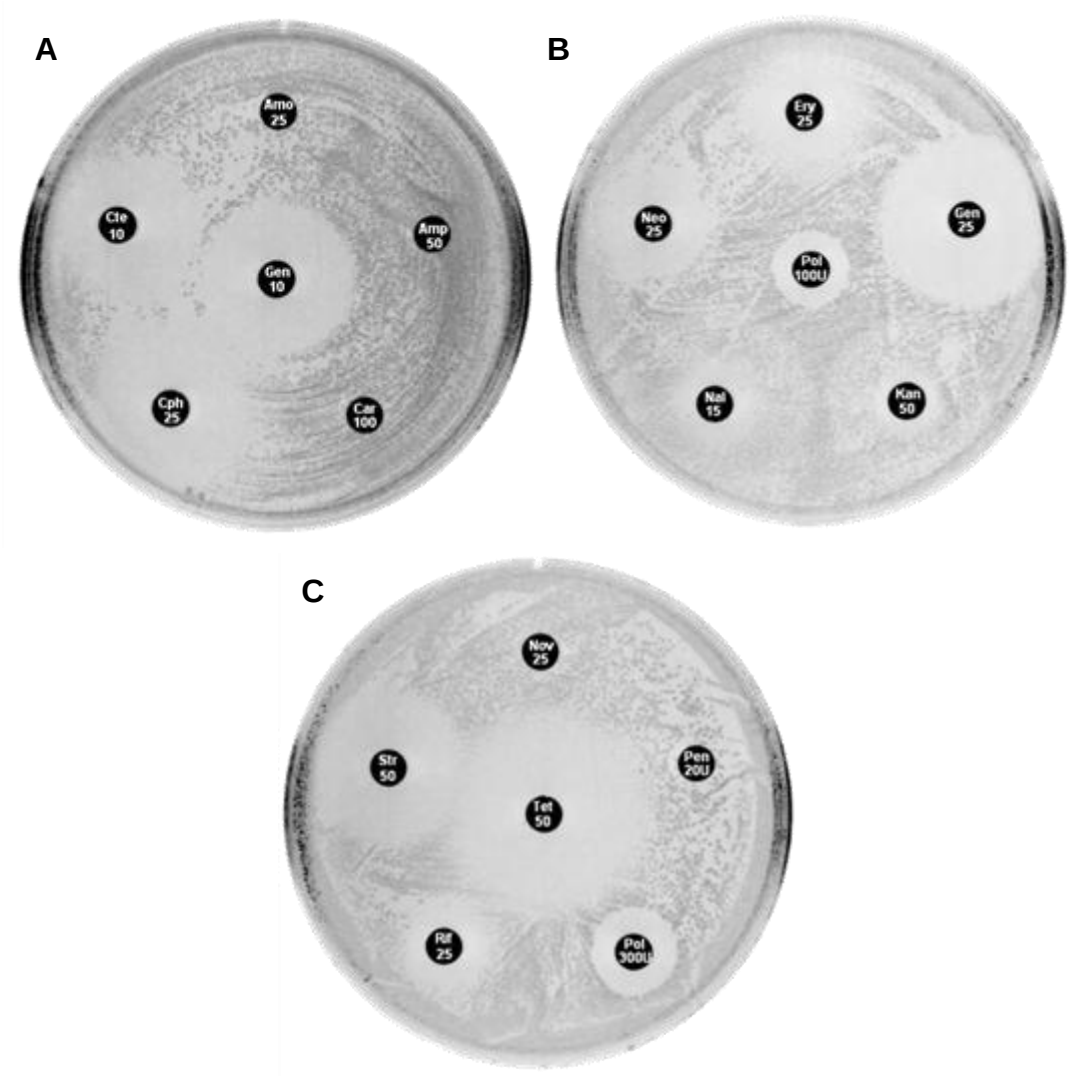



**Figure 5.2.** An example of the 18 antibiotic test disks arranged on three inoculated plates. Three-letter abbreviations and antibiotic dose (in µg unless otherwise stated) have been overlayed. Amo -amoxicillin, Amp -ampicililn, Car -carbenicillin, Cph -chloramphenicol, Cte -chlortetracycline, Gen -gentamicin, Ery -erythromycin, Kan -kanamycin sulphate, Nal -nalidixic acid, Neo -neomycin, Pol -polymyxin B, Nov- novobiocin, Pen -Penicillin G, Rif -rifampicin, Str -streptomycin sulphate, Tet -tetracyline.

### *5.2.3 Preliminary feeding assay*

In an initial bioassay, antibiotics were incorporated into an artificial diet (Section 2.1.2) for administration to the slugs by feeding, using a modified method from Walker (1997). Field collected slugs were initially starved for 3 days (Days -4 to -1) to purge the gut of transient bacteria, after which all slugs were fed on an antibiotic-free diet for 24 hours (Day -1 to 0) before commencement of antibiotic treatment for the remainder of the assay (Days 0 to 16). Three groups each containing 20 slugs were fed diets containing equal quantities of the antibiotics chloramphenicol and tetracycline to a final concentrations of 1, 5, and 10 mg.g $diet^{-1}$. A further control group of 20 slugs was fed on an antibiotic-free diet. Slugs were housed in individual 9 cm Petri dishes which were lined with two sheets of Whatman No.1 filter paper moistened with 1.5 ml of sterile distilled water. A sector of approximately 60° was cut from the filter paper to allow the diet to be placed directly onto the Petri dish to prevent the antibiotic being absorbed into the filter paper. Filter paper was replaced every two days and Petri dishes were changed every four days.

Pre-weighed fresh diet was offered daily to the slugs and uneaten diet from the previous day was removed and weighed so that diet and antibiotic intake could be determined. Daily measurements of slug mass and faeces dry mass were also recorded. At four day intervals, (Days -4, 0, 4, 8, 12, 16) one slug per group was sacrificed and dissected as per section 2.1.3 and colony counts performed to assess the bacterial content of the slug gut in response to antibiotic treatment. Briefly, the masses of the excised whole digestive tracts were recorded then the tissue homogenised to a smooth paste with 100 µl of sterile quarter-strength Ringer's solution (Oxoid, UK) using a sterile polypropylene pestle. Sterile Ringer's solution was added to make a total volume of 1 ml and a 10-fold dilution series prepared by transferring 100 µl of crude gut homogenate to 900 µl dilution blanks of Ringer's solution. Cultures were prepared on plate count agar (Oxoid) by spreading 200 µl of dilutions from $10^{-2}$ to $10^{-6}$ onto the plates using a sterilised glass spreader rod. Plates were incubated at 20 °C for two days after which colonies were counted on plates containing around 50 to 300 colonies. The colony counts were converted to colony forming units per mg tissue (cfu.mg $tissue^{-1}$).

### *5.2.4 Data analysis*

#### *Mass Data*

Slug mass, food intake and faeces data were used to calculate a number of nutritional indices describing the feeding status and food utilisation of the slugs (Mehrotra *et al.*, 1972; Reese & Beck, 1978; Scriber & Slansky, 1981; Blake & Wagner, 1984). The consumption index and growth rate are measures of the food ingestion and growth of the slug relative to the average mass of the slug during the feeding period and both carry the unit $days^{-1}$. They are calculated thus;

$$\text{Consumption index } (CI) = \frac{Food\ ingested\ (wet\ mass)}{T.A}$$

$$\text{Growth rate } (GR) = \frac{Mass\ gained\ by\ slug\ (wet\ mass)}{T.A}$$

Where T.A is a product of the duration of feeding (T) and the mean of the initial and final mass of the slug (A).

The approximate digestibility or assimilation efficiency represents the proportion of the ingested food that is digested and absorbed by the slug. It is calculated from dry masses of food ingested and faeces produced by the slug. Dry masses were obtained by collecting the faeces and uneaten food on pre-dried and pre-weighed 2 cm circles of filter paper and drying at 60 °C for 24 hours before re-weighing both paper and sample.

$$\text{Approximate digestibility } (AD) = \frac{Food\ ingested\ (dry\ mass) - Faeces\ (dry\ mass)}{Food\ ingested\ (dry\ mass)}$$

To assess the ability of the slug to utilise the food it consumes, efficiency of conversion of ingested (ECI) and digested (ECD) food was calculated. These indices are dimensionless and therefore have no units. They describe the mass gained by the slug over the period of feeding as a percentage of the mass of the food that is ingested and digested, to show how efficiently food is converted to biomass.

$$ECI = \frac{Mass\ gained\ by\ slug\ (wet\ mass)}{Mass\ of\ food\ ingested\ (wet\ mass)} \times 100$$

$$ECD = \frac{Mass\ gained\ by\ slug\ (wet\ mass)}{Mass\ of\ food\ ingested\ (wet\ mass) - Faeces\ mass(wet\ mass)} \times 100$$

### Summary Statistics

To analyse slug growth during the assay, two response features were applied to summarise the data for each individual slug and this was followed by a simple analysis of variance of the response. This method of applying summary statistics to serial measurements is described in Matthews *et al.* (1990). In the current study, the two response features used were the mean for each slug across all measurements and the regression coefficient of the response on time. Respectively the two features describe differences in the mean masses and differences in the rate of change of the masses of slugs from different treatment groups. Summary measures were compared using analysis of covariance (ANCOVA) in Genstat, with the slug mass prior to antibiotic administration as the covariate.

### Survival Data

The Cox proportional hazard model was adopted for the analysis of the slug mortality data. The model assumes an underlying hazard function which describes the risk of a terminating event (in this case death of the slug) occurring for members of the control group at any point in time (Collett, 2003). The hazard function for a treatment group relative to the control is expressed as a proportion of this baseline hazard function taking into account the rate of death at all time points for which death is observed. Using survival data, the proportional hazard function was calculated in the statistical package Genstat (Payne *et al.*, 2009).

## *5.2.5 Preliminary injection assay*

It was apparent from the results of the preliminary feeding bioassay that an alternative method of administration of the antibiotics should be sought owing to unfavourable palatability of the antibiotic diet (Section 5.3.2). This second preliminary study (based on the method described by Richards *et al.* (2008) tested the injection of antibiotics and increased the sample size in an attempt to reduce the large variation in the data of the previous assay. Three antibiotics, chloramphenicol, gentamicin and tetracycline were administered in pair-wise combinations to target a broad range of bacteria and reduce the bacterial content of the

slug gut as much as possible whilst still allowing comparisons between different antibiotics to be made. The three antibiotic combinations (chloramphenicol and gentamicin, chloramphenicol and tetracycline, gentamicin and tetracycline) were prepared to final concentrations of 5 mg per ml in sterile slug saline (70 mM NaCl, 2.0 mM KCl, 4.7 mM $MgCl_2$, 4.9 mM $CaCl_2$, 5.0 mM glucose, 5.0 mM HEPES; pH 7.6 (Ito *et al.*, 2000; Richards *et al.*, 2008). A Hamilton microlitre syringe (Hamilton Company, Reno, NV, USA) fitted with a 30 gauge needle was used to inject the antibiotics into the haemocoel through the foot of the slug near the tail, taking care not to insert the needle so deep as to damage the internal organs. A new sterile disposable needle was used for each slug. The injected volume was adjusted according to slug mass such that each slug received a dose approximating to 0.2 mg per gram of body mass. Three control groups were used, one saline control injected with sterile saline only, one non-injected control which was not touched with a needle and one needlestick control in which members were penetrated with a needle but nothing was injected. Each of the antibiotic treatment groups and the saline control comprised 70 individual slugs whilst the non-injected and needlestick controls comprised 30 slugs each, making a total of 340 slugs in the assay.

Slugs were purged for four days (Days -4 to 0) after collection from the field to reduce the amount of transient bacteria in the gut. On day zero treatment was administered and slugs were placed into individual 9 cm Petri dishes which were lined with Whatman Number 1 filter paper disks moistened with 1.5 ml of sterile distilled water. Slugs were stored in an incubator at 15 °C with a 12 hour light:dark period. In this assay, slugs were fed with 2 cm diameter leaf disks of Chinese cabbage. The leaf disks were surface-sterilised by first washing in 70% ethanol for 2 minutes, 0.1% sodium hypochlorite solution for 5 minutes and rinsing twice in sterile distilled water for 2 minutes. The disks were then dried in a class two microbiological safety cabinet for 20 minutes and stored at 4 °C in sterile Petri dishes. Fresh leaf disks were offered to the slugs on alternate days. Petri dishes were replaced every four days at which point slug mass, food intake and faeces mass were also recorded. To obtain dry masses, remaining food and faeces were transferred to pre-dried and pre-weighed 1.5 ml microcentrifuge tubes and then dried at 60 °C for 24 hours before weighing again. Slug mortality was recorded daily.

On days -4, 0, 1, 3, 7 and 21, ten slugs from each antibiotic treatment and from the saline control were selected at random and killed and dissected as per section 2.1.3 so that the size of the gut bacterial population could be estimated. As previously described, ten-fold serial

dilutions of the slug gut homogenate were prepared and the $10^{-3}$, $10^{-4}$ and $10^{-5}$ dilutions were plated onto nutrient agar, incubated for two days at 20 °C and the colonies counted.

### *5.2.6 Injected antibiotic bioassay*

The preliminary assays were followed by a main assay which was run on two separate occasions to obtain two replicate datasets. These assays followed the same protocol as the previously described preliminary injection assay (Section 5.2.5) with a number of modifications. The sample size of 70 slugs per treatment group proved unmanageable and so was reduced to 20 slugs per treatment group resulting in a total of 120 slugs in the first "July" assay. In the "August" assay a total of 290 slugs were involved, which included an additional 40 per antibiotic treatment group and an extra 50 in the saline control group to allow for the sacrifice of slugs for estimations of the gut bacterial load on days -4, 0, 1, 3, 5, 11 and 20. All treatments and controls were kept the same as the previous assay but instead of a single antibiotic dose on day 0, antibiotic was administered on three occasions on days 0, 4 and 8 in an attempt to maintain antibiotic in the slugs for a prolonged period. Measurements of slug mass, food intake and faeces mass were increased in frequency to every second day. Mortality was again recorded daily.

Leaf disks as a food source proved to be unsuitable as they were difficult to surface sterilise and production was time consuming and impractical in a large experiment as they needed to be prepared fresh. The leaf disks had a tendency to begin to decompose within 24 hours and it was suspected that this affected palatability and perhaps contributed to high mortality in all groups in the preliminary injection assay. Instead, the agar-based diet described in section 2.1.2, this time without antibiotic, was utilised in these main assays. Agar could be made in large volumes in advance of the experiment and autoclaved to sterilise before dispensing into sterile Petri dishes for storage at 4 °C.

## 5.3 Results

### *5.3.1 Antibiotic susceptibility testing*

Sensitivity of bacteria isolated from ten slugs was tested against 16 antibiotics. Mean zones of inhibition (ZOI) in sensitivity disk tests on nutrient agar ranged from 0 mm to 31.5 mm (Figure 5.3) with drugs of the β-lactam group of antibiotics exhibiting very low or zero ZOIs. Particularly good activity was observed from antibiotics of the aminoglycoside group, namely gentamicin, kanamycin sulphate, neomycin and streptomycin sulphate. The antibiotics to which the slug-associated bacteria were most sensitive and therefore producing the largest ZOIs were chloramphenicol, gentamicin and tetracycline which reinforces the decision to use these antibiotics in the bioassays. These findings are comparable to similar antibiotic sensitivity testing conducted on bacteria isolated from *D. reticulatum* by Walker (1997) and on the snail *Helix aspersa* by Watkins and Simkiss (1990).

### *5.3.2 Preliminary feeding assay*

Feeding slugs an agar-based diet containing the antibiotics chloramphenicol and tetracycline was successful in reducing the bacterial counts obtained from the gut (Figure 5.4). The initial starvation period, from field collection (Day -4) to day 0 when antibiotic administration was commenced, saw a 25-fold decrease in bacterial count in the slug gut. This level was further reduced after antibiotic administration such that bacteria were undetectable in slug gut extracts from all antibiotic treatment groups on day 4. Bacterial counts remained low for the majority of the assay but began to rise in the 10 mg treatment group on day 16. The control group which did not receive any antibiotic showed a ten-fold increase in bacterial counts in the gut post-starvation from day 0 to day 4, but this subsequently reduced by 120-fold to what can be considered negligible levels by day 8. This could be due to the slugs being fed a sterile diet resulting in an eventual natural purging of bacteria from the control slugs despite not being treated with antibiotic.

Slugs that were fed a high dose of antibiotic (5 mg.g diet$^{-1}$ and 10 mg.g diet$^{-1}$) lost body mass over the course of the assay whereas the control and low dose groups (1 mg.g diet$^{-1}$) appeared to remain the same or increase in mass (Figure 5.5). In fact, regression analysis (Section 5.2.4) of the rate of change in mass of the slugs showed that the control group

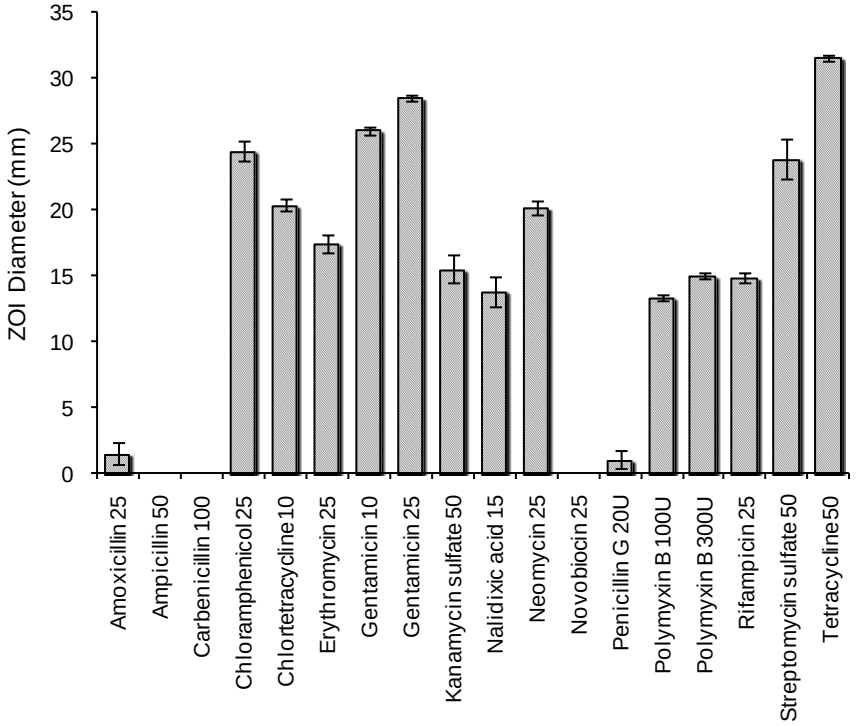



**Figure 5.3.** Mean diameter of zone of inhibition (ZOI) in millimetres when culturable slug gut bacteria was tested for sensitivity to 16 antibiotics. Antibiotic dose is quoted as μg per disk unless otherwise stated. Error bars show the SEM of two replicates from each of ten slugs.

exhibited an overall negative growth whilst the low dose treatment group were the only slugs with an overall positive rate of change to their mass (Regression coefficients -0.00155 and 0.00369 respectively, $p<0.001$, ANCOVA), showing that an antibiotic in the diet may actually be beneficial to slug health at low dosage. This is further reflected in the nutritional indices (Table 5.1). The approximate digestibility (AD) was around 3-fold higher for slugs in the control and low dose treatment groups than for high dose slugs, indicating that less of the ingested food was absorbed by the high dose groups (Table 5.1).

