Investigation of Environmental Reservoirs of *Strongyloides stercoralis* as Targets for Utilising Nematophagous Fungi as Potential Biocontrol Agents

A thesis submitted for the degree of

Doctor of Philosophy

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DECLARATION

I certify that this thesis does not incorporate without acknowledgement any material previously submitted for the degree or diploma in any university; and that to the best of my knowledge and belief it does not contain any material previously published or written by another person except where due reference is made in the text.

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THESIS ABSTRACT

This thesis presents an investigation into the environmental reservoirs of *Strongyloides stercoralis* in Australia and aimed to develop methods to reduce numbers of infective larvae in these areas as part of an integrated multifaceted approach to control the parasite.

This work develops a framework for further investigations into a neglected human parasite in an Australian context using native fungi to reduce larval loads in environmental reservoirs. This was conducted by attempting to develop a real-time PCR assay for larval detection in environmental samples, the subsequent aims were to isolate native nematophagous fungi from Northern Territory soils, assess nematophagous efficacy in faecal-soil matrices and assess the toxicity of nematotoxins from nematophagous fungi on *Strongyloides ratti*.

This work investigates the limitations of working with an organism that is prevalent in remote and isolated areas with limited access to samples. Sample storage and transit methods require development to improve DNA yields and numbers of viable organisms as these were a distinctive constraint in the study.

Soil samples were collected from the Northern Territory in Australia and the sprinkle plate used as the technique to isolate potential nematophagous fungi. Characterization of the isolates proved difficult due to the lack of clarity between predatory activity, saprotrophic activity and weak predatory activity. It was concluded that none of the isolates were strictly nematophagous fungi and were weakly or opportunistically predating nematodes

Predatory activity of nematophagous fungi has been attributed to both biological competition and induced by low nutrient substrates. *S. stercoralis* are known to occur in soil-faecal matrices, therefore this work aimed to assess the efficacy of nematophagous fungi in these substrates. *A. oligospora* and *C. elegans* were used as indicator organisms in a soil-faecal matrix. *A. oligospora* demonstrated the ability to effectively trap and remove 99.9% of *C. elegans* in a canine faecal matrix, however, in a soil-faecal matrix trapping efficacy was only 61.2%. Providing clarity on the potential applicability of nematophagous fungi to control nematodes in high nutrient substrates.

The nematotoxic substances produced by *P. ostreatus, P. ostreatus* var. columbinus and *H. clelandii*, which aid in their nematophagous processes were investigated for their toxicity to *C. elegans* and *S. ratti.* The purpose of this work was to identify if extracts had potential for use as an environmental control measure for free-living *S. stercoralis* larvae. These extracts proved effective at immobilising *C. elegans* larvae but the toxicity towards *S. ratti* was inconclusive.

This research is one of very few studies investigating the effect of fungi on *S. stercoralis* and provides a framework for more directed research in this area.

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DEDICATION

This thesis is dedicated to Francis O'Donahoo.

Frank your passion is contagious and I was truly honoured to work with someone who cared so greatly and genuinely for what they do. You lived and breathed your research and made sure you were always in touch with the real issues. The world through your eyes never saw *Strongyloides* as a disease that could be tackled in a laboratory or by clinicians alone. You saw the problem for how it was and worked hard to communicate this through multiple avenues, whether you were dressed up as a dog telling a story to kids in communities or presenting your research at a conference. Thank you for inspiring and educating, I am glad you inspired me.

This thesis is for you and me both Goldilocks!

Rest In Peace Frank.

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1. Chapter One: General Introduction

This introduction is composed of two published articles, which shape the scope of this research and introduce the reader to strongyloidiasis in Australia and suggests a novel method for incorporating, into an integrated management approach for this disease, environmental control. Each paper is introduced and summarised to provide context within the work as a whole. This preliminary research was used to inform the direction of this study which is summarised in the research aims and directions at the end of this chapter.

The following section is a published journal article authored by Michael J Taylor, Tara A Garrard, Francis J O'Donahoo and Kirstin E Ross in *Research and Reports in Tropical Medicine*, published 20 August 2014, Volume 2014:5, Pages 55—63. (Taylor et al., 2014)

This article highlights the unknown areas about Strongyloidiasis and sets the scope of the research in this thesis. Many of the clinical components of this disease are well understood and treatments are readily available, however, with an estimate of 100-370 million cases worldwide the persistence of this disease must be questioned. The article explores the transmission of the disease and why infection is occurring in the first place. This includes the intricacies of the free-living life-cycle and the factors that favour development as well as infection in other host organisms and the environmental sources of infection. The knowledge gaps in these crucial areas are

key to why there is such high prevalence of an easily treatable disease and highlights the concept that treatment and control are separate principles.

This was a joint authored publication which was developed as a result of a literature search conducted by Tara Garrard and extensive discussions with all authors. All authors contributed to the introduction and conclusions. The Strongyloides' life cycle and Clinical treatment of strongyloidiasis sections were authored by Tara Garrard. The detection in clinical and environmental samples section was put together by Tara Garrard and Michael Taylor. Tara contributed to and wrote components of Prevalence: significantly underestimated?, Lack of ivermectin resistance, Environmental reservoirs and Is *S. canis* a separate species sections.

1.1. Human strongyloidiasis: identifying knowledge gaps, with emphasis on environmental control

Michael J Taylor, Tara A Garrard, Francis J O'Donahoo and Kirstin E Ross in *Research and Reports in Tropical Medicine*, published 20 August 2014, Volume 2014:5, Pages 55—63. (Taylor et al., 2014)

1.1.1. Abstract

Strongyloides is a human parasitic nematode which is poorly understood outside a clinical context. This paper identifies gaps within the literature, with particular emphasis on gaps that are hindering environmental control of *Strongyloides*.

The prevalence and distribution of *Strongyloides* is unclear. An estimate of 100-370 million people infected worldwide has been proposed, however inaccuracy of diagnosis, unreliability of prevalence mapping and the fact that strongyloidiasis remains a neglected disease suggests that the higher figure of over 300 million cases is likely to be a more accurate estimate.

The complexity of *Strongyloides* lifecycle means that laboratory cultures cannot be maintained outside of a host. This currently limits the range of laboratory based research which is vital to controlling *Strongyloides* through environmental alteration or treatment.

Successful clinical treatment with anthelminthic drugs has meant that controlling *Strongyloides* through environmental control rather than clinical intervention has been largely overlooked. These control measures may encompass alteration of the

soil environment through physical means such as desiccation or removal of nutrients; or through chemical or biological agents.

Repeated anthelminthic treatment of individuals with recurrent strongyloidiasis has not been observed to result in the selection of resistant strains, however, this has not been explicitly demonstrated and relying on such assumptions in the long term may prove to be short sighted. It is ultimately naive to assume that continued administration of anthelminthic will be without any negative long term effects.

In Australia strongyloidiasis primarily affects Indigenous communities, including communities from arid central Australia. This suggests that the range of *Strongyloides* extends beyond the reported tropical/subtropical boundary. Localized conditions that might result in this extended boundary include accumulation of moisture within housing due to malfunctioning health hardware inside and outside the house and the presence of dog faecal matter in or outside housing areas.

1.1.2. Key learning points

- Human strongyloidiasis is a neglected disease requiring immediate research attention, particularly in Australia where Indigenous Australians are disproportionally affected.

- The parasite *Strongyloides stercoralis* is often still referred to as a tropical disease despite publications demonstrating that this is not the case.

- An overemphasis has been given to clinical interventions rather than environmental control in regards to preventing infections. This is of concern as there is the potential for reinfection and development of resistance to currently administered drugs.

- Significant knowledge gaps exist in the understanding of the organism's lifecycle and environmental reservoirs. These gaps are hindering research into controlling *Strongyloides* through environmental alteration or treatment.