**Table 5.1.** Mean consumption index (CI), growth rate (GR), approximate digestibility/assimilation efficiency (AD) and efficiency of conversion of ingested food (ECI) for slugs fed on a diet containing antibiotic at three concentrations. Within each column, different letters in superscript denote statistical differences at the 5% level (ANOVA).

| Treatment | Mean Nutritional Indices | | | |
|---|---|---|---|---|
| | CI $days^{-1}$ | GR $days^{-1}$ | AD | ECI |
| Control | $0.011^{a}$ | $0.011^{b}$ | $49.3^{a}$ | $5.110^{a}$ |
| 1 mg.g $diet^{-1}$ | $0.011^{a}$ | $0.020^{a}$ | $53.6^{a}$ | $16.035^{a}$ |
| 5 mg.g $diet^{-1}$ | $0.004^{b}$ | $-0.002^{c}$ | $15.1^{b}$ | $-21.657^{a}$ |
| 10 mg.g $diet^{-1}$ | $0.004^{b}$ | $-0.002^{c}$ | $17.7^{b}$ | $-36.840^{a}$ |

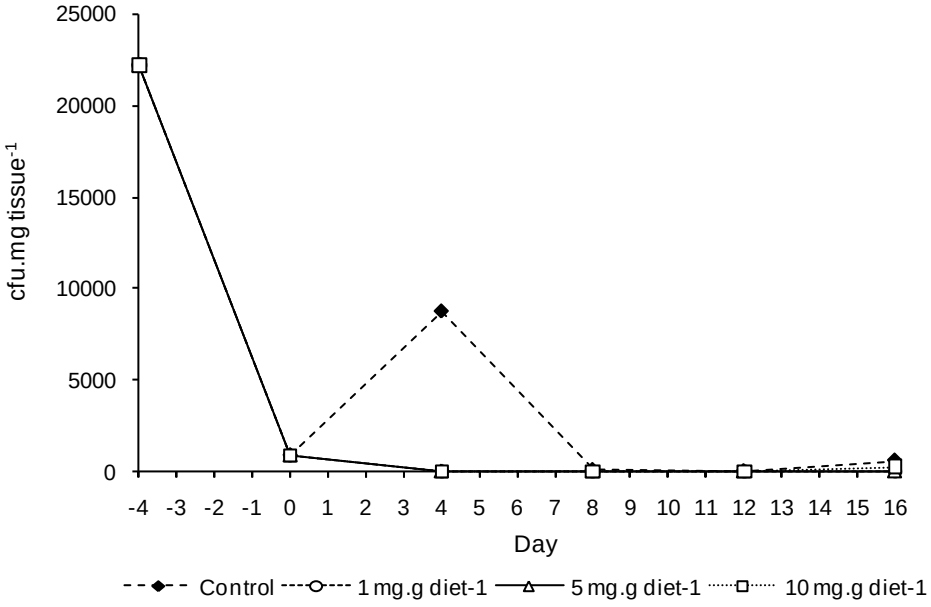



**Figure 5.4.** Bacterial colony counts performed on the gut extracts of slugs sacrificed at 4-day intervals during the antibiotic feeding assay. Slugs were field collected at day -4 prior to being starved for three days, and then fed an antibiotic-free diet for 24 hours before antibiotic treatment with chloramphenicol and tetracycline was commenced on day 0. Bacterial counts are expressed as colony forming units per mg of slug tissue (cfu.mg tissue$^{-1}$).

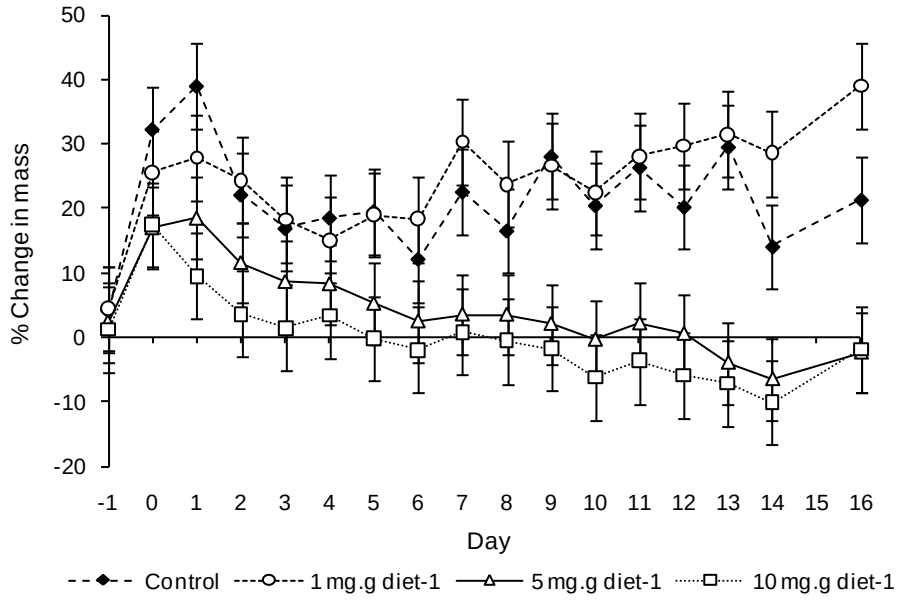



**Figure 5.5**. Mean percentage change in body mass relative to starting mass of slugs treated with a combination of chloramphenicol and tetracycline incorporated into an agar-based diet in three different total concentrations. Error bars show SED ($p<0.005$, ANOVA).

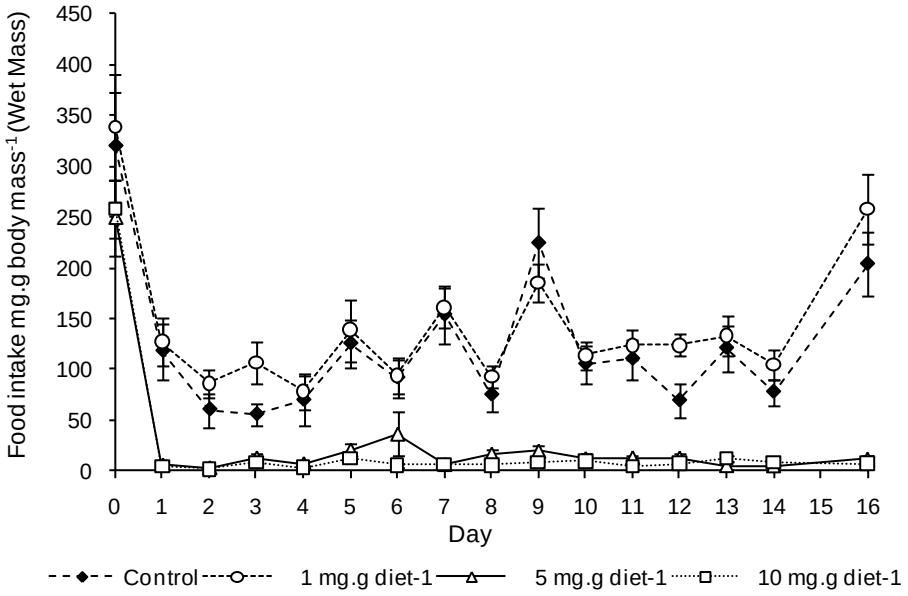



**Figure 5.6.** Mean food intake (wet mass) of slugs treated with a combination of chloramphenicol and tetracycline incorporated into an agar-based diet in three different total concentrations. Error bars show SEM.

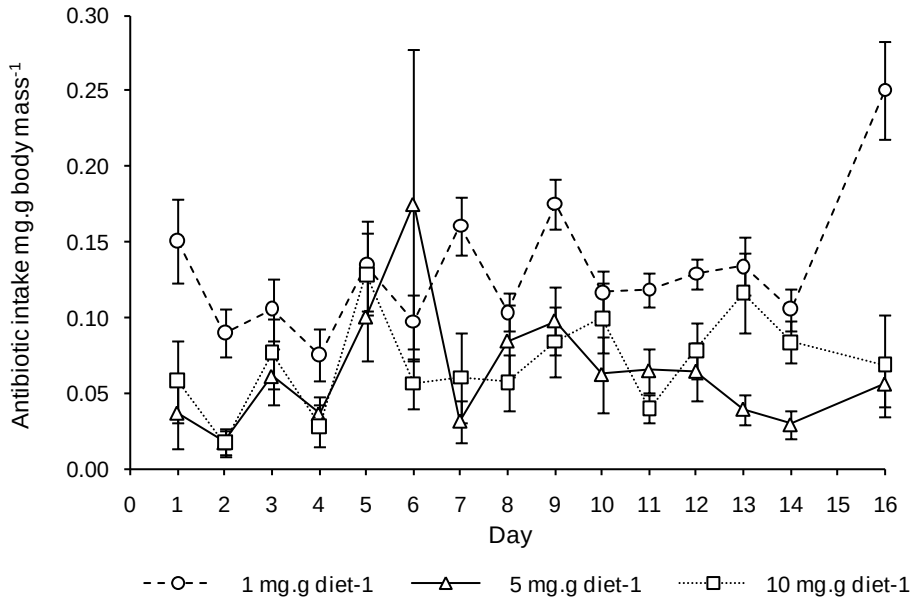



**Figure 5.7.** Mean antibiotic intake of slugs treated with a combination of chloramphenicol and tetracycline incorporated into an agar-based diet in three different total concentrations. Error bars show SEM.

Despite being seen to lose mass when fed antibiotic in large doses (Figure 5.5) it is unlikely that this was due to the disruption of a beneficial interaction between the slug and possible bacterial symbionts. Food intake in the two high dose groups was considerably lower than in the control and low dose slugs which shows that inhibition of feeding is the cause of this loss of body mass (Table 5.1, Figure 5.6). Instead, this highlights reduced palatability of the diets containing high doses of antibiotic and thus the slugs were deterred from eating by the taste and/or smell of the diet. Furthermore, due to reduced food intake in the high dose treatment groups, overall antibiotic intake was at its highest in the slugs of the low dose group which were observed to increase in mass throughout the assay (Figure 5.7), therefore antibiotic intake and subsequent putative disruption of symbioses is not responsible for reduction in slug body mass. Slug mortality throughout this assay was not affected by the administration of antibiotics. Only three slugs died throughout the assay, two from the low dose group and one from the control group which correspond to 16.7% and 8.3% mortality respectively.

### *5.3.3 Preliminary injection assay*

Injection of the antibiotic was tested as an alternative to administration by feeding. A reduction in slug gut bacteria was observed in all antibiotic treatment groups 24 hours after injection of the antibiotic (Figure 5.8). The reduction of bacterial counts was greatest where gentamicin and tetracycline were administered in combination suggesting complimentary activity and reflecting the results of the antibiotic sensitivity tests carried out previously (Figure 5.3). On day three (72 hours after injection) there was still a significant difference between bacterial counts obtained from gut extracts of antibiotic treated slugs compared to control slugs, but by day eight bacterial counts had returned to a level similar to the control group thus injection of antibiotic is successful in reducing bacteria in the slug gut for between four and eight days. It is important to add that the size of the reduction in bacterial numbers in the slug gut was not as great as achieved by feeding the antibiotic to the slug where bacteria were near-undetectable in plate counts on nutrient agar (Figure 5.4).

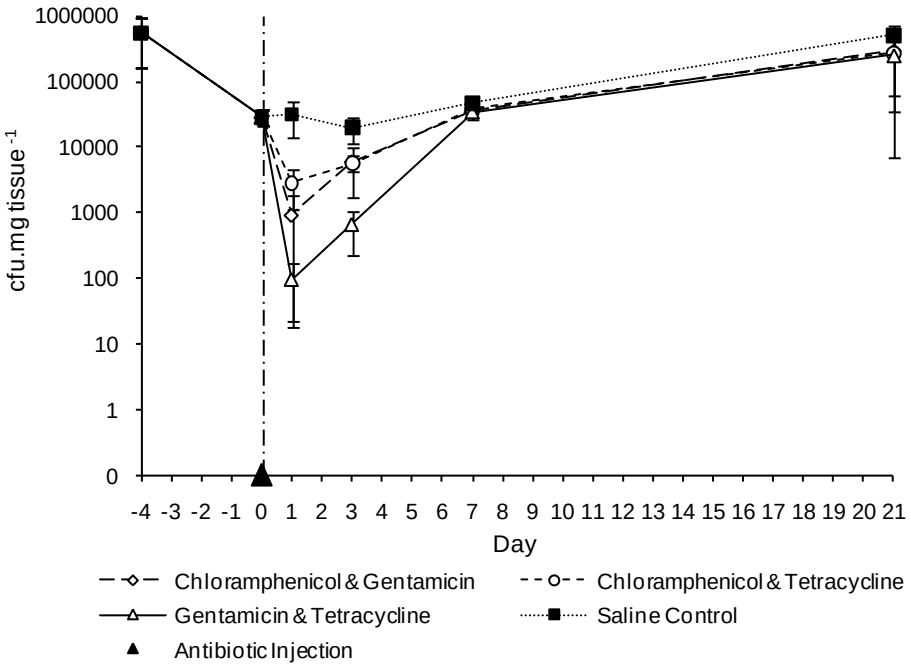



**Figure 5.8.** Bacterial counts performed on gut extracts from slugs treated with antibiotic. Slugs were field-collected at day -4, starved for 4 days then injected with one of three combinations of antibiotics on day 0. Counts are expressed as colony forming units per mg or slug gut tissue (cfu.mg tissue$^{-1}$). Error bars show SEM. Data are plotted on a log scale.

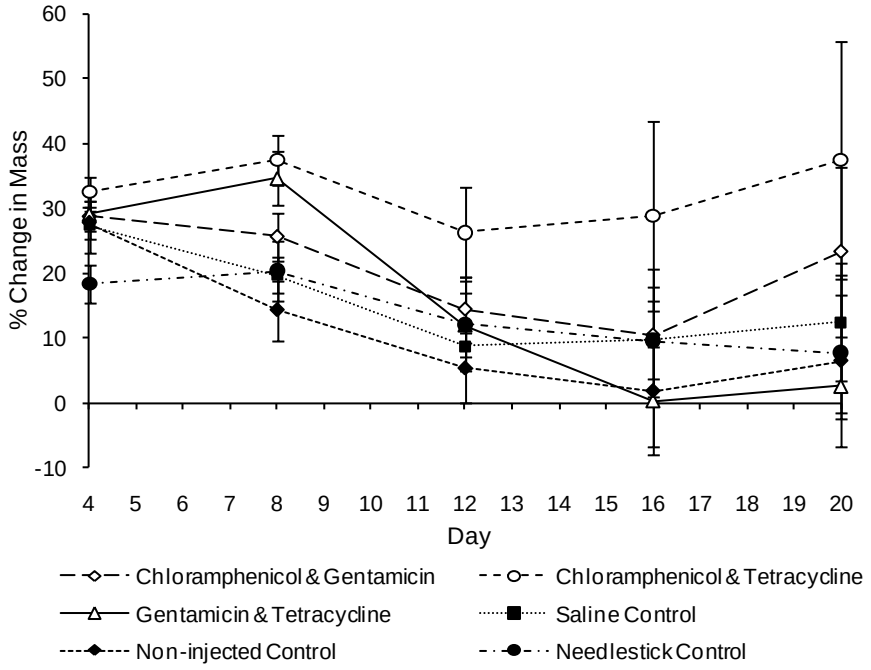



**Figure 5.9.** Mean percentage change in body mass relative to starting mass of slugs injected with antibiotics in a preliminary trial of injection. Error bars show SEM.

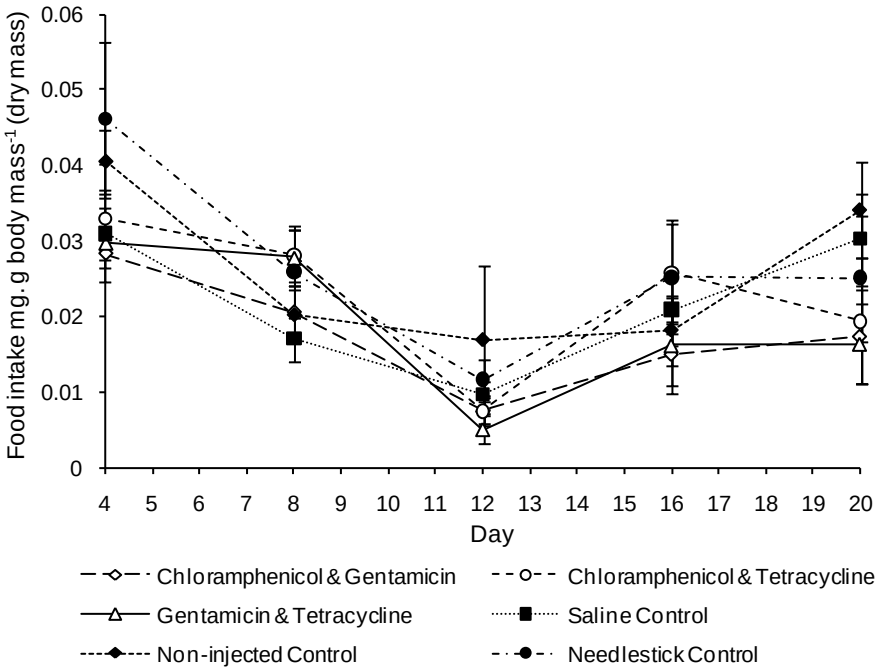



**Figure 5.10.** Mean food intake expressed in milligrams of food (dry mass) per gram of body mass (mg.g bodymass$^{-1}$) for slugs treated with antibiotic in a preliminary trial of injection. Error bars show SEM.