1.1.3. Key Words

Strongyloides stercoralis, Strongyloidiasis, environmental control, parasitology, nematode

1.1.4. Introduction

Humans are hosts to two species of the parasitic nematode *Strongyloides, Strongyloides stercoralis* and *S. fuelleborni* (separated into two sub-species, *S. f. fuelleborni* [in Africa] and *S. f. kellyi* [in Papua New Guinea]). (Cox, 2002) Strongyloidiasis is caused by infection by either of these species. The important species in human infection is *S. stercoralis**.

(*unless otherwise indicated, the remainder of this paper will refer to *Strongyloides*, indicating any *Strongyloides* spp. capable of causing strongyloidiasis)

In Australia, a history of successful clinical treatment with anthelminthic drugs (Fisher et al., 1993, Johnston et al., 2005) has meant that controlling *Strongyloides* through environmental control rather than clinical intervention has been largely overlooked. However in light of reinfection rates in endemic areas, coupled with concern about the potential for development of anthelminthic resistance, environmental control should be given greater attention, either by altering the soil environment; through physical means such as desiccation or removal of nutrients; or through chemical or biological control.

Strongyloides has a complex lifecycle with early research identifying the unique alternation between the free living and parasitic stages of *Strongyloides*. (Leuckart, 1883) Furthermore, in 1905 Looss (Looss, 1905) demonstrated the mode of infection – through the skin – by infecting himself and finding *Strongyloides* in his faeces 64 days later, and Fülleborn (Fülleborn, 1914) reported how the parasite moves through the human body to end up in the intestine. (Faust, 1933) Despite this early understanding of the infective nature of *Strongyloides*, and its prevalence, significant gaps in our understanding of *Strongyloides* still exist. These gaps are impacting on our ability to control infection rates globally. In this paper we present an overview of our current understanding of *Strongyloides*, and summarise the gaps in our knowledge, with particular emphasis on those gaps that are preventing better environmental control of *Strongyloides*.

1.1.5. Methods

The authors reviewed the body of literature to identify knowledge gaps which may be hindering progress in the environmental control of *Strongyloides.* Journal indexing services (Google Scholar, PubMed, Ingenta Connect) were queried for publications from the last 25 years which represented the current best practice or best knowledge in terms of treatment, diagnosis, epidemiology and microbiology of *Strongyloides* and strongyloidiasis. Efforts were directed towards obvious gaps in the literature which represented significant barriers to the understanding of the organisms' survival in the environment and where research may be directed to address these gaps. The thorough description of these gaps forms the basis of this manuscript. Where appropriate, historical context was provided by older and seminal publications within the field.

1.1.5.1. Strongyloides' lifecycle

The lifecycle of *Strongyloides stercoralis* incorporates complex host mediated (homogonic) and free living environmental (heterogonic) processes. The parasite has the ability to reproduce indefinitely within the host (Ly et al., 2003) if not treated with anthelminthic.

Human infection occurs when filariform larvae penetrate the skin. These larvae then enter the venous system where they migrate through the right atrium and ventricle of the heart and then to the lungs and occupy the bronchi and trachea. From this

region of the respiratory system larvae are coughed up and subsequently swallowed. Larvae pass through the digestive system until they reach the small intestine where they submerge themselves in the intestinal mucosa. (Ly et al., 2003) Embedded worms undergo further development into predominantly adult females which are capable of parthenogenic (asexual) reproduction. (Ashton et al., 1998) Adult females drive an autoinfective lifecycle, whereby eggs are laid in the gut. These eggs hatch and develop into male and female rhabditiform larvae and both eggs and larvae are excreted in faeces. Rhabditiform larvae then develop into filariform larvae and either re-penetrate the gut lining, the skin surrounding the perianal region or environmentally – a new host. (Ericsson et al., 2001)