Slug mass and food intake were unaffected by antibiotic treatment with all controls and treatments exhibiting similar values (Figures 5.9 and 5.10). It is notable that food intake in all groups showed a steady decline from days four to twelve (Figure 5.10). As this happens also to control slugs, it is not due to effects of the antibiotic treatment, however it may be due to the phenomenon of "neophilia" whereby slugs feed upon foods more readily when they are novel, and a subsequent reduction in consumption follows later (Frain & Newell, 1982; Whelan, 1982; Cook *et al.*, 2000). After day twelve, consumption began to increase again as the slugs were forced to eat the only food available.

Mortality was high in this assay, with between 87.5% and 92.5% mortality recorded in the three antibiotic treatment groups by day 21 (Figure 5.11). The controls exhibited a slightly lower mortality (65% to 80%) although there were no significant differences between the hazard ratios of each of the groups compared to the saline control (p=0.181) and estimates of the Kaplan-Meier survivor function showed no significant difference between the groups at the 95% confidence level. Most mortality occurred between days eight and twelve (Figure 5.11), which corresponds to the point at which bacterial counts in the antibiotic treated slugs returned to the same level as the controls (Figure 5.8).

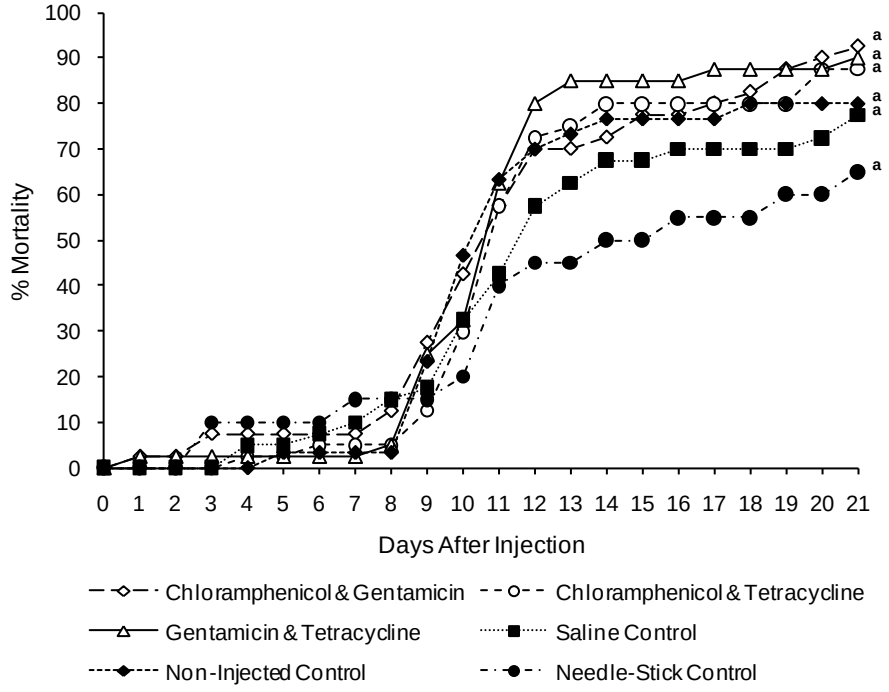



**Figure 5.11.** Percentage mortality of slugs treated with antibiotic in a preliminary trial of injection. Different letters represent significant differences at the 5% level (Kaplan-Meier).

### *5.3.4 Injected antibiotic assay*

This main bioassay was repeated on two separate occasions and was intended to provide two combinable datasets however differing patterns of mortality in control groups in the two assays meant that it was not possible to combine the two datasets for analysis. The two assays will be referred to as "July" and "August" when describing the results. There was an overall higher mortality in August than in July with mean mortality of 69% and 33% respectively (Figure 5.12, Figure 5.13). In July, there were no observed deaths in the chloramphenicol and gentamicin group and only one death in the chloramphenicol and tetracycline group (Figure 5.12). In both assays it is notable that the three controls suffered greater mortality than the antibiotic treated groups, suggesting a benefit of antibiotic treatment, perhaps in suppressing a bacterial pathogen. Survival data were fitted to the Cox proportional hazard model which assigns individuals in the experiment a hazard function which describes the likelihood of death occurring at any given time relative to an assumed baseline hazard (Section 5.2.4). With the saline control as the reference, all groups in July with the exception of the needlestick control have a negative hazard function which indicates a lower likelihood of death occurring in these groups compared to the saline control ($p<0.001$) (Table 5.2). The hazard functions quoted in table 5.2 are multiplicable in magnitude, where a hazard function of -8, for example, represents four times lower risk of death than a hazard function of -2. In August all groups had negative hazard functions relative to the saline control and all three antibiotic treatment groups had significantly lower hazard functions than the controls (Table 5.2). This indicates reduced mortality in antibiotic-treated slugs. In contrast to the preliminary injection assay, mortality begins to occur much earlier, mostly from day two to six in July and day one to eight in August compared to day eight to twelve in the preliminary assay (Figures 5.11, 5.12, 5.13).

**Table 5.2.** Proportional hazard functions relative to the saline control of groups of slugs injected with antibiotics in July and August bioassays. Within each column, difference letters indicate significant differences ($p<0.001$)

| Treatment | Hazard Function | |
|---|---|---|
| | July | August |
| Chloramphenicol & Gentamicin | -8.7 [a] | -1.313 [a] |
| Chloramphenicol & Tetracycline | -2.71 [b] | -1.297 [a] |
| Gentamicin & Tetracycline | -1.248 [b c] | -2.102 [a] |
| Non-injected Control | -0.313 [c d] | -0.23 [b] |
| Needlestick Control | -0.461 [d] | -0.443 [b] |

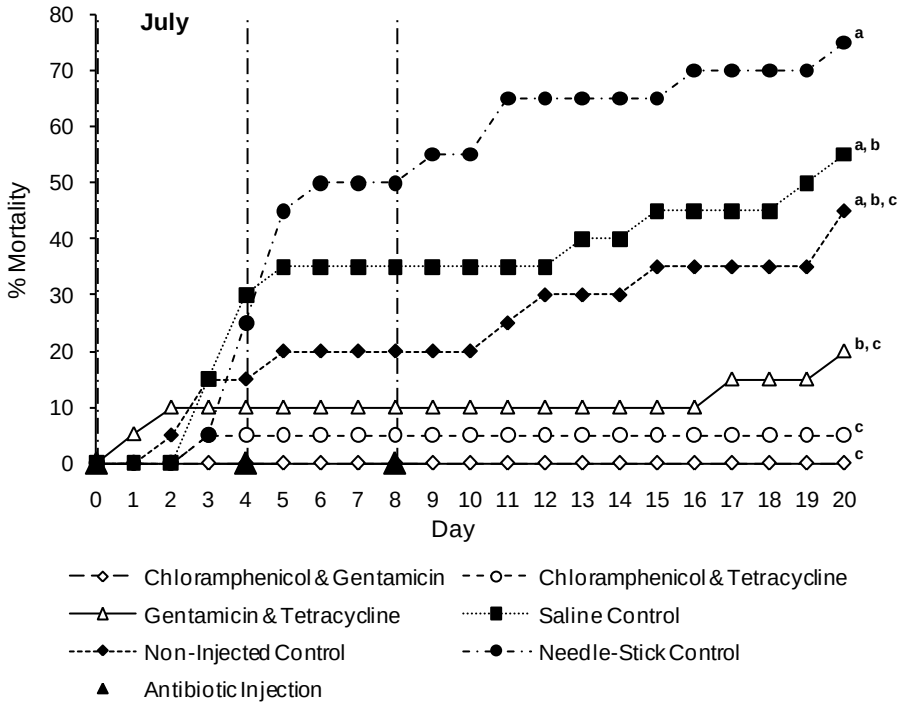



**Figure 5.12.** Percentage mortality of slugs injected with antibiotics in the **July** bioassay. Treatment was administered on days 0, 4 and 8 after an initial 4-day starvation period (Days -4 to 0). Different letters indicate significant differences at the 5% level (Kaplan-Meier).

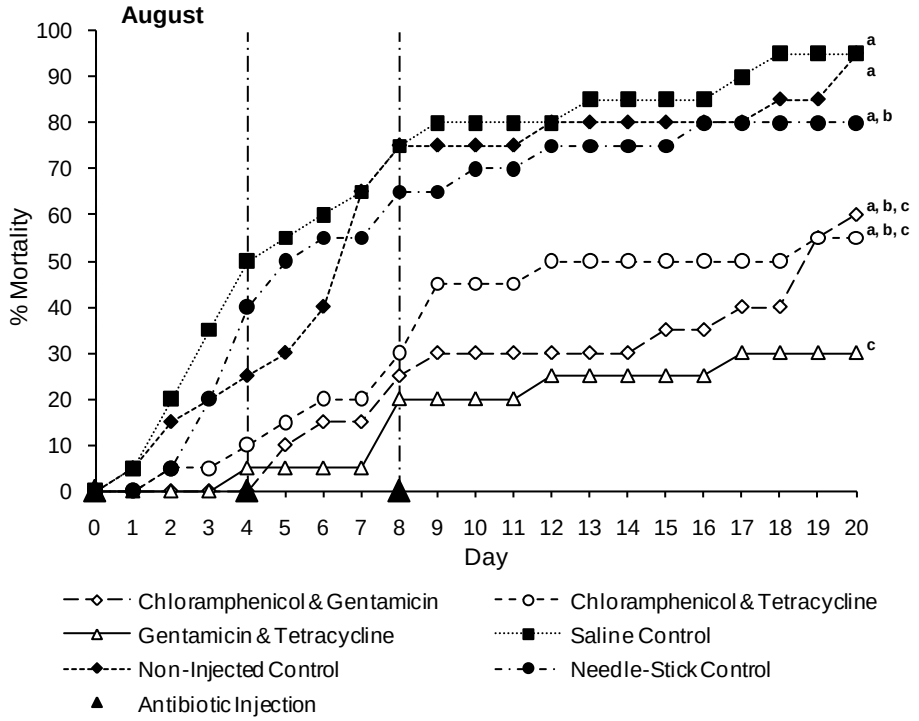



**Figure 5.13.** Percentage mortality of slugs injected with antibiotics in the **August** bioassay. Treatment was administered on days 0, 4 and 8 after an initial 4-day starvation period (Days -4 to 0). Different letters indicate significant differences at the 5% level (Kaplan-Meier).

The August assay contained extra slugs in the antibiotic treatment and saline control groups to allow for the sacrifice of a sample from each at various times throughout the assay in order to conduct bacterial colony counts on gut extracts. As in the preliminary experiments, a large reduction on colony counts was observed after the initial starvation period where bacterial counts at collection (Day -4) of 1 million colony forming units per mg of tissue (cfu.mg tissue$^{-1}$) were decreased to 38000 cfu.mg tissue$^{-1}$ at the time of antibiotic administration (Day 0) (Figure 5.14). From day 0 bacterial counts in the antibiotic treated slugs remained at this reduced level for the duration of the experiment while bacterial counts were seen to rise again in the saline control group peaking at eight times that of the chloramphenicol and gentamicin group on day three. The bacterial count in the saline control fell after day three to a level similar to the antibiotic groups by day five where it remained until the end of the experiment at day 20. A real-time quantitative PCR assay was used to compare the counts of viable bacteria with the total bacterial DNA present in the slug gut as it is widely understood that culture-dependant techniques are likely to sample only a small proportion of the total bacteria (Torsvik *et al.*, 1990). The results closely resemble those from the bacterial plate counts and adequately show the disparity between the bacterial content of control and treated slugs between days zero and five (Figure 5.15).

Slugs that died during the August experiment were dissected and their gut extracts assessed for total bacterial content using the 16S rDNA qPCR assay (Section 2.2.2) as a comparison with healthy slugs. A greater amount of bacterial 16S rDNA was detectable in slugs that were sacrificed compared to dead slugs (Figure 5.16). Within all three antibiotic treated groups, there was no significant difference in the amount of bacterial 16S rDNA detectable in sacrificed or dead slugs (Figure 5.17). Sacrificed slugs in the saline control group showed a mean bacterial 16S rDNA content up to six times that of the antibiotic treated groups and six times greater than slugs immediately after collection from the field, showing that conditions of the assay promoted bacterial proliferation where antibiotic was not present (Figure 5.17).

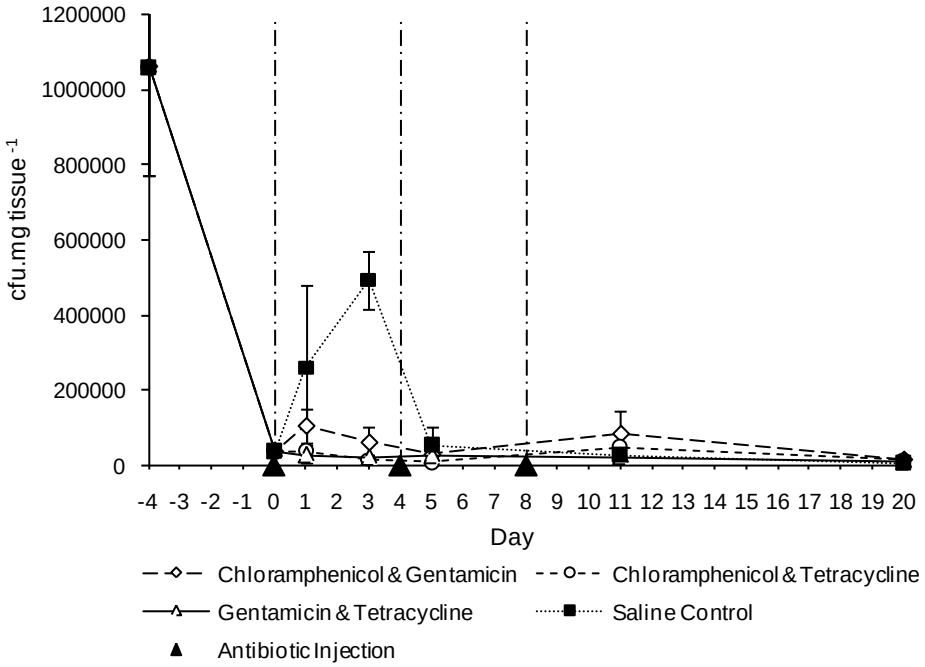



**Figure 5.14.** Bacterial colony counts performed on gut extracts from slugs injected with antibiotics. Bacterial counts are expressed as colony forming units per mg of slug gut tissue (cfu.mg tissue$^{-1}$). Error bars show SEM.

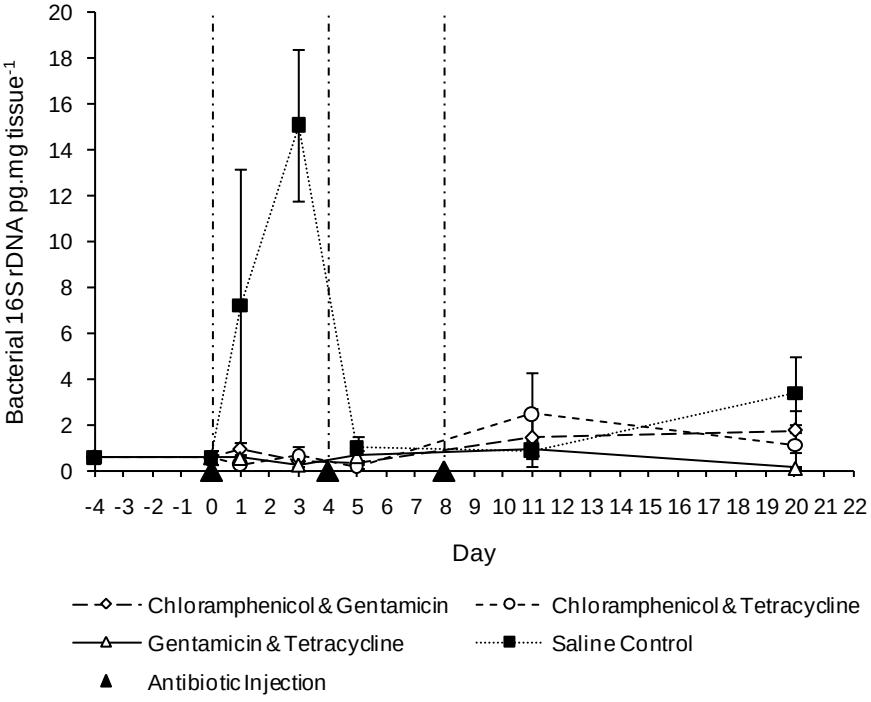



**Figure 5.15.** Real-time quantitative PCR assay of 16S rDNA in gut extracts from slugs injected with antibiotics. Slugs were field-collected on day -4 and following an initial starvation period (Days -4 to 0), treatment was repeated three times on days 0,4 and 8. Error bars show SEM.

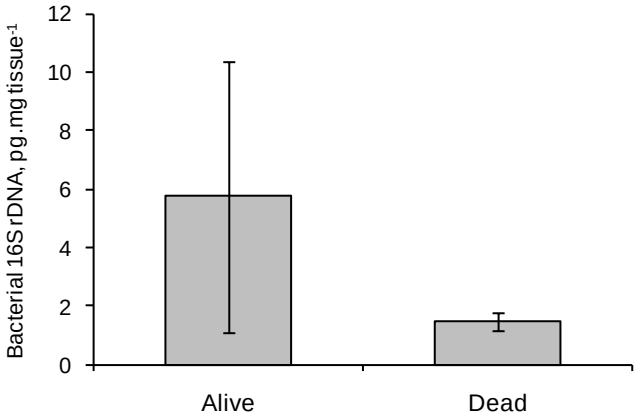



**Figure 5.16.** Estimated mean quantity of bacterial 16S rDNA (picograms per mg tissue) by qPCR, present in the gut extracts of all slugs that either were sacrificed (Alive) or died (Dead) during the August injected antibiotic bioassay. Error bars show SEM.

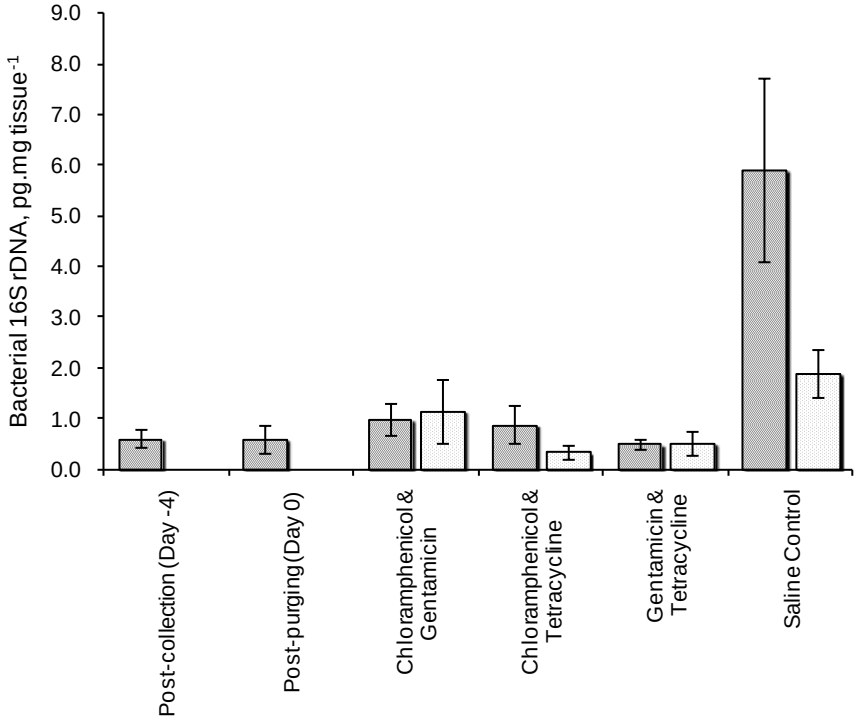



**Figure 5.17.** Estimated mean quantity of bacterial 16S rDNA (picograms per mg tissue) present in gut extracts from slugs that either were sacrificed (▣), or died (□) during the August injected antibiotic bioassay. Error bars show SEM.

Slug mass was variable in both assays with fluctuating measurements observed every two days. In July, although differences in mean slug mass between groups were significant only at the 5% level ($p=0.038$), analysis of the rate of change in mass showed that all slugs experienced a small loss of mass with the antibiotic groups losing mass at a significantly lower rate than the saline control ($p<0.001$) (Table 5.3A, Appendix 5.1). Rates of change in mass of the non-injected and needlestick controls were intermediate and not significantly different to either of the other groups. Mean slug masses in August revealed significant differences between the two tetracycline containing treatments, the needlestick control and the saline and non-injected controls ($p<0.001$) (Appendix 5.1). The rates of change in mass were negative for all groups except the saline and non-injected controls however this difference was not significant ($p=0.083$) (Table 5.3A).

**Table 5.3.** Summary measures describing the daily mean and rate of change of **A**) slug mass, **B**) food intake and **C**) mass of faeces produced by groups of slugs in July (**J**) and August (**A**) antibiotic bioassays. Within each column, difference letters indicate significant differences at the appropriate confidence level (ANCOVA).

| **A** | **Slug Mass (g)** | | | |
|---|---|---|---|---|
| | **Mean Daily** | | **Rate of Change** | |
| | **J** | **A** | **J** | **A** |
| Chloramphenicol & Gentamicin | $0.592^{ab}$ | $0.604^{c}$ | $-0.0009^{ab}$ | $-0.0096^{b}$ |
| Chloramphenicol & Tetracycline | $0.569^{b}$ | $0.667^{ab}$ | $-0.0035^{ab}$ | $-0.0055^{ab}$ |
| Gentamicin & Tetracycline | $0.616^{a}$ | $0.673^{a}$ | $0.0009^{a}$ | $-0.0046^{ab}$ |
| Saline Control | $0.565^{b}$ | $0.603^{c}$ | $-0.0147^{c}$ | $0.0027^{a}$ |
| Non-injected Control | $0.574^{b}$ | $0.629^{bc}$ | $-0.0080^{bc}$ | $0.0024^{a}$ |
| Needlestick Control | $0.577^{b}$ | $0.640^{abc}$ | $-0.0056^{ab}$ | $-0.0048^{ab}$ |
| p value | 0.038 | 0.003 | <0.001 | 0.083 |

| **B** | **Food Intake (g)** | | | |
|---|---|---|---|---|
| | **Mean Daily** | | **Rate of Change** | |
| | **J** | **A** | **J** | **A** |
| Chloramphenicol & Gentamicin | $0.0238^{a}$ | $0.0155^{c}$ | $-0.0007^{a}$ | $-0.0004^{a}$ |
| Chloramphenicol & Tetracycline | $0.0239^{a}$ | $0.0196^{a}$ | $-0.0006^{a}$ | $-0.0010^{c}$ |
| Gentamicin & Tetracycline | $0.0216^{a}$ | $0.0206^{a}$ | $-0.0004^{a}$ | $-0.0004^{ab}$ |
| Saline Control | $0.0213^{a}$ | $0.0209^{a}$ | $-0.0008^{ab}$ | $-0.0002^{a}$ |
| Non-injected Control | $0.0222^{a}$ | $0.0187^{ab}$ | $-0.0013^{c}$ | $-0.0006^{ab}$ |
| Needlestick Control | $0.0214^{a}$ | $0.0170^{bc}$ | $-0.0013^{bc}$ | $-0.0008^{bc}$ |
| p value | 0.295 | <0.001 | <0.001 | 0.003 |

**Table 5.3.** Continued.

| C | Faeces Mass (g) | | | |
|---|---|---|---|---|
| | Mean Daily | | Rate of Change | |
| | J | A | J | A |
| Chloramphenicol & Gentamicin | 0.0085[a] | 0.0048[b] | -0.0004[ab] | -0.0003[c] |
| Chloramphenicol & Tetracycline | 0.0087[a] | 0.0070[a] | -0.0003[a] | -0.0004[c] |
| Gentamicin & Tetracycline | 0.0079[a] | 0.0075[a] | -0.0002[a] | -0.0001[ab] |
| Saline Control | 0.0063[a] | 0.0067[a] | -0.0003[a] | 0.0001[a] |
| Non-injected Control | 0.0075[a] | 0.0052[b] | -0.0006[b] | -0.0001[ab] |
| Needlestick Control | 0.0072[a] | 0.0049[b] | -0.0006[b] | -0.0002[bc] |
| p value | 0.101 | <0.001 | 0.004 | 0.002 |

Patterns of food intake were similar in July and August although slugs in July on average consumed more food than in August (Table 5.3B, Appendix 5.2).Within the July values there were no significant differences between groups in regression analysis of the mean food intake ($p=0.295$). In August there were significant differences in mean food intake between groups however there was no notable pattern to this ($p<0.001$). All groups exhibited an overall decline in food consumption as the trial progressed in both July and August (Table 5.3B) There were differences in the rate of change of the food intake in July between antibiotic treated slugs and those in the non-injected and needlestick controls ($p<0.001$) (Table 5.3B) however these differences were small and probably not of great significance with respect to the effects of antibiotic treatment upon slug feeding status. In August there was slight evidence of differences in the rate of change of food intake between groups ($p=0.003$) however, as mentioned earlier, no patterns were discernable so as to indicate a positive or negative effect of antibiotic treatment.

As one might expect, data on the mass of faeces produced by the slugs follows similar patterns to the food intake. Faeces production in August was generally lower and showed greater variability between treatment groups than in July (Appendix 5.3). For example regression analysis of the mean faeces production of each group revealed no significant differences between groups in July ($p=0.101$), whereas in August the tetracycline containing antibiotic treated groups and the saline control produced significantly more faeces than the non-injected control, needlestick control and chloramphenicol and gentamicin treated slugs ($p<0.001$) (Table 5.3C). In July all treatment groups exhibited a decline in the rate of faeces

production, presumably coupled to the decline in food intake, with the chloramphenicol and tetracycline, the gentamicin and tetracycline and the saline control groups exhibiting a significantly lower rate of reduction in faeces production than the non-injected and needlestick controls ($p=0.004$). In August, the saline control was the only group that showed a positive rate of change of faeces production and this was significantly greater than the chloramphenicol and gentamicin, the chloramphenicol and tetracycline and the needlestick control groups ($p=0.002$).

## 5.4 Discussion

The antibiotics used in the bioassays were chosen by testing bacteria extracted from the slug alimentary tract against a variety of common antibiotics. Of those tested, the greatest inhibition of bacterial growth was achieved by antibiotics of the tetracycline (tetracycline, chlortetracycline), chloramphenicol and aminoglycoside (gentamicin, streptomicin sulphate, kanamycin sulphate) classes. These antibiotics all target protein synthesis by inhibiting the ribosome at one of a number of sites. In contrast, bacteria from the slugs were resistant to β-lactam antibiotics (amoxicillin, ampicillin, penicillin, carbenicillin) which target cell wall synthesis, for which resistance is well documented (Zapun *et al.*, 2008; Kong *et al.*, 2010). Rifampicin is used to create aposymbiotic insects in some studies of insect symbioses, but it was not very effective in the concentration used here (Douglas, 1996; Akman Gunduz & Douglas, 2009).

The choice of antibiotics for such a bioassay is important as it is necessary to utilise drugs with activity against the bacteria thought to be important to the organism of interest. In this case, a lack of prior knowledge as to which bacteria may be important in *D. reticulatum* dictated a more general approach. The broad spectrum antibiotics, chloramphenicol, gentamicin and tetracycline were used in order to knock out as large a proportion of the slug-associated bacteria as possible, which would hopefully include a bacterial symbiont should it exist. If more was known about a bacterium of interest, it may, to an extent, be possible to select narrower range antibiotics to knock out certain groups of bacteria but in reality antibiotics are not very selective and therefore sensitivity testing remains the most practical method of determining suitable antibiotics for a bacterial target. An obstacle in the selection of antibiotics lies in the fact that in many intimate symbiotic associations, the bacterial symbiont cannot be cultured *in vitro* (Moran & Telang, 1998) therefore removing the possibility of conducting antibiotic susceptibility testing and limiting the researcher to broad spectrum antibiotics when studying the aposymbiotic organism. In the context of this study, if a beneficial symbiont does exist in the slug, and it is unculturable outside of the slug, it would not be possible to conclude from these tests whether or not this organism in particular is susceptible to the drugs administered in the bioassays. In response to this, in the latter assays pair-wise combinations of chloramphenicol, gentamicin and tetracycline were used in an attempt to broaden the range of bacteria that could be removed from the slugs. The use of these antibiotics is in line with other studies of bacterial symbioses in invertebrates (Prosser & Douglas, 1991; Heddi *et al.*, 1993; Walker *et al.*, 1999; Visotto *et al.*, 2009).

The antibiotics selected for these bioassays were successful in reducing both the culturable and unculturable bacteria in the gut of *D. reticulatum* (Figures 5.14, 5.15). A real-time quantitative PCR (qPCR) assay was used to estimate the quantity of DNA of the bacterial 16S rRNA gene present in the slug gut and this was compared to plate count data. Similar patterns were obtained for each set of data showing that the qPCR assay was suitable for quantifying bacterial DNA, however it was limited in its effectiveness as no direct comparison could be drawn between the qPCR data in picograms of bacterial DNA, and the plate count data in colony forming units per mg of slug tissue (cfu.mg tissue$^{-1}$). In order to achieve such a comparison, it would be necessary to have prior knowledge of the composition of the bacterial community and 16S rRNA gene copy numbers for each bacterium. It is important to point out that some bacterial species may possess multiple copies of the ribosomal operon, which may lead to an overestimation of bacterial abundance in qPCR (Farrelly *et al.*, 1995; Klappenbach *et al.*, 2000; Crosby & Criddle, 2003). This issue has been discussed several times in the literature, and as yet no studies offer a suitable solution (Crosby & Criddle, 2003; Castillo *et al.*, 2006). Instead it is generally advised that caution should be exercised when handling data and quoting bacterial abundance, but it is widely accepted that any errors due to variations in rRNA copy number outweigh the level of uncertainty often experienced in culture-based methods. Real-time PCR quantification of bacterial DNA stands as a useful alternative to plate counts in comparative studies of bacterial concentrations and provides considerable time savings, generating results within 12 hours.

Viable cell counts return results that are on the whole poorly reproducible, due to clumping of bacterial cells. Large errors can be introduced as a combined result of uneven distribution of cells in a suspension and because only very small samples are taken when producing spread plates. Even with the best technique, errors of ±90% are not uncommon for counts of 10,000-100,000 per ml (Collins *et al.*, 2004) and indeed in this study large variation was inherent in the plate count data (Figures 5.8, 5.14, 5.15). Plate counts are expressed as colony forming units (cfu), due to the likelihood that each colony counted may not have originated from a single cell and they are therefore not directly comparable to the number of bacterial cells present in the sample. Additionally, bacteria that are counted on a solid culture are limited to those that are able to grow freely in the selected media, culture conditions and culture time with unculturable organisms not being included. Fastidious or slow growing bacteria are also easily missed if colonies are not sufficiently large at the time of counting (Staley & Konopka, 1985; Amann *et al.*, 1995).

The bacteria in the slug gut appear to be largely a transient population. At the time of collection from the field, slugs regularly have a large number of bacteria in their gut but starvation for four days is sufficient to reduce this count by as much as 25-fold (Figures 5.4, 5.14). When purged slugs are fed a sterile antibiotic-free diet and kept in relatively clean conditions, this low background level of bacterial remains and does not return to field levels. The fact that a small background population of bacteria remains in the slug and is not simply flushed out when a sterile diet is consumed could indicate a symbiotic relationship and there is certainly scope for further research in this area.

No significant detrimental effect of antibiotic treatment on the fitness and survival of the slugs in these assays could be discerned and so this study is unable to present any evidence suggesting a bacterial symbiont is present in *D. reticulatum* and important for its normal healthy function. On the other hand, neither has this experiment disproven this hypothesis. The main notable observation was that often slugs treated with antibiotic had a lower mortality than control slugs, suggesting a suppression of pathogenic bacterial infections. Real-time quantitative PCR of bacterial 16S rDNA in the gut of slugs does not indicate an increased bacterial load in dead slugs, that may be indicative of the presence of a bacterial pathogen (Figures 5.16, 5.17). All efforts were made to prevent the growth and transmission of pathogens during bioassays, slugs were contained throughout the assays in individual sterile Petri dishes, moisture was provided by sterile distilled water, the agar-based diet was autoclaved during preparation and forceps used to handle the slugs and food were alcohol-sterilised. Ruling out introduction of a pathogen during the assay, it could be concluded that a pathogen was already on the slugs when they were collected from the field. One of the best documented pathogens of slugs is the parasitic nematode *Phasmarhabditis hermaphrodita* which infects slugs and introduces the bacterium *Moraxella osloensis* which proliferates and causes death of the slug (Wilson *et al.*, 1993; Wilson *et al.*, 1995; Tan & Grewal, 2001). It is possible that *P. hermaphrodita* infections already established in the field, or nematodes co-transported with the slugs could be accountable for some slug deaths in these bioassays.

One difficulty in bioassays where invertebrates are treated with antibiotics is in establishing whether any subsequent effects on fitness and survival of the organism are due to the disruption of symbioses or by toxicity of the drug. In this study however, it can be concluded that the antibiotics, in the concentrations used, were not detrimental to the normal health of the slug as untreated control slugs had similar or higher mortality than treated slugs.

It proved difficult to obtain steady measurements of slug mass throughout the experiment for a number of reasons. Firstly, the slugs studied in the July and August bioassays consumed on average 20% of their mass which caused a considerable variation in body mass. Futhermore, food intake in these slugs followed a similar pattern of regular fluctuation over a four-day cycle (Appendices 5.1, 5.2). This shows that the slugs did not feed with a regular daily pattern but consumed more food on the first two out of every four days despite food being offered every two days. Presumably variation in slug mass measurements is also affected by when the measurements are made in relation to when the slug has fed or defecated. This fluctuation in the food intake was first observed in the preliminary feeding assay (Figure 5.6) and in an attempt to overcome this, in later assays measurements of slug mass, faeces mass and food intake were made less frequently, every four days in the preliminary injection and every two days in the final July and August assays but the effect still remains. These bioassays used near-adult slugs which were perhaps not ideal where food intake and change in slug mass were the main variables monitored. In mature slugs, one would expect that natural growth and change in mass may be relatively static, which indeed seemed to be the case in this experiment. In the future, bioassays should focus on juvenile slugs, either hatched from eggs in the laboratory, or field-collected, as these slugs are more likely to demonstrate a regular pattern of growth in control groups and provide more valuable slug mass data. Walker (1997) was able to demonstrate clear growth in juvenile slugs (2.2- 4.3 mg) during a bioassay which studied the effect of proteinase inhibitors on slug growth. Walker ran bioassays for 36 days which enhanced the differences in growth rate between treatment groups. Also of note, is that Walker reported fluctuating food intake similar in pattern to that observed in the present study (Figures 5.6, 5.10, Appendix 5.2) suggesting that this may be a behavioural effect.

In this experiment it has not been possible to demonstrate the presence of a bacterial symbiont in *D. reticulatum* that is crucial to the normal survival of the slug. Given that the well characterised obligate symbioses, where the symbiont is contained within mycetocytes in the host, are all in insects, it is possible that such obligate symbioses do not occur in other invertebrates, especially those consuming broad generalist diets. For example, aphid (*Aphidoidea*), tsetse fly (*Glossinidae*) and sucking lice (*Anoplura*), all consume very narrow diets of plant phloem sap and vertebrate blood (Nogge, 1976; Sasaki-Fukatsu *et al.*, 2006). These two diets are deficient in essential amino acids and B vitamins respectively (Akman Gunduz & Douglas, 2009). In contrast, slugs are generalist feeders consuming plant foliage, seeds, and occasionally exhibiting cannibalistic behaviour (Port & Port, 1986) so there is not

likely to be a niche for a symbiont with a nutritional role. Where a symbiont is not required for nutrition they may perform other functions. Symbioses between marine invertebrates and chemoautotrophic bacteria appear widespread (Cavanaugh, 1994). Chemoautotrophs are capable of synthesising all their nutritional requirements from inorganic sources such as hydrogen sulfide, using carbon dioxide as a carbon source. In symbiosis, these bacteria enable their hosts to survive in reducing environments such as around deep sea hydrothermal vents, mangrove swamps, and sewage outflows, by detoxifying sulfide, a potent metabolic inhibitor (Cavanaugh, 1994). The host provides sulfide and oxygen to the symbiont which in turn provides carbohydrates for the host. The bivalve mollusc *Solemya velum*, is so dependent upon its symbiont that it has a severely reduced digestive system capable only of ingesting very small particles (Krueger *et al.*, 1992). When the antibiotic tetracycline is administered to the velvet bean caterpillar, *Anticarsia gemmatalis*, protease, serine-protease and lipase activity in the insect gut is reduced suggesting a microbial contribution to these metabolic functions (Visotto *et al.*, 2009). Although not crucial to normal survival and development, the gut bacteria of *A. gemmatalis* may confer resistance of the pest to plants that are rich in protease inhibitors such as soy bean (Visotto *et al.*, 2009).