Due to the complexity of *S. stercoralis'* lifecycle, (CDC, 2013) laboratory cultures cannot be maintained outside of a host and subsequently only a single heterogonic cycle has been observed. (Lok, 2007) There is no clear consensus backed by empirical evidence demonstrating which factors cause the differentiation of rhabditiform *S. stercoralis* into females, males or filariform larvae. Early research demonstrated varied effects of temperature on larval development and exposure to faecal dilutions. (Shiwaku and Chigusa, 1988, Berezhnaia et al., 1991) However more recent research has found that in temperatures below 34˚C larvae molt four times and develop into free-living sexually mature adults and in temperatures above 34˚C larvae molt twice and develop into infective filariform larvae. (Nolan et al., 2004, Schär et al., 2013b) Chemosensory factors may also influence larval differentiation. Chemosenory and thermosensory neurons are contained in the amphids in nematodes. Research into

the function of the amphids has shown several classes of theses neurons. The skin penetrating larvae in *Strongyloides stercoralis* have been shown to be thermotaxic moving upwards on a thermal gradient, this is regulated by the paired ALD class neurons. (Lopez et al., 2000) *S. stercoralis* has been demonstrated to be chemotaxic, moving towards the chemical markers present in sweat. Developmental switching (switching between alternative free-living developmental pathways) has been shown to be controlled by the ASF and ASI chemosensory amphidial neurons. (Ashton et al., 1998) Similarly the development of infective larvae has been shown to be controlled by similar molecular genetic mechanisms as *C. elegans* (via the AGE-1 region) in *S. stercoralis* via a structural homologue of the AGE-1 region called Ss-AGE-1. (Stoltzfus et al., 2012) Therefore a chemical agent is likely to be involved in the mediation of differentiation of *S. stercoralis* larvae.

As homogonic *S. stercoralis* primarily exists in tissues and periodically in faeces, it seems likely that a chemical element present in faeces either inhibits or induces larval differentiation. Adult forms and eggs are excreted in faeces to either carry out a free-living sexual reproductive cycle or immediately seek new hosts. This requires that eggs either become filariform larvae or produces males to mate with females, and differentiation must occur at an advantageous juncture in order to provide the highest likelihood of survival. It has been demonstrated that cholesterol and other sterols play a hormonal or signaling role in larval development in the related rhabditiform nematode *Caenorhabditis elegans.* (Matyash et al., 2004, Castelletto et al., 2009) It seems plausible that sterols may be a key signaling molecule in the

development of *S. stercoralis* larvae, and may go so far as to account for differences in host specificity given the formation of distinctly different faecal sterols in various mammals. (Leeming et al., 1996) Siddiqui et al. (Siddiqui et al., 2000) present evidence of a receptor which they hypothesize supports steroids triggering hyperinfection of *Strongyloides*. This is supported by Wang et al., (Wang et al., 2009) who suggest that ligand-binding to treat disseminated *Strongyloides* may be pharmacologically possible.

The complexity of *Strongyloides*' life cycle might be a reason for the limited published bioassays assessing the nematode's susceptibility to environmental challenges. Tests rely on *Strongyloides* extracted from faeces and are often problematic. *Strongyloides* need to be extracted from faeces for each set of bioassays, which presents both ethical and occupational safety problems. The lack of research into maintaining *Strongyloides* in the laboratory impacts on our ability to assess the *Strongyloides*' susceptibility to environmental control potential such as desiccation, or chemical and biological control possibilities.