In conclusion, *D. reticulatum* has a largely transient bacterial population within its gut which can be reduced up to 25-fold by purging for four days. Treatment with the antibiotics chloramphenicol, gentamicin and tetracycline can further reduce gut bacterial load through administration by either feeding or injection, however ingestion provides the greatest reduction due to better targeting of the drug to the gut region. Antibiotic treatment is not detrimental to slug fitness and survival so there is no immediate evidence of the presence of a potential mutualistic symbiont in *D. reticulatum*. Slugs treated with antibiotic did in fact display lower mortality than control slugs suggesting the possible presence of a bacterial pathogen which may warrant future investigation. In future studies, continued rigorous bioassays, using feeding as the administration method should be continued to try to elucidate an important relationship between the slug and its gut bacteria. Should such mutualistic bacterial symbionts be found in *D. reticulatum*, a role for the bacterium could be elucidated by studying the changes in microbial communities of the gut in response to antibiotic treatment. By coupling molecular microbial analyses with bioassays, one can try to understand the interactions between the symbiont and the host slug which may highlight potential means for targeting this relationship in biocontrol.

# Chapter 6

# General Discussion

## 6.1 Summary of the Study

Despite being well-studied since the early 20th century and the release of several different commercial controls, slugs continue to pose a threat to UK agriculture. There are several pest species of which the grey field slug, *Deroceras reticulatum*, is the most important due to its ide distribution and because it is the only slug that remains active at very low temperatures putting crops at year-round risk of damage (Port & Port, 1986). Many different crops can be damaged by slugs, the most common being wheat, oilseed rape and potatoes, vegetables and soft fruits. Slugs have also been implicated as potential vectors in the transmission of plant pathogens such as downy mildew (Wester *et al.*, 1964), bacterial rots of carrots and potatoes (Dawkins *et al.*, 1985; Dawkins *et al.*, 1986), and vertebrate parasites such as the sheep lungworm (Rose, 1960). As the climate trends towards milder, wetter winters, and warmer, drier summers, it is unclear how slug populations might respond, but it is likely that the geography of the problem may shift within the UK to more northerly latitudes (Willis *et al.*, 2006). A general move towards minimum tillage in agriculture and increased autumn sowing neglects what were traditionally valuable cultural controls for slug pests.

The slug parasitic nematode *Phasmarhabditis hermaphrodita*, marketed as the products Nemaslug® and Nemaslug® Xtra (Becker Underwood, UK), is the only novel slug control to come to market in the last 15 years. In this time it has failed to experience significant use due primarily to its high cost, up to 10 times the cost of chemical molluscicides, which limit it primarily to use in high value crops such as potatoes, fruit, vegetables and flowers (Blake, 2009). Slug pellets containing one of three active chemicals (Metaldehyde, methiocarb, ferric phosphate) can achieve effective slug control, but their use can be difficult, requiring the optimisation of a number of factors including weather, the time of application in relation to crop stage, time of year and slug activity. Furthermore, pellets broadcast on the soil surface are ineffective at controlling slugs that are not surface-active, and non-target effects of chemicals toward beneficial organisms such as earthworms and carabid beetles (slug predators) are undesirable.

Chemical controls are under greater scrutiny as the European Union implements the EU Pesticide Directive 91/414. Under this legislation thiodicarb was withdrawn from use in 2008, and methiocarb is due for review again by September 2011 and as such its future is uncertain due to its toxicity in line with maximum residue levels (MRLs) in food and water for human consumption. Metaldehyde, which accounts for 80% of molluscicides

applications in the UK, has much lower toxicity but has itself been the focus of very recent concern over its discovery in water courses at levels exceeding those set down in drinking water standards by the EU. This problem is exacerbated because metaldehyde cannot be removed from water in conventional treatment processes. A concerted effort in recent stewardship initiatives has seen a reduction in metaldehyde entering water courses in 2009 but further improvement must be achieved. A ban on metaldehyde could result in a considerable increase in the cost of slug control because the alternative chemicals are at least double the cost of metaldehyde pellets. Considering that metaldehyde currently accounts for over 80% of molluscicide applications in the UK, this represents a significant increase in slug control costs which could impact upon profit margins and tempt some farmers to take risks that may result in serious yield losses in the long term.

To this end, there is significant need for innovation in the field of slug control so that farmers have sufficient options at their disposal to allow continued and effective control of slugs and protection of the long term sustainability of UK farming. This study set out to investigate the bacteria associated with the digestive tract of *D. reticulatum*. By characterising the slug gut microflora and assessing the importance of these bacteria for optimum slug fitness and survival this study attempted to identify potential symbionts which may be suitable targets for a novel biological slug control product.

Prior to this study, little attention had been paid to the bacteria associated with gastropod molluscs (Elliott, 1970; Watkins & Simkiss, 1990; Walker, 1997) and little was known about the types and diversity of bacteria present. In the late 1990s Anthony Walker conducted some PhD research at IACR Long Ashton, Bristol, into the possibility of controlling slugs by inhibiting slug digestive enzymes with particular focus on proteolysis (Walker *et al.*, 1996; Walker, 1997; Walker *et al.*, 1998). He briefly studied the contribution that protease-secreting bacteria in the digestive tract of *D. reticulatum* made to digestion in their host, concluding that although a large population of these bacteria existed in the digestive tract, they are not required for protein digestion or the normal survival and fitness of *D. reticulatum* (Walker *et al.*, 1999). In further work, Walker *et al.*(1999) produced transgenic Arabidopsis thaliana which expressed OC-IΔ86, an inhibitor of the major cysteine proteinase in the slug gut. When this transgenic leaf tissue was fed to juvenile slugs, a 31% reduction in growth was observed as well as an increase in mortality, demonstrating the potential role that transgenic plants could take in an integrated pest management system for slugs (Walker *et al.*, 1999).

Comparing the sensitivity to antibiotics of bacteria isolated from the slug gut in the present study, with similar studies by Walker (1997) and Watkins and Simkiss (1990) (Snail, *Helix aspersa*), revealed results in agreement with both previous studies suggesting a bacterial population of similar composition (Chapter 5, Section 5.3.1). Bacteria isolated from the slug gut were identified using API-20E test strips (BioMérieux) and 16S rRNA gene sequencing. The bacteria associated with the digestive tract of *D. reticulatum* belonged to genera that have been previously identified in soil, water and other environmental samples (Chapter 3). All were either members of the phyla *Proteobacteria*, *Firmicutes* or *Bacteroidetes*. Symbiotic bacteria in other invertebrates are commonly *Proteobacteria* of the *Gamma-* and *Beta-proteobacteria* classes (Moran & Telang, 1998).

Several of the bacterial genera isolated from the gut of *D. reticulatum* are of documented medical importance. The five species of *Pseudomonas* have all been recorded in medical cases as have some *Serratia* species (Saito *et al.*, 1989; Hejazi & Falkiner, 1997; Kerr & Snelling, 2009). These are all reported as opportunistic infections but non-the-less pose a risk to human health given the close proximity of slugs and produce destined for human consumption and it may be of epidemiological importance to be aware of potential reservoirs of human pathogens. Other bacteria identified in this study can transmit antimicrobial agents that elicit biological control of some plant pathogens. *Rahnella* species may be applicable in the biological control of bacterial pathogens of fruit trees, vines and tomatoes. While these plants are not normally likely to harbour large numbers of slugs, *Pseudomonas* species producing the antibiotic 2,4-diacetyl-phloroglucinol (DAPG) offer potential for biocontrol of wheat pathogens (Cook, 2003; Siasou *et al.*, 2009). The fungal root disease "take-all" is responsible for large global yield losses in wheat but *Pseudomonas* have been shown to reduce the severity of the disease by producing DAPG. Siasou et al. (2009) demonstrated that DAPG production was only observed when the causative agent *Gaeumannomyces graminis var. tritici* (Ggt) was present in the soil and observed an increase in magnitude of production when both Ggt and mutualistic arbuscular mycorrhizal fungi (AMF) were present in the soil. These examples of the biocontrol potential of slug associated bacterial species should not be readily overlooked, especially with regards to any role that slugs may play in the transmission of these bacteria in the field. This highlights the importance of fully understanding the interactions of microbial communities in the environment and of being aware that manipulating bacteria to control one pest may have the undesired affect of exacerbating a different crop protection issue.

Using ribosomal intergenic spacer analysis (RISA) it has been possible to compare bacterial communities in the digestive tract of *D. reticulatum* from different locations (Chapter 4). Biological control offers many benefits to agriculture, but for a control to be commercially viable it must be universally applicable. To this end, any bacterial target for biocontrol of the slug must have a broad geographical distribution. RISA demonstrated that slugs from different locations contained diverse bacterial communities. These communities appeared to be largely transient and were greatly reduced through starvation and feeding a sterile diet (Chapter 5, Section 5.3.2). Slugs from the same location shared greater similarity with each other than they did to slugs from different locations, but no highly conserved RISA band sizes could be identified (Chapter 4, Section 4.3.2). It is likely that the bacteria living in slugs resembles those in the soil at particular locations, and as soil nutrient content, chemical pollutants and plants can all alter soil microbial communities, these findings are perhaps unsurprising (Girvan *et al.*, 2003).

Field-collected juvenile slugs were reared for 28 days in sterile conditions but real-time quantitative PCR (qPCR) showed that bacterial DNA present in the slug gut was not significantly reduced (Chapter 4, Section 4.3.3). Although every effort was made to keep conditions as sterile as possible, complete sterility was neither achieved nor expected. Rather, it was intended to reduce bacterial load of the gut to minimum levels with the hypothesis that transient bacteria would be purged from the gut and only symbiotic organisms would remain. In future work, it would be extremely valuable to research a means of producing slug eggs free of exogenous bacteria which could then be reared into gnotobiotic slugs to assess the fitness and survival of the slug in total absence of the bacteria. The majority of obligate symbionts studied are vertically transmitted down the maternal line (Bright & Bulgheresi, 2010) and one would hypothesise that if *D. reticulatum* possessed an obligate symbiont, it ought to be detectable in slug eggs, and similarly if these symbionts could be removed from the egg, any effect on slug development may become apparent.

Bioassays in which antibiotics were administered to slugs (Chapter 5) did not provide any direct evidence of the presence of a bacterial symbiont in *D. reticulatum*, but neither has this hypothesis been disproven. If symbiotic bacteria do exist in and are important for the normal fitness and survival of *D. reticulatum*, it is unlikely that they have been disrupted in bioassays in this study. However, considering that slugs consume a comparatively broad diet compared to other organisms with known obligate symbioses these findings may be justified. The only detrimental effects of antibiotic treatment were observed when slugs were fed a

high dose of antibiotic ( $\geq$ 5 mg.g diet$^{-1}$) (Chapter 5, Section 5.3.2). However, in this instance the weight loss exhibited by the slugs was shown to be due to reduced food intake as a result of reduced palatability of the diet. Antibiotic administration by feeding at a dose of 1 mg.g diet$^{-1}$ was able to achieve a greater overall reduction in the culturable bacterial load of the gut as measured with plate counts, than injection of the antibiotic. This is presumably a result of the antibiotic more easily accessing bacteria in the gut when antibiotics are ingested as opposed to injected into the haemocoel which require diffusion or transport into the gut reducing efficacy. Higher rates of mortality in control slugs than antibiotic treated slugs were often recorded which suggests the presence of a bacterial pathogen. *Moraxella osloensis*, a symbiont of the slug-parasitic nematode *Phasmarhabditis hermaphrodita* is the only documented slug pathogen (Tan & Grewal, 2001). The presence of *M. osloensis* was not investigated in bioassays but neither was it identified in any slugs during 16S rDNA sequencing or cloning and sequencing of 16S-23S RISA bands.

Slugs used in bioassays were all near-adult in size and development, and thus perhaps not the ideal choice for use in bioassays where natural growth and change in mass may be quite static. Future research may be improved by using small juvenile slugs in bioassays which are more likely to demonstrate an overall growth for control groups. This does however limit the times of year when bioassays may be conducted depending on the availability of juveniles. From experience, juvenile slugs are most often found in large numbers in January to March, but it can be difficult to locate juvenile slugs in early spring as cold temperatures limit their activity and they take refuge in the soil.

This study has focussed mostly on the digestive tract of *D. reticulatum*, including the crop, oesophagus, intestine and digestive gland. The decision to study this part of the slug in particular was largely because previous research had looked at this area, and Walker (1997) reported large numbers of bacteria associated with these organs. Walker (1997) suggested that as these areas were involved in digestion, they may be the most likely location of microbial agents that afforded nutritional benefits to the slug. There may however be some merit in studying parts of the slug other than the digestive system. Indeed bacteria have been studied in the mucus of *D. reticulatum* with regards to its ability to harbour bacteria of importance in plant pathology (Dawkins *et al.*, 1985; Dawkins *et al.*, 1986) and it would be interesting to understand more about the bacteria on the slug surface.

In the laboratory, administering antibiotics to insects that possess obligate symbionts causes a reduced growth rate and inhibits reproduction, but the lifespan of the insect is only moderately reduced (Douglas, 1996). This would limit a symbiont-based biocontrol to only reducing pest populations over a prolonged period of time rather than causing immediate death of an insect, which may only suit certain pest control situations. If such a control was possible in *D. reticulatum*, early appetite suppression would be a requirement, similar to the action of the parasitic nematode *P. hermaphrodita*. If on the other hand, removal of a symbiont only caused a moderate reduction in life span with little effect on feeding and reproductive activity, the product would not be suitable for either reducing slug damage in crops, or lowering slug populations in a field in the long term.

Even though this study does not provide direct evidence that an obligate symbiont is present in *D. reticulatum* and important for its fitness and survival, some alternative means of biological control can be suggested. Further and more detailed studies of the bacterial community composition in the field slug with particular emphasis on micro-manipulation of individual species may identify an opportunistic bacterium whose growth under normal conditions is inhibited by more dominant bacteria. Biological control may be elicited by manipulating more dominant phylotypes such that this opportunistic fraction is able to proliferate to harmful levels in the slug. Furthermore it would be desirable to study in detail the interactions between bacteria within the slug and between the slug and bacteria to try to elucidate any important pathways that may be exploitable.

It is also important to consider the ecological niche of the slug when thinking about slug control. Slugs may transmit bacterial biological control agents, whose importance in plant disease control may not yet be recognised (El-Hendawy *et al.*, 2005; Farzaneh *et al.*, 2007; Siasou *et al.*, 2009). Any novel slug control that exploits slug-associated bacteria must be careful not to disrupt the potential benefits afforded by these biocontrol bacteria without first fully understanding the extent to which they are effective in the field. In contrast, *D. reticulatum* also harbours bacteria that are of clinical importance to humans and manipulation of the slug gut bacteria must not allow, through suppression of competition by other bacteria, the proliferation of human pathogens such that the slug presents a hazard to humans by transmitting pathogens to the food chain.

In any host:symbiont system, it is critical to study the mechanism of interaction between symbiont and host in order to fully understand the function of the symbiont. In gaining

greater understanding of the pathways of interaction, it may be possible to effect biocontrol by manipulating aspects of these pathways rather than by directly targeting the symbiont. Similarly, symbiont transmission could be a target for biocontrol. The status of a bacterium as beneficial, harmless or pathogenic can be dependent on environmental factors, therefore it is essential to understand the underlying dynamics of the microflora of the slug and the mechanisms of interaction between both parties. For example, it may be possible to exploit chemical cues that cause a shift in the balance of microorganisms in the slug such that other opportunistic and previously inhibited bacteria may proliferate to pathogenic levels. The *Bacillus thuringiensis* (bt) toxin which is used as an insecticide and can also be expressed by transgenic crops, is only active against caterpillars of the gypsy moth in the presence of *Enterobacter* sp., part of the normal gut flora (Broderick *et al.*, 2006). Both the toxin and bacteria are harmless on their own, but work synergistically as the bt toxin disrupts the gut epithelium allowing the bacteria to enter the tissues where they cause septicaemia and death. This is an example of a completely harmless commensal symbiont which can, under the right circumstances, become harmful to its host, further highlighting the importance of understanding the underlying mechanisms of symbiosis.

At a time where the future of current chemical controls is threatened by pesticide legislation, it is important that research focuses on ways in which to make remaining controls (namely nematodes and ferric phosphate-based products) more effective. Reformulation may improve the longevity of bait pellets, while advancements in manufacturing may help to reduce the cost of product and improvements in the delivery method may ensure that slugs can encounter the product more readily. At the same time, continued improvements in metaldehyde formulations could improve its efficacy such that applied rates may be reduced, further improving the prospect that this chemical control may avoid regulatory action. There is disparity between the perceived slug threat and actual damage sustained by crops. In a detailed study sponsored by Defra and HGCA which paid particular attention to the risk-assessment and forecasting of slug damage, farmers were encouraged to routinely trap slugs and to apply pellets in response to increased activity (Glen *et al.*, 2006). Studies such as this are crucial to the effective and targeted use of molluscicides especially at such a time when farmers are forced to think twice about metaldehyde application so as to use a field's pellet allowance wisely (Metaldehyde Stewardship Group, 2010). It is important that future research monitors how the slug threat may shift in response to climate change (Willis *et al.*, 2006) and alter the advice to farmers accordingly so that farming practice may stay in control of the slug threat.

## 6.2 Review of Methods Used

This project demonstrated a number of different techniques in the characterisation and ecological study of microbial communities present in environmental samples and the invertebrate gut. These methods are each critically reviewed in detail in the corresponding chapters of this thesis, but a brief summary here follows.