1.1.5.2. Prevalence: Significantly underestimated?

Strongyloidiasis is widespread within tropical and subtropical areas around the world. Based on ratios of prevalence of other helminthes, an estimate of 370 million people infected worldwide has been proposed, (Bisoffi et al., 2013) making strongyloidiasis a more common infection than malaria, which has an estimated

infection level of 219 million cases (uncertainty range: 154 - 289 million) (World Health Organization, 2012). Other more conservative estimates suggest a global infection level of 100 million people, (Bethony et al., 2006) although researchers believe that this figure is grossly underestimated, due to the infection mimicking other illnesses with symptoms such as diarrhea, abdominal pain, septicemia, and vomiting, and is therefore often misdiagnosed (Rahim et al., 2005, Leang et al., 2004). The inaccuracy of diagnosis (Krolewiecki et al., 2013) and the unreliability of prevalence mapping (Bisoffi et al., 2013) coupled with the fact that *Strongyloides* infection is often not tested for, suggests that the higher figure of over 300 million cases is likely to be a more accurate estimate. Publications regarding the infection rate and incidence of strongyloidiasis have been described as 'patchy' and 'virtually non-existant' and have been highlighted as key gaps in knowledge which would provide insight into how frequently and how rapidly people are reinfected after successful treatment (Schär et al., 2013b). The prevalence of strongyloidiasis in Australia is equally unknown, with extremely varied estimates ranging from >1%-60% depending on the community tested and the diagnostic tools used (Kukuruzovic et al., 2002, Meloni et al., 1993, Prociv and Luke, 1993, Flannery et al., 1993). A 20 year retrospective survey of remote communities in Queensland discovered fluctuating prevalence which correlated with both the wet season, where prevalence increased from 12% to 27.5%, and thiabendazole treatment after which prevalence fell to 7% for approximately 4 years (Prociv and Luke, 1993). This work highlighted the effective use of anthelmintics to treat persistent strongyloidiasis and lower re-infection rates, but suggested that without changes to failed infrastructure eradication may not be possible.

The health consequences of *Strongyloides* infections range from asymptomatic light infections to chronic symptomatic strongyloidiasis, and finally uncontrolled multiplication of the parasite (hyperinfection) and potentially life-threatening dissemination of larvae to all internal organs among individuals with compromised immune systems (Vadlamudi et al., 2006). Dissemination and hyperinfection has been replicated with *S. stercoralis* in dogs and shown to be a model for the human course of the infection (Schad et al., 1984, Grove et al., 1983). Immunocompromised dogs were shown to be highly susceptible to hyperinfection and disseminated strongyloidiasis. Similarly, hyperinfection has also been induced in gerbils using *S. stercoralis* (Nolan et al., 1993). Marcos et al. (2008) suggest that severe strongyloidiasis has a high mortality rate (up to 80%) because the diagnosis is often delayed. This relates to its nonspecific presentation and the host's immunocompromised status. Most immunocompetent individuals who develop strongyloidiasis have asymptomatic chronic infections that result in negligible morbidity. Immunosuppressed individuals are most vulnerable, with mortality rates being highest amongst these groups, (Vadlamudi et al., 2006, Ferreira, 2003) with Indigenous Australians suffering high rates of non-communicable disease (Vos et al., 2009, Condon et al., 2004), which increases the infection risk and worsens outcomes compared with non-Indigenous Australian (Einsiedel et al., 2012).

1.1.5.3. Detection in clinical and environmental samples

Detection of *Strongyloides* in clinical samples can be classified broadly as either molecular, incorporating either PCR or qPCR, (Hanan Z. Rayan, 2012) immunological methods, (Ahmad et al., 2013) or microscopic methods which include the Koga plate method, (Koga, 1991), the Baerman technique (Khieu et al., 2013, Baermann, 1917) and the Katz thick smear. (Katz et al., 1972, Kongs et al., 2001, de Kaminsky, 1993)

Microscopic techniques provide conditions that separate intact, living nematodes from clinical samples (primarily faeces). These methods all suffer from primarily three main limitations; the identification of *Strongyloides* using morphological features can be subjective; working with live *Strongyloides* in an uncontained system is a biosafety hazard; and the larval load in stool varies greatly (Dreyer et al., 1996, Mansfield and Schad, 1992, Montes et al., 2010). Identification ambiguities and safety concerns may be mitigated with sufficient training and an appropriate laboratory setup, however variations in larval load is highly dependent on the parasite's lifecycle stage, host health and treatment status (Mansfield and Schad, 1992, Dreyer et al., 1996, Montes et al., 2010). Ultimately this may produce results where there are insufficient larvae in stool to visually confirm infection resulting in false negatives. Additionally, these techniques can take up to 48 hours for results to be available.

Molecular methods allow for the detection of *Strongyloides* based solely upon the presence of target DNA sequences, removing the analyst as a subjective source of bias. Samples (not limited to stool) have their DNA compliment extracted and primers are added that anneal specifically to target *Strongyloides* sequences. A range of

primer sets incorporating both conventional qPCR and probe based detection systems have been described (Hanan Z. Rayan, 2012, Moghaddassani, 2011, Repetto et al., 2013, Schär et al., 2013a, Sultana et al., 2013b, Verweij et al., 2009). Serological detection methods are available which detect either proteins or antibodies in the blood plasma using an enzyme-linked immunosorbent assay (ELISA). (Ahmad et al., 2013)

These methods need significant refinement to achieve reliable, inter-sample quantitation, but molecular methods inherently have several advantages over microscopy directed diagnostic tools. Once DNA extraction has occurred, samples are entirely non-infective, and samples need not be stored to retain viability of the helminthes present. Samples may be frozen or otherwise heat killed and should remain PCR competent, even with low numbers of larvae present due to the sensitivity of the method. However, the size of the sample processed may lead to false negatives in samples with low larval loads. Sample processing and detection may occur in a matter of two to three hours.

Environmental detection currently remains an understudied area of research and efforts should be made to better understand the role of environmental reservoirs of *Strongyloides*. Molecular detection methods used in clinical analyses are potentially readily transferable to environmental samples including soil and animal faeces, with little addition or modification to the method, however, the presence of compounds inhibitory to PCR may significantly reduce or halt the progress of the reaction. This is

an area that needs to be addressed to allow us to understand and map the distribution of *Strongyloides* in the environment.

1.1.5.4. Clinical treatment of strongyloidiasis

Strongyloidiasis is commonly treated with anthelmintics such as ivermectin and albendazole. Ivermectin is a broad spectrum macrocyclic lactone which inhibits the motility of the nematode by increasing the opening of glutamate-gated chloride channels causing paralysis of pharyngeal pumping (Ikeda, 2003, Martin, 1997). Albendazole inhibits the formation of microtubules by selectively binding to β-tubulin (Martin, 1997). Albendazole is usually prescribed at 400 mg for 3 days and has a 38% efficacy (Datry et al., 1994). Ivermectin had 83% efficacy when 150-200 μg/kg was administered in a single dose (Ikeda, 2003, Datry et al., 1994). Further work has supported ivermectin's preferential administration, demonstrating an efficacy of 96% when administered at 200 μg/kg, increasing to 98% after a follow up treatment 2 weeks after the initial dose (Zaha et al., 2002). Alternative anthelmintics; thiabendazole, cambendazole and mebendazole, can be used but are significantly less effective than ivermectin (Marti et al., 1996, Gann et al., 1994, Bisoffi et al., 2013).

1.1.5.5. Lack of ivermectin resistance

Repeated treatment of individuals with recurrent strongyloidiasis has not been demonstrated to result in the selection of resistant strains. We theorise that this

process is inhibited by *Strongyloides*' clonal lifecycle within a host. During the host-bound parthenogenic lifecycle, all infective individuals are clonally propagated and as such the rate of mutation and the rate at which novel genetic information is introduced is low. As a result, only limited adaptation is possible and so treatment with anthelmintics tends to be successful, even with repeated doses over an extended period of time with no reported instances of resistance in humans (Keiser et al., 2008). However, this has not been explicitly demonstrated either in a laboratory setting or through close monitoring of treatment-resistant individuals and relying on such assumptions in the long term may prove to be short sighted.