*Identification of bacteria by phenotypical and genotypical methods*

Bacterial isolates cultured from gut extracts from *D. reticulatum* were identified using the API identification system (BioMérieux) (Chapter 3). Of the 24 isolates tested using the API 20E test strip, less than half returned a profile match with a bacterium in the API database, and only five of these reported greater than 80% ID. API mostly was only able to identify bacteria to the genus level. This highlights one of the main problems with the API system when used in this context. Because the database primarily contains bacteria of medical importance, or very common environmental bacteria, the identification of rarer environmental bacteria is hindered. Furthermore, there are a number of different test strips for identification of different groups of bacteria which requires that the researcher knows a little about the organism beforehand to allow selection of the correct test strip. In this study the API 20E strip, for gram negative enteric bacteria was selected as the most appropriate for the majority of the bacterial types found in soil or the invertebrate gut, but it is possible that some of the unidentifiable profiles may have been produced by bacteria that were outside of this group. These factors mean that API is not an ideal choice for a large-scale study of bacteria especially when the query organisms may belong to number of different groups. Due to the subjectivity in interpretation of the colour changes in the test strip, the user may not feel that the profile is reliable. Better control over the number of bacterial cells used in the tests would improve the tests. Some of the uncertainty introduced by variation in cell numbers, incubation time and subjectivity of interpretation could be alleviated by running the tests in triplicate, but this would have considerable cost and time implications. The Biolog system (Biolog Inc. Hayward, CA, USA) was suggested as an alternative to API for phenotypical identification. It is suitable for identification of a wide range of clinical and environmental bacteria owing to a larger database, and can be automated which would facilitate replication of tests and high-throughput studies. Successful use of phenotypical identification methods require the organism of interest to be culturable, and so is not very applicable to the study of the vast majority of soil bacteria and obligate symbiotic bacteria (Torsvik *et al.*, 1990; Moran & Telang, 1998).

Molecular identification of the bacteria isolated from *D. reticulatum* in this study offered considerable time savings and provided identifications that were more reliable and could be trusted more than those from API. Although this study only sequenced the 16S rRNA genes from cultured bacteria, the technique could be combined with a 16S rDNA clone library in order to obtain the sequences of unculturable bacteria in a sample. This would however require the sequencing of multiple clones, and could be costly while providing no guarantee of an exhaustive identification of all species present. In many cases, it is possible to achieve an identification to species level and sometimes strain level with 16S rDNA sequencing, which represents greater resolution than was achieved by API in this study. The vast amount of bacterial 16S rDNA sequence data deposited in databases such as Genbank make this a particularly attractive approach for the identification of rare or little-studied bacteria, allowing for the phylogenetic alignment of sequences and inference of clone phylogeny even if an exactly matching sequence has not been previously deposited.

*Ribosomal intergenic spacer analysis and Agilent 2100 Bioanalyzer*

RISA was selected as a means of comparing bacterial communities in *D. reticulatum* from different locations because of its high resolution and ability to produce microbial fingerprints cheaply, simply and quickly. It was possible to obtain a fingerprint from a slug gut extract including DNA extraction in a single working day. One of the main advantages of RISA compared to T-RFLP (Chapter 4, Section 4.1) is the ability to excise RISA bands from a gel for cloning and sequencing. There is however a drawback to this as there is a relatively small amount of 16S-23S ITS sequence data available in Genbank in comparison to 16S rDNA sequences. This makes identification of bacteria more difficult from ITS sequences as was demonstrated in this study (Section 4.3.3). However, with the database continually increasing in size, this problem may be overcome at a later date.

All molecular fingerprinting methods are subject to biases from sample preparation right through to final fingerprint interpretation (Section 4.4). Those pertaining to genome size, genome number and ribosomal gene copy numbers present the greatest challenges to microbial ecologists, demanding a certain level of compromise to be adopted when interpreting microbial community data (Farrelly *et al.*, 1995). Multiple copies of the ribosomal operon can result in a bacterial species abundance being overestimated while interoperonic heterogenetity may result in multiple bands from a single species and overestimation of richness. In addition to these factors, differences in genome length between species may result in their 16S genes being present in different molar concentrations

within a standard quantity of template DNA. It is thought that multiple copies of the ribosomal operon allow a bacterium to be more adaptable to changing environmental conditions, the increased capacity for protein synthesis allowing faster growth. It is these bacteria however that are also fast-growing and over-estimated in culture-based techniques (Crosby & Criddle, 2003). Considering these assumptions that must be made when inferring microbial diversity from molecular fingerprints, these methods are best suited to comparative studies, measuring the changes in a population in response to a perturbation. It is still widely agreed that molecular fingerprinting offers a great improvement on culture-dependent techniques.

This project demonstrated the novel application of ribosomal intergenic spacer analysis (RISA) in the study of bacterial communities present in *D. reticulatum* which included using the Agilent 2100 Bioanalyzer, an instrument that does not appear to have been used for this application in the past (Chapter 4). Using the Bioanalyzer presents a viable and cost-effective alternative to costly sequencing machines while circumventing the technical difficulties in setting up, running and staining large polyacrylamide sequencing gels. However using this instrument was found to compromise the data analysis and the inference of diversity due to lower sizing precision than achieved by DNA sequencing machines. This would not be a problem for routine sizing of products from conventional PCR or molecular protocols with few fragments and where sizing to within 20 bp is acceptable. However for molecular fingerprint analysis this creates some technical problems when comparing community profiles as a difference of only a few base pairs in size calling could result in two identical ribotypes being treated as separate OTUs when calculating diversity. Binning of fingerprint data can help to alleviate some of these problems but cannot remove all doubt from the analysis. Despite these drawbacks, the Bioanalyzer represents a very valuable alternative to agarose gel electrophoresis in the nucleic acid fragment analysis, and may also be used for proteins and for cell counting by using different chips.

## 6.3 Application of Bacteriophage in Slug Control

An important question still to answer is how a bacterial symbiont may be used for the basis of a biological slug control, and how selective removal of such a symbiont may be achieved in the field. Large-scale use of conventional antibiotics in the environment would be unacceptable and potentially destructive towards natural microbial balance as well as the

more obvious concerns regarding antibiotic resistance. Effects would likely be serious, complex and unpredictable. Alternatively, the naturally occurring viruses of bacteria, bacteriophages, possess a number of characteristics that lend themselves to function as a biocontrol agent.

Bacteriophages (now commonly shortened to phage) are the intracellular viruses of bacteria and are found throughout the world wherever their host bacteria thrive. At an estimated $10^{31}$ phage globally (Wommack & Colwell, 2000), they are the most abundant biological entities on earth. Bacteriophages were first discovered almost a century ago in work conducted independently by Frederick Twort and Felix d'Herelle in 1915-17, yet their properties have yet to be fully exploited. Phage therapy is probably the most widely recognised potential application of bacteriophage, in which administered phage infect pathogenic bacteria within the patient, multiply within the host cells and following cell lysis phage progeny are released and infect and kill neighbouring bacterial cells (Pirisi, 2000; Skurnik & Strauch, 2006). The naturally occurring antibacterial properties of bacteriophage could however find application in more broad-ranging fields wherever bacteria pose a problem. This may include agriculture where antibiotics are currently used in environmental prophylaxis to prevent food-borne pathogens such as *E.coli* O157:H7 in cattle or *Salmonella* in eggs from entering the food chain (Stone, 2002b) or as described here, in the direct or indirect biological control of pests organisms.

Early phage therapy was hailed a miracle cure and received much attention in the 1920s and 1930s but this was to be short-lived in the West at least, being widely rejected by Western doctors by the end of the 1940s (Bradbury, 2004). This could be attributed to a lack of knowledge about phage biology and phage:host systems and the discovery of chemical antibiotics eventually eclipsed phage research (Fischetti *et al.*, 2006). Early phage researchers somewhat underestimated the degree of specificity exhibited by phage for their host, typically to the strain level, which meant that patients were often treated with phage incapable of infecting the causal bacteria. In some cases, phage preparations were administered to treat infections that were not caused by bacteria (Bradbury, 2004). Long after its abandonment by the western world, phage therapy research continued in the Soviet Union with particular success treating infections of external wounds and a number of marketed formulations saw general use particularly so in the military (Stone, 2002a). Now, with the increasing emergence of bacterial resistance to antibiotic drugs, attention is again turning to phage technology (Fischetti *et al.*, 2006). Together with a greater understanding of

phage genetics and biology and the prospect of genetically engineering phage, they are likely to offer real advances in the control of bacteria (Levin & Bull, 2004).

As per antibiotics, bacterial resistance to phage is also a problem, and just as bacteria can acquire mutations that confer resistance to antibiotics, mutations in important receptors on the cell surface can render them refractory to infection by bacteriophage (Comeau & Krisch, 2005). Unlike antibiotics however, phage are capable of evolving alongside the bacteria and can likewise acquire mutations that enable them to overcome newly acquired bacterial resistance (Pirisi, 2000). It is after all, detrimental to an obligate parasite to cease to be able to infect its host, and bacterial resistance becomes a selection factor for phage evolution (Levin & Bull, 2004). Despite this, large-scale application of phage could, as with antibiotics, result in the emergence of bacterial strains that are resistant to multiple phage strains limited in further evolution by the number of potential receptors on the surface of the bacterial cell. The arrival at this point can be prolonged by utilising phage cocktails whereby a mixture of different phage with differing receptor molecules are co-administered, thus requiring two or more independent mutations in the bacterial receptor molecules for the bacteria to evade infection (Levin & Bull, 2004).

A key advantage of bacteriophage is that they exhibit very narrow host ranges, often being infective at the species or strain level. This however, could be a disadvantage for phage therapy where phage specificity would demand a vast library of phage from which a suitable therapeutic agent could be selected, based on pathogen identification. In biocontrol, this specificity would prevent some of the non-target effects observed with chemical molluscicides, which should improve the environmental acceptability of such a control making it compatible with integrated pest management systems.

As with clinical products, there would be obvious licensing constraints involved in getting any new clinical therapeutic to market and the large number of phage strains and the complexities of their specific relationships with their host bacteria will make this a particularly difficult process. Each phage would have to be characterised in detail so that all aspects of its biology, genomics, and interactions with its host were understood. Presenting a particular obstacle is the phenomenon of phage conversion whereby phage can carry lethal genes or virulence factors giving them the power to turn a non-pathogen into a pathogen, clearly an undesirable asset (Alisky *et al.*, 1998; Garcia-Aljaro *et al.*, 2006). Detailed

genomic analysis is essential to ensure that phage are not carrying any such virulence factors which could have serious implications should they enter the food chain through crops.

The life cycle of a phage should also be determined. Strictly lytic phages, which kill the bacterial cell by multiplying within the cell and lysing the cell membrane, would potentially present the lowest risk of gene transfer and may be best for phage therapy or biocontrol. These phage may confer self-propagating properties to a slug control whereby virus progeny released from lysed bacterial cells can continue to infect other bacteria in the vicinity such that a small initial dose of phage may be suitable. A lytic phage-based biocontrol may also be transmissible if virus particles are excreted in the faeces or sloughed off in mucus which in turn may be encountered by other slugs that would be subsequently infected themselves. This is a unique characteristic that is not possible with chemical slug controls.

Lysogenic phage are capable of integrating their genetic material into the host genome where it can be carried asymptomatically as a prophage until such a point that environmental conditions stimulate the phage to enter into a lytic cycle resulting in lysis of the host cell and release of virus particles. Lysogenic phages do have a potential application whereby they could be used to deliver lethal genes to a bacterium resulting in death of the bacterial cell (Fischetti *et al.*, 2006). This non-lytic killing of bacteria would be particularly desirable for human therapy as it would circumvent the risk of toxic shock in the aftermath of cell lysis (Fischetti *et al.*, 2006), and in slug biocontrol it may prevent long-term persistence of introduced phage in the environment, and subsequent problems associated with this.

Despite the promising prospects for phage therapy, there is still a general reluctance to develop phage technology in the west. This can be contributed in some part to the issue of obtaining clear intellectual property rights (Skurnik *et al.*, 2007). Since the technique of phage therapy has been around for many years, it itself cannot be patented and it may be difficult to patent individual phage due to the vast number in existence and the likelihood of there being several different phages with similar host ranges. Without patents in place, large companies have no incentive to enter lengthy and costly clinical trials (Clark & March, 2006). This said however, phage research is expanding in the west and in recent years a number of new companies have set up (Pirisi, 2000; Bradbury, 2004). There are calls for the international scientific community to share their knowledge on phage, particularly with the idea of western investment in already established phage research in eastern Europe (Alisky *et al.*, 1998). Obtaining regulatory approval may also be a problem for phage research but it is

hoped that the natural abundance of phage will stand in their favour especially given that they are natural commensals of the human body (Breitbart *et al.*, 2003).

In summary a phage-based slug control could bring a number of advantages over current chemical controls. Occurring naturally in the field environment, and because of their very narrow host range, phage would be environmentally acceptable and compatible with integrated pest control systems. Non-target effects may be small given the specificity of phage to the strain or species level. In addition to this, a phage bio-control has the potential to be self-propagating as the phage undergo natural growth and replication within host bacteria in the slug. A slug whose gut microflora is infected with phage may exhibit some infectivity as phages are excreted in faeces or carried in the body mucus, presenting the possibility of transmission from slug to slug and increasing the number of ways in which a slug may come into contact with the control. Bacteriophages persist wherever the host bacterium lives and although it is not likely that phage numbers will be sustained in the soil at sufficiently high levels so as to remove the need for seasonal applications, they may remain in the soil at low levels and provide protection for a longer period than is currently achieved with slug pellets. Particular attention would be required when developing a delivery method for such a control as the poor ability of phage to diffuse through the heterogeneous matrix of the soil (Goodridge, 2004) is likely to be a limiting factor, especially when slugs are deep in the soil in drier weather. However, large-scale phage production would be relatively simple and cheap so it would not be difficult to apply large quantities of phage to the soil to ensure inundation of the upper soil layers with phage. Clearly thorough testing would be required to assess the efficacy, non-target effects, and delivery method before a treatment could reach commercial production.

## 6.4 Conclusion

In conclusion, this study has investigated the *D. reticulatum* gut microflora and asked whether any of these bacteria are beneficial to the slug and important for normal survival and function. Bacteria cultured from *D. reticulatum* are from common soil genera, many of which are of have been of medical importance, or of potential value in biocontrol of plant pathogens. A high diversity and geographical variation within slug gut microbial communities was demonstrated by molecular fingerprinting, but no highly conserved bacterial ribotypes, that may be indicative of a conserved bacterial symbiont, could be identified. Administration of antibiotics to slugs in bioassays succeeded in reducing the

bacterial load of the slug gut, but were not able to demonstrate any subsequent effects on slug fitness and survival, and so prove inconclusive in the search for a bacterial symbiont in *D. reticulatum*. The following points should be considered in future research in this field;

Further bioassays should use younger slugs and administration of antibiotic by feeding. An attempt to monitor changes in the diversity and composition of the gut microflora when antibiotics are administered may also allow any physiological or developmental effects on the slug to be coupled to changes in the microbial associations.

If beneficial bacteria can be identified in *D. reticulatum*, elucidating a role for the symbiont and mechanism of interaction with the slug may allow control by means other than simply removing the symbiont.

An in-depth analysis of unculturable bacteria in *D. reticulatum* using 16S rDNA sequencing would provide a comprehensive picture of the slug microflora constituents allowing for the identification and phylogenetic study of putative symbionts. This could be expanded to include bacteria from different locations in the slug.

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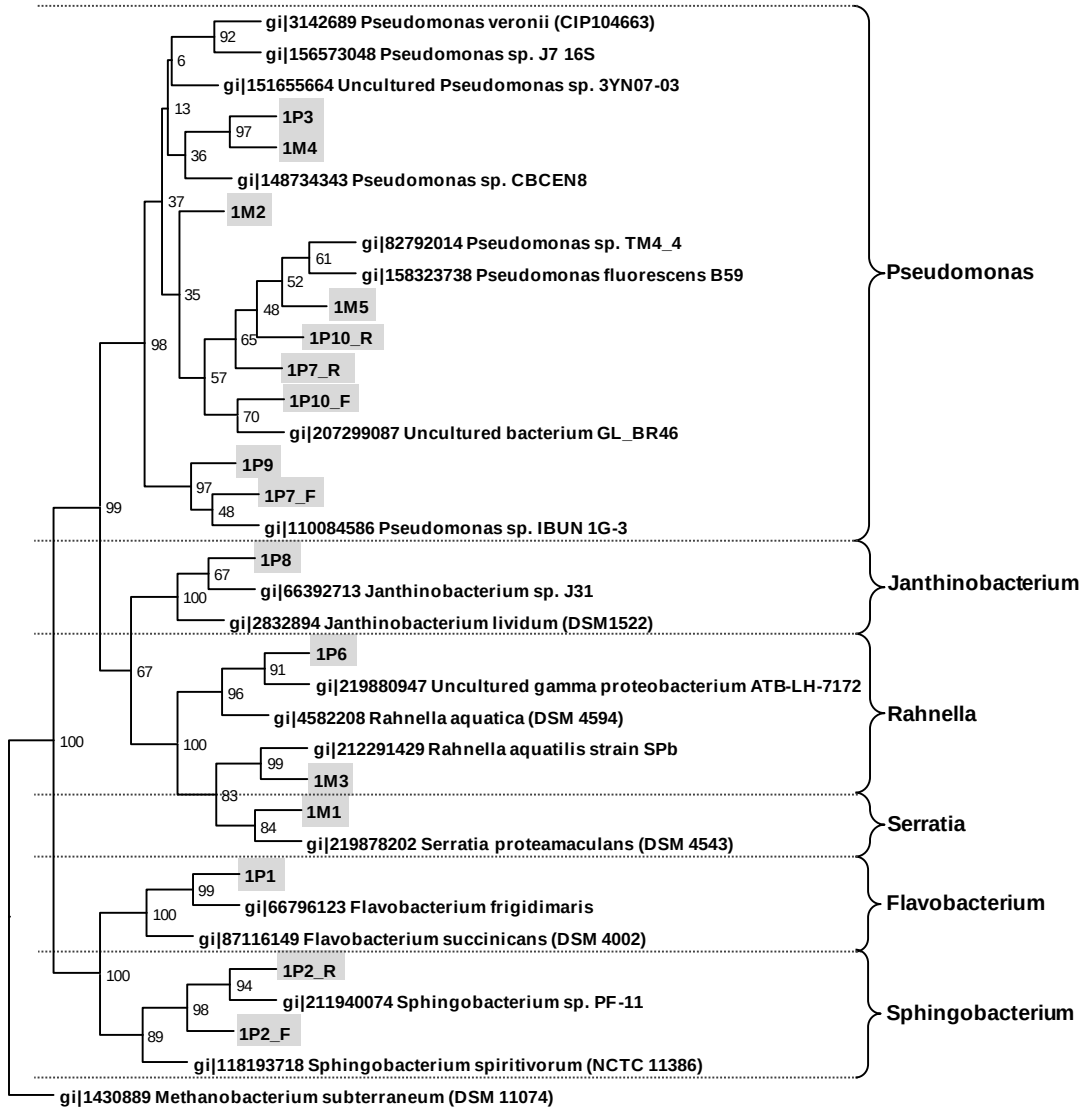
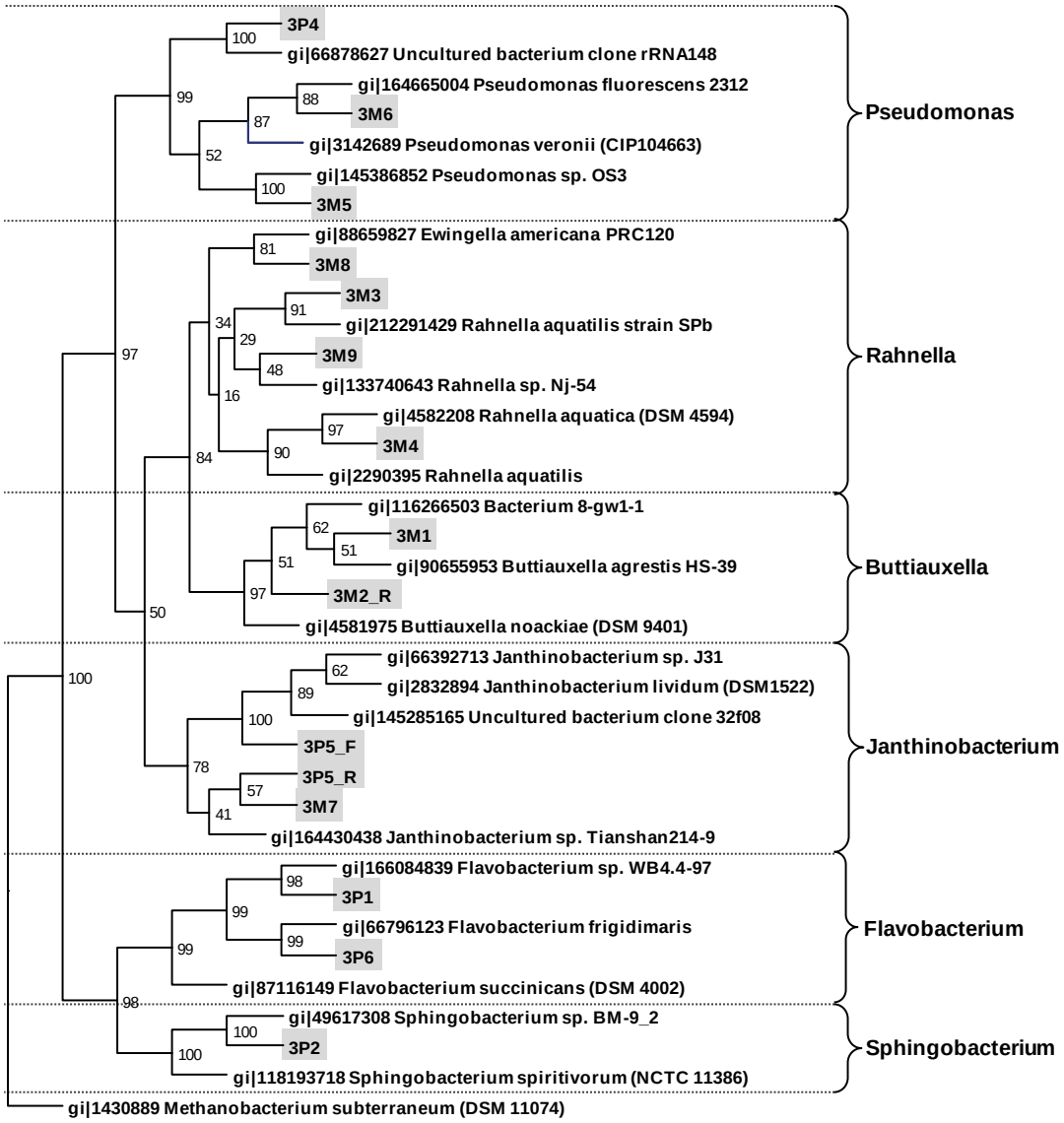
# Appendices

## Appendix 3.1



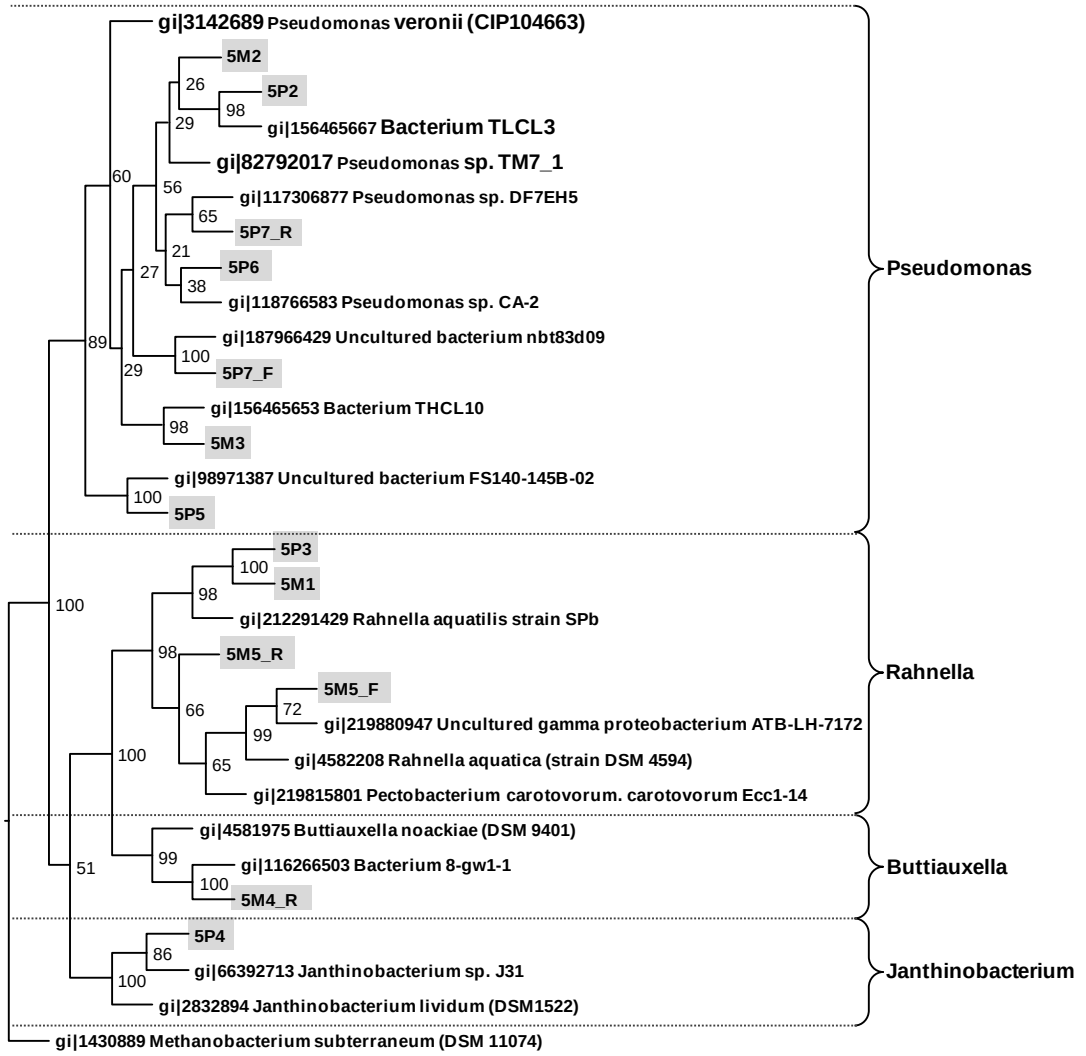

Phylogenetic tree by the maximum likelihood method of the partial 16S rRNA gene sequences of bacterial isolates from slug MRW001 and their closest matches from the Genbank Nucleotide Database. The numbers at the nodes represent the number of times that node was present in 100 bootstrap trees. Tree was assembled using the Phylip phylogenetic software package (Felsenstein, 1989) and images produced with FigTree (http://tree.bio.ed.ac.uk/software/figtree/).

## Appendix 3.2



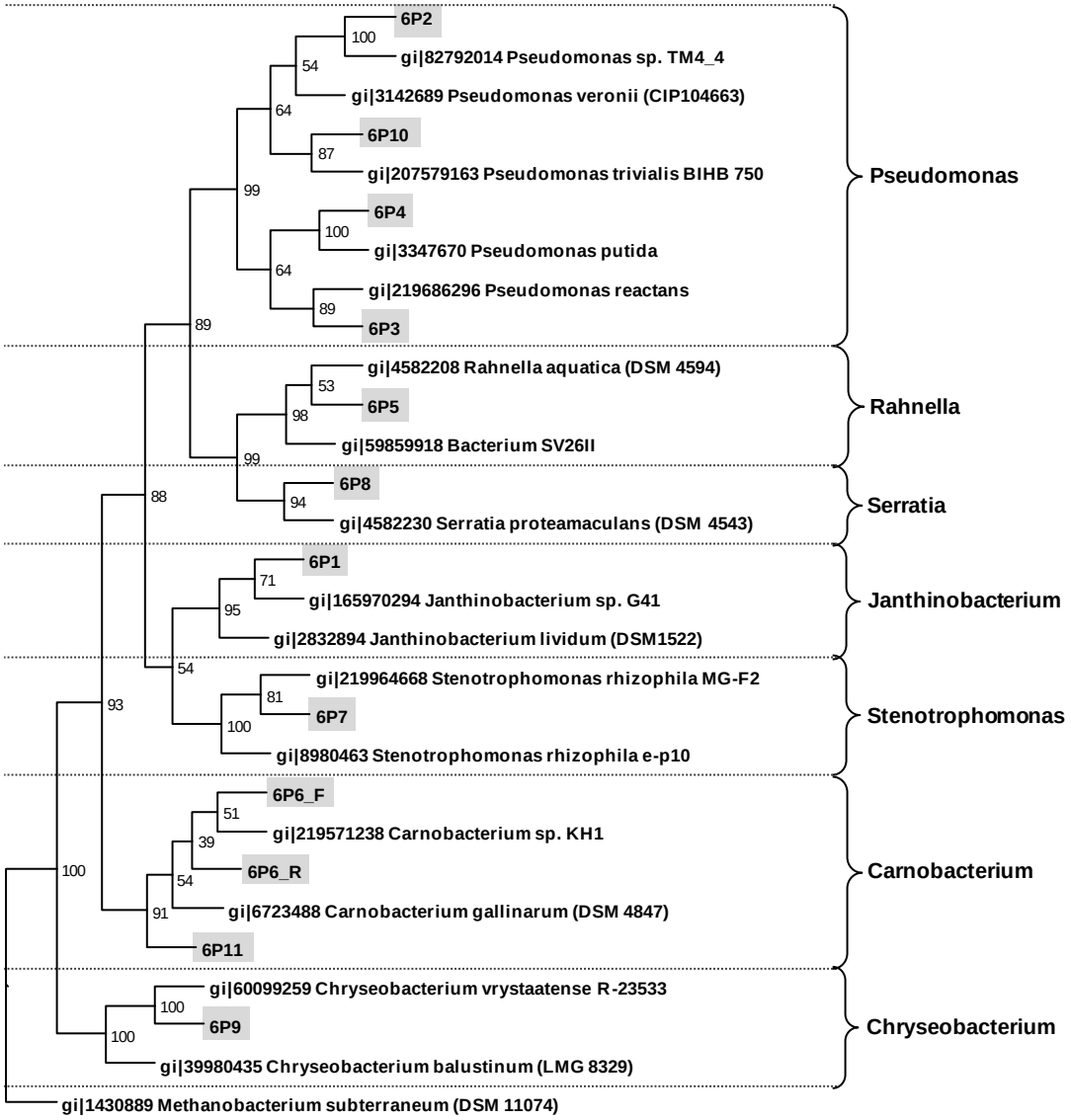

Phylogenetic tree by the maximum likelihood method of the partial 16S rRNA gene sequences of bacterial isolates from slug MRW003 and their closest matches from the Genbank Nucleotide Database. The numbers at the nodes represent the number of times that node was present in 100 bootstrap trees. Tree was assembled using the Phylip phylogenetic software package (Felsenstein, 1989) and images produced with FigTree (http://tree.bio.ed.ac.uk/software/figtree/).

## Appendix 3.3




Phylogenetic tree by the maximum likelihood method of the partial 16S rRNA gene sequences of bacterial isolates from slug MRW005 and their closest matches from the Genbank Nucleotide Database. The numbers at the nodes represent the number of times that node was present in 100 bootstrap trees. Tree was assembled using the Phylip phylogenetic software package (Felsenstein, 1989) and images produced with FigTree (http://tree.bio.ed.ac.uk/software/figtree/).

## Appendix 3.4




Phylogenetic tree by the maximum likelihood method of the partial 16S rRNA gene sequences of bacterial isolates from slug MRW006 and their closest matches from the Genbank Nucleotide Database. The numbers at the nodes represent the number of times that node was present in 100 bootstrap trees. Tree was assembled using the Phylip phylogenetic software package (Felsenstein, 1989) and images produced with FigTree (http://tree.bio.ed.ac.uk/software/figtree/).

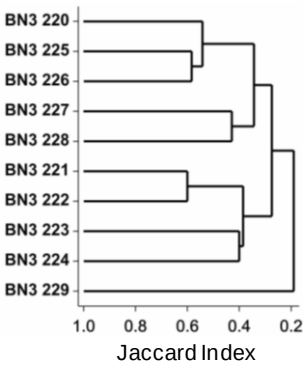
## Appendix 4.1

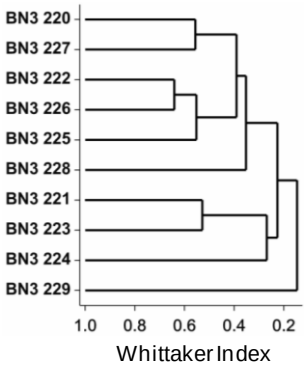
Excel macro for binning RISA fingerprint data as described in section 4.2.5. Thanks to Mr M. Mitchell for assistance in writing this code.

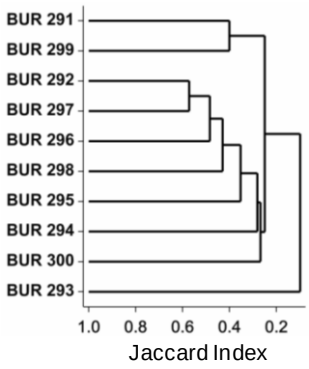
```
Sub BinningMacro()

Sheets("bin1").Select
Call bin
Sheets("bin2").Select
Call bin
Sheets("bin3").Select
Call bin
Sheets("bin4").Select
Call bin
Sheets("bin5").Select
Call bin
Sheets("bin6").Select
Call bin
Sheets("bin7").Select
Call bin
Sheets("bin8").Select
Call bin
Sheets("bin9").Select
Call bin
Sheets("bin10").Select
Call bin
'Repeated for 40 bins
End Sub

Function bin()
End Function
With ActiveSheet.Range("a1").CurrentRegion
  .Resize(.Rows.Count - 2, .Columns.Count - 2).Offset(2, 2).ClearContents
  A = .Value
  With CreateObject("Scripting.Dictionary")
    For i = 3 To UBound(A, 1)
      For ii = A(i, 1) To A(i, 2)
        .Item(ii) = i
      Next
    Next
    B = Sheets("data").Range("a1").CurrentRegion.Value

    For ii = 1 To UBound(B, 2) Step 2
        For i = 3 To UBound(B, 1)
          B(i, ii) = Application.Round(B(i, ii), 0)
          If .exists(B(i, ii)) Then
            A(.Item(B(i, ii)), ii + 2) = B(i, ii)
            A(.Item(B(i, ii)), ii + 3) = B(i, ii + 1)
          End If
        Next
```

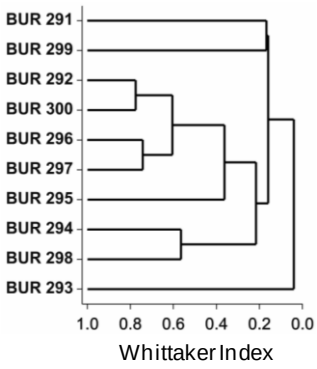
## Appendix 4.2

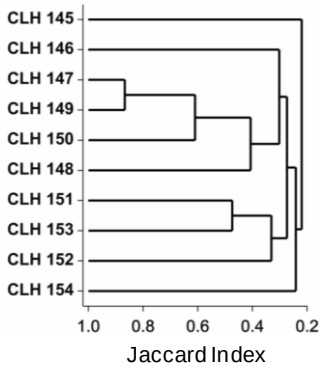
Dendrograms showing similarity of RISA profiles of slug gut microbial communities in the gut of the field slug *Deroceras reticulatum*. Dendrograms were produced in Genstat using cluster analysis by UPGMA method of Jaccard and Whittaker similarity indices (Section 4.2.3) and show ten individuals per location (nine for TAP) as denoted by the three letter location abbreviation as listed in table 4.1.