Furthermore, the repeated administration of ivermectin and specifically mass drug administrations (MDA) may lead to the formation of resistant population in other parasitic organisms, such as sarcopic mites (*Sarcoptes scabiei*) which historically have demonstrated the formation of resistance (Pasay et al., 2006, Coskey, 1979, Moberg et al., 1984) and have begun to show resistance to ivermectin treatment (Currie et al., 2004). Studies of related soil and veterinary helminthes have raised concerns on the formation of resistance (Albonico et al., 2004, Geerts and Gryseels, 2000, Geerts and Gryseels, 2001) and proposed the need for monitoring for resistance, (Albonico et al., 2004) or have begun to suggest the formation of resistance to benzimidazole compounds (Prichard, 2007). It is ultimately naive to assume that continued administration of anthelmintics will be without any negative long term effects, particularly without exploring prevention strategies which incorporate environmental control.

1.1.5.6. Geographical distribution: questioning a strict tropical/subtropical range

Genta (Genta, 1989) reviewed literature reporting the prevalence of *S. stercoralis* among various populations on five continents and found the following risk groups: *"residents of and emigrants from any developing country and southern, eastern, and central Europe; travellers and veterans returning from endemic areas; natives and residents of the Appalachian region in the United States and local endemic areas in other countries; and institutionalized persons*" (Genta, 1989). Historical studies have also demonstrated that the range of *Strongyloides* is not strictly limited to tropical/sub-tropical regions, with case reposts from urban areas in non-tropical regions (Amir-Ahmadi et al., 1968, Nauenberg et al., 1970). Despite the existence of literature indicating that strongyloidiasis is not confined to a tropical distribution it often still perceived and treated as such (Olsen et al., 2009, Greaves et al., 2013).

In Australia, the literature indicates the primary burden of the disease is borne by Indigenous communities of northern Australia (M et al., 2003, Prociv and Luke, 1993). Strongyloidiasis in Australian Indigenous populations has been primarily attributed to individuals and communities who inhabit tropical and subtropical areas of Australia. However, growing evidence suggests that the nematode is more widespread than previously thought within Indigenous populations, although further research is required to map infection outside the tropical and subtropical zones

(Johnston et al., 2005). From routine laboratory results and epidemiological surveys *Strongyloides* is now known to be spread more widely than was previously thought, particularly in Aboriginal communities in arid regions of central Australia (Einsiedel and Fernandes, 2008, J, 2009, J, 2013) although this clinical evidence is not yet supported in the literature. For example, *The 8th National Workshop on Strongyloidiasis* (p 27-28) listed unpublished data of infection rates of between 2 – 58% in Indigenous communities, including 32% in one community in 2007, and 15% in another in a 2005 survey (Speare et al., 2013). Australia's unique assortment of geographical features ranging from tropical to arid presents an opportunity to better understand the climactic limitations of *Strongyloides'* geographic distribution.

1.1.5.7. Environmental reservoirs

Unlike other diseases such as malaria, *Strongyloides* infection responds readily to chemotherapy (Bisoffi et al., 2013, Marti et al., 1996, Gann et al., 1994). Possibly as a result of this ease of clinical treatment, tackling environmental reservoirs as a means of controlling *Strongyloides* infection has been overlooked. Soil and faeces are assumed to be the environmental reservoirs of *Strongyloides* and Grove (1990) points out that the most effective control measures against human helminths has been the installation and usage of safe waste disposal systems (Grove, 1990). Few clinicians have sought an environmental solution to transmission. Durrhiem (2013) suggests that a solution to *Strongyloides* transmission might be wearing footwear, however, lack of cultural acceptance of wearing shoes, particularly in a hot climate, might make

this simple approach to interruption of transmission not possible. There is a general consensus that enforcing, or even educating for, behaviours that are counter to culturally accepted norms, will not be successful. (Resnicow et al., 1999, Kreuter et al., 2003)

As noted above, in Australia, Strongyloidiasis primarily affects Indigenous communities, particularly those remote Indigenous communities in the Northern Territory. There are a number of reasons for this, including malfunctioning health hardware[1] inside the house, malfunctioning health hardware outside the house, the presence of dog faecal matter in or outside housing areas and the close relationship between dogs and humans.