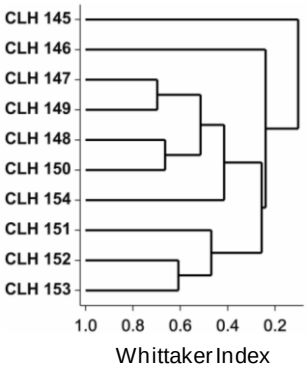


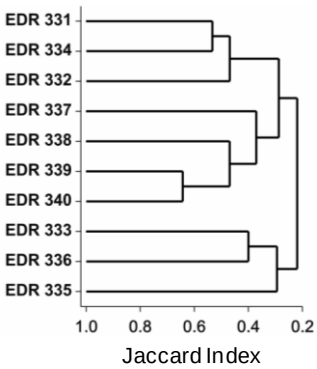



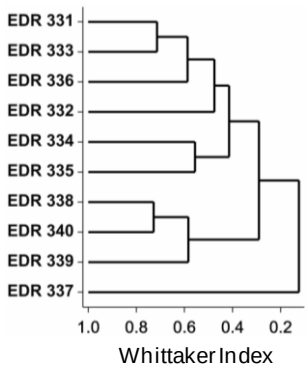



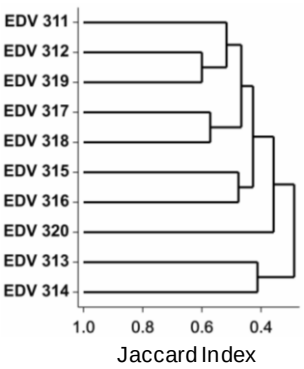



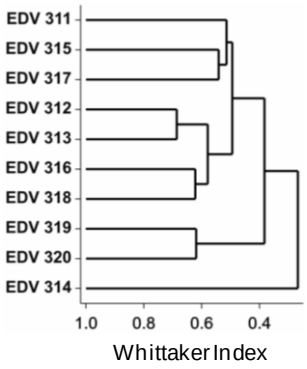

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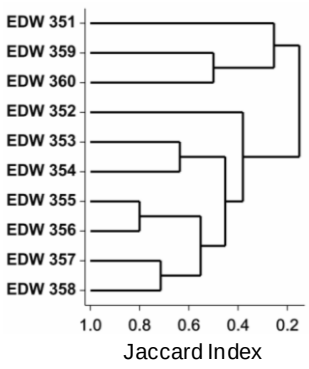
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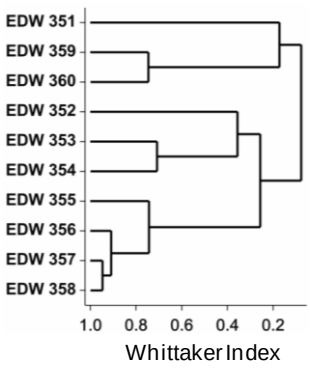
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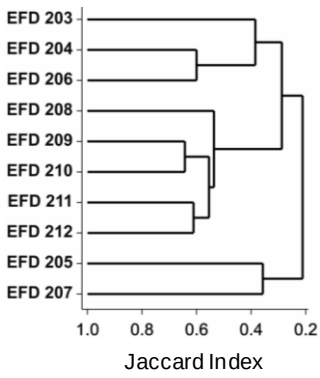
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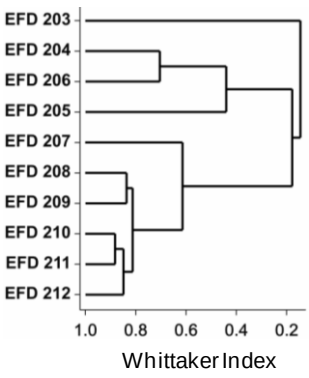
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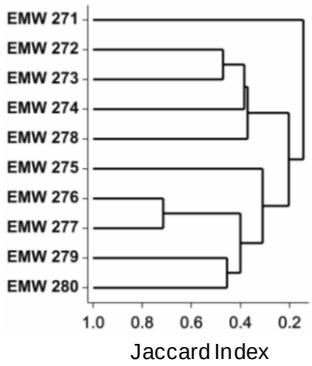
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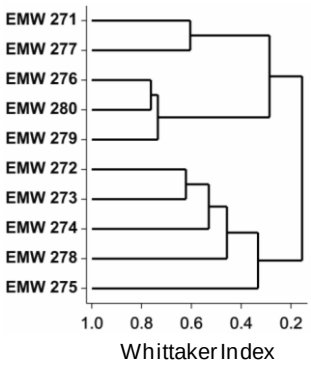
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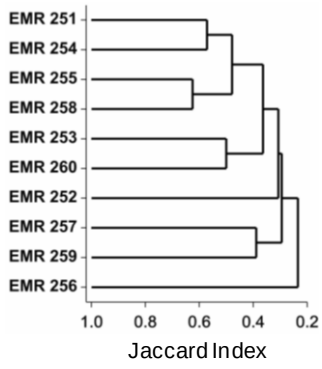
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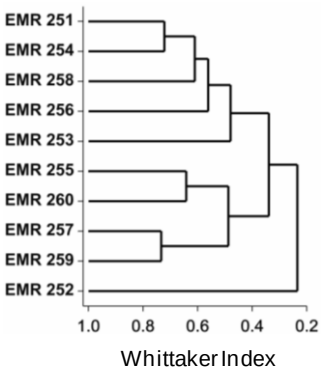
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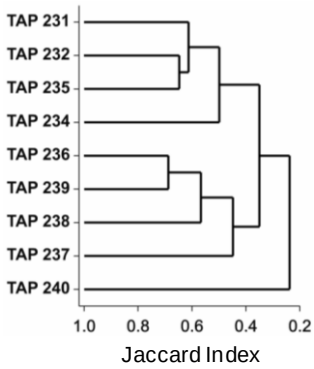
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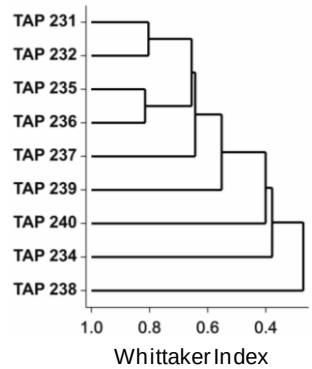
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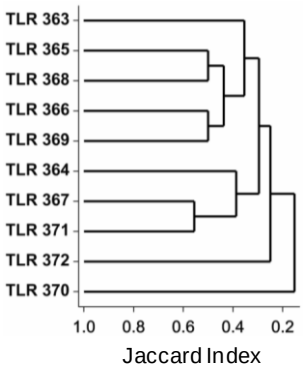
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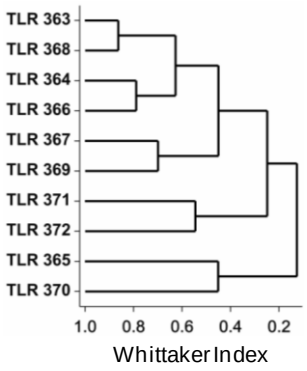
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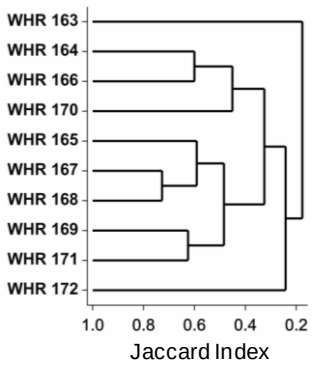
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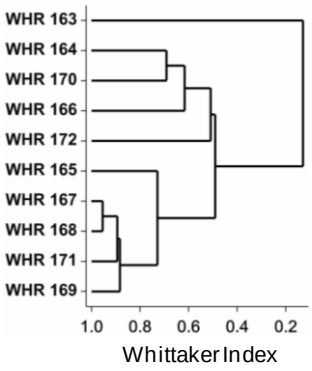
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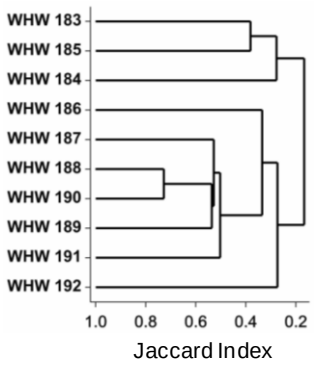
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WHW 184
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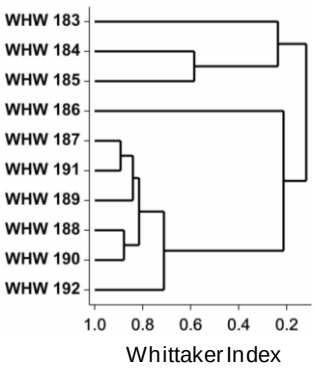
1.0 0.8 0.6 0.4 0.2
Jaccard Index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WHW 186
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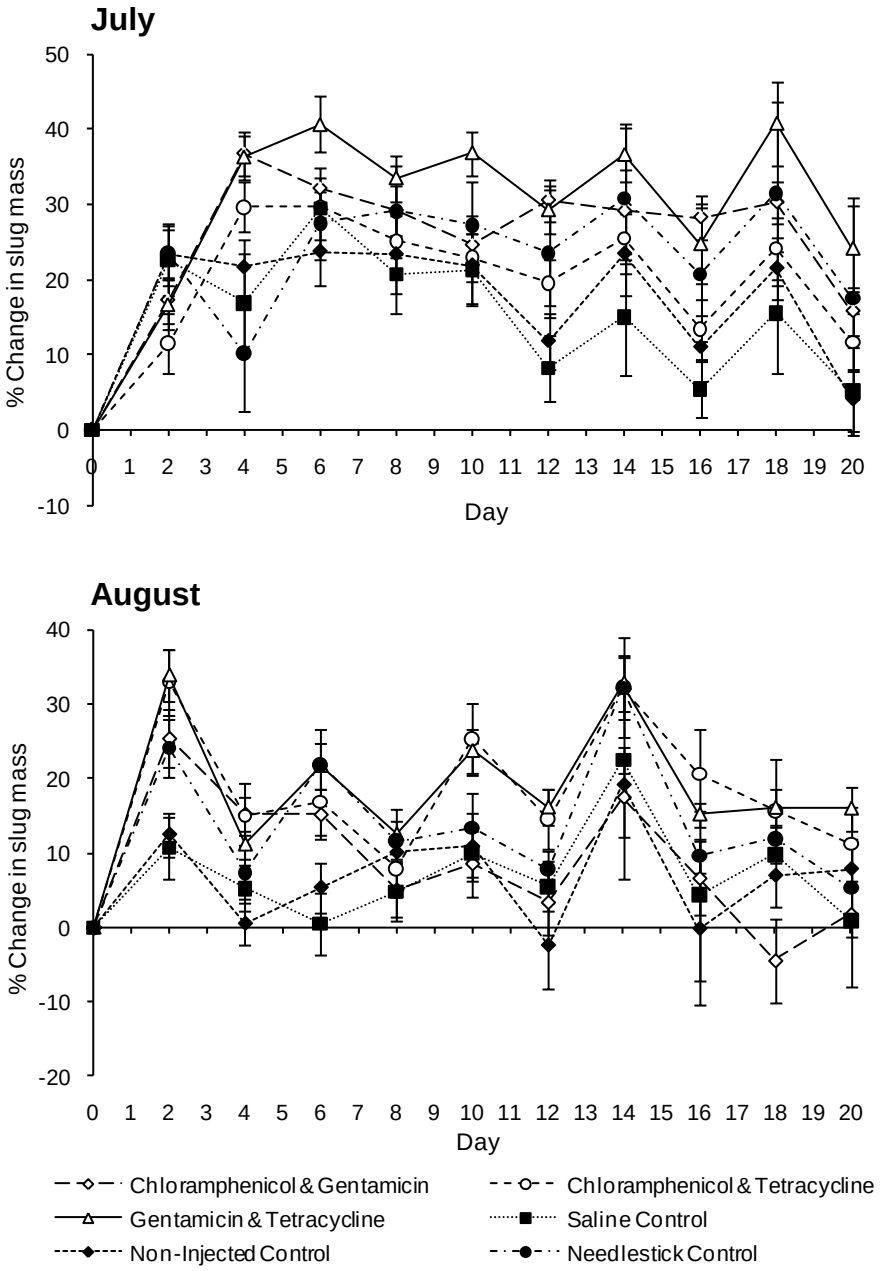
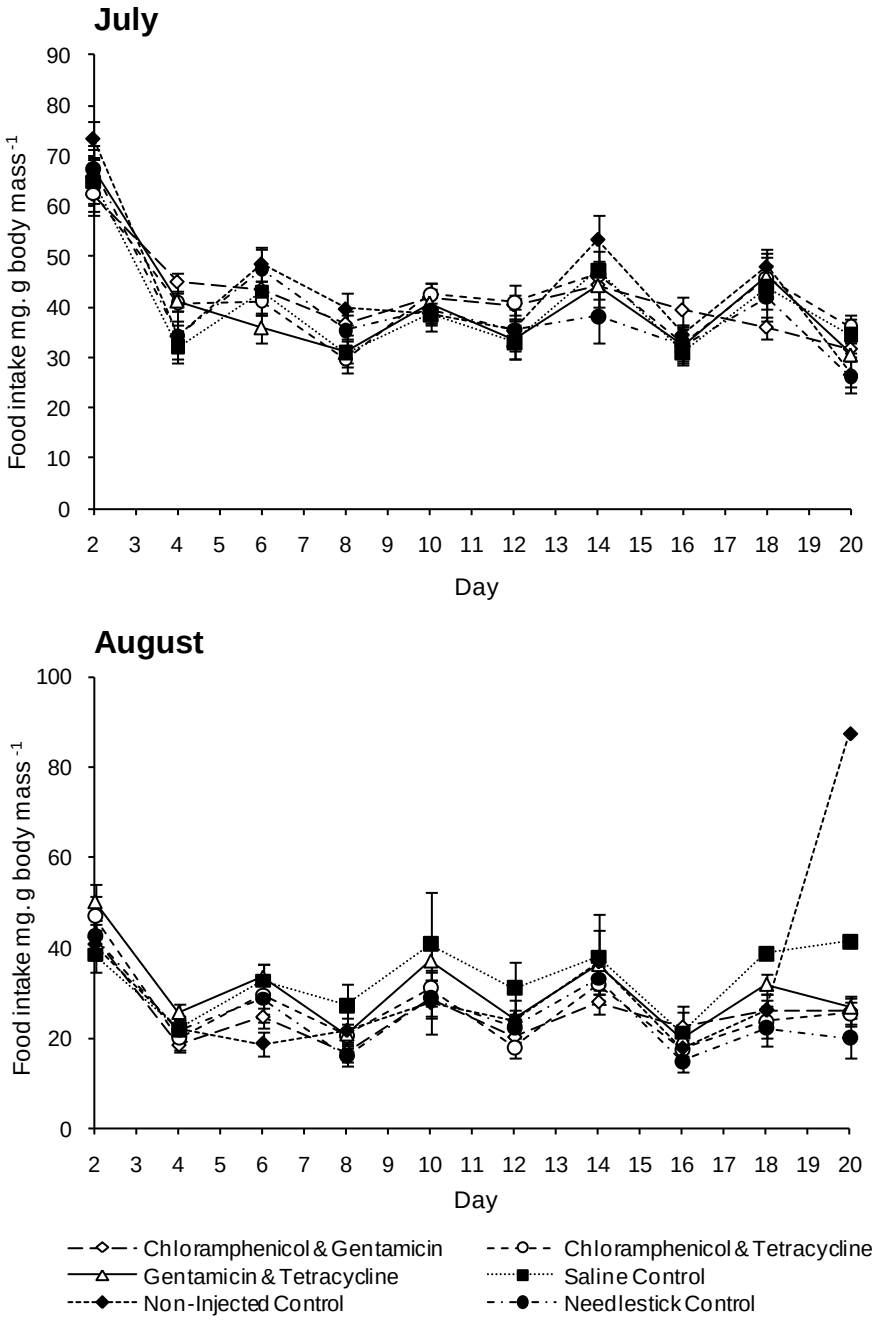
WHW 192
1.0 0.8 0.6 0.4 0.2
Whittaker Index

## Appendix 5.1



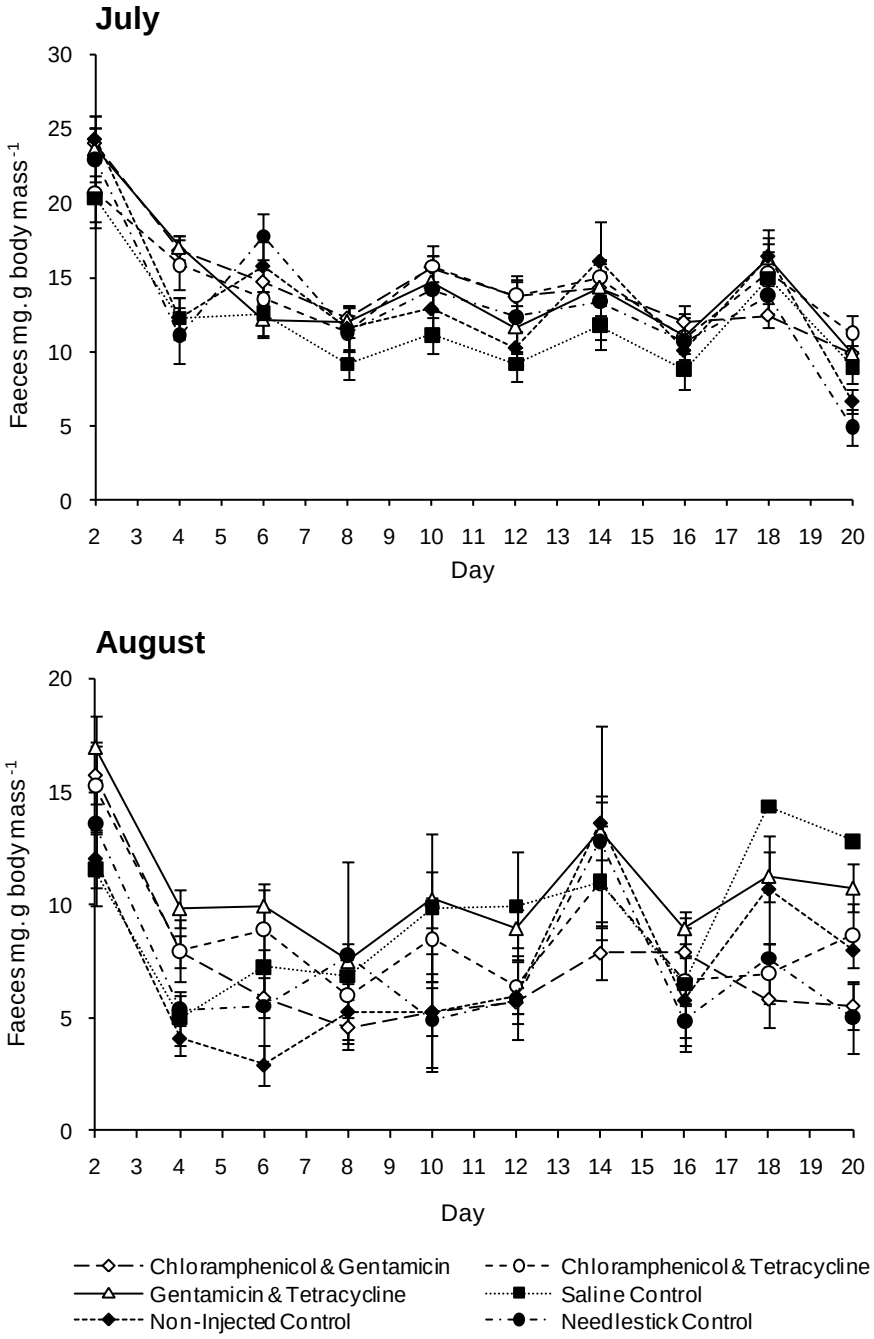

Mean percentage change in slug mass relative to the starting mass for slugs treated with antibiotic in the July and August bioassays. Error bars show SEM.

## Appendix 5.2




Mean food intake for slugs treated with antibiotic in the July and August bioassays. Error bars show SEM.

## Appendix 5.3




Mean faeces production in mg per g of body mass (mg.g bodymass$^{-1}$) for slugs treated with antibiotic in the July and August bioassays. Error bars show SEM.