If the wet areas of a house (bathrooms, laundry, kitchen) are not functioning properly, leaking taps and/or poor drainage results in moisture being present for extended periods. The role of failed and poor infrastructure in strongyloidiasis transmission has been previously noted, but remediation has not extensively explored as a potential control method (Prociv and Luke, 1993). Indigenous houses often have lower levels of working housing infrastructure such as water and wastewater disposal (Pholeros, 1993, Australian Indigenous HealthInfoNet, 2010). Inside, this means moisture is retained for extended periods in the bathroom, laundry

[1] The term "health hardware" has been used to describe safe electrical systems, toilets, showers, taps, kitchen cupboards and benches, stoves, ovens and fridges collectively. it is estimated that 60% of the work required to improve these facilities is due to lack of routine maintenance and repairs (Pholeros, 1993).

and kitchen areas. Outside, prolonged water retention occurs as a result of leaking or malfunctioning rain water tanks and septic systems. This retention of moisture inside and outside the home may mimic other confirmed environmental reservoirs, (Nithiuthai et al., 2004, Bakir et al., 2003, Mintz et al., 1995, Prociv and Luke, 1993) and provides an environment that could sustain the environmental life cycle stage of *Strongyloides*, possibly for extended periods (although the survival time of *Strongyloides* in the environment has not been quantified).

In Aboriginal communities, dogs and people live in close proximity (Walton et al., 1999) and the close relationship between dogs and people has been documented (Gaskin, 2007, Schrieber, 2012). High numbers of dogs have been reported in Indigenous communities, for example, Bradbury and Corlette (2006) reported over 50% of homes housing three or more dogs, and 10% of homes exceeding 8 dogs per household). J. Driver (Environmental Health Officer, Department of Health, Northern Territory) and J. Kennedy (Child and Family Health Nurse, Department of Health, Northern Territory) (personal communications, 11/10/13) confirm the presence of dog faeces inside homes. While the presence of dogs and dog faeces does not necessarily provide a source of infection for *Strongyloides*, preliminary evidence for infective transfer from dogs to humans has been established. (Sprott et al., 1987)

Another study of environmental reservoirs surveyed garbage collectors in Brazil and concluded that contact with garbage or sewage may be associated with infection

with intestinal parasites, with workers surveyed having acquired strongyloidiasis. (Clark et al., 1984, Gomes et al., 2002)

Controlling *Strongyloides* by addressing the environmental factors that play a role in transmission, in addition to treating the infection once it occurs should be a priority for researchers.

1.1.5.8. Is *S. canis* a separate species?

The volume of published material on the existence of a canine specific *Strongyloides* species is at best scant, with journal indexing services queried (Google Scholar, PubMed, Ingenta Connect) returning less than 15 articles mentioning *Strongyloides canis*. Similarly, searches of the National Centre for Biotechnology Information (NCBI) contain no submitted sequences from *Strongyloides canis*. It is conceivable that a canine adapted species of *Strongyloides* exists and has yet to be thoroughly characterized, however the distinct possibility exists that canine infections are primarily caused by *S. stercoralis* (Augustine and Davey, 1939, Galliard, 1950, Gaskin, 2007, Júnior et al., 2006, Mbaya et al., 2009). Sequencing of internal transcribed spacer regions 1 and 2 as per Sultana et al (2013a) may demonstrate a genetic and possible taxonomic basis for the classification of a canine specific *Strongyloides* species but at present the volume of published material does not support this.

This is a crucial area of research for several reasons. Firstly, if dogs are harbouring human *Strongyloides*, their role as a reservoir for human infection needs to be understood, particularly for Indigenous Australian communities, where dogs play an important cultural role. Secondly, from a research perspective, laboratory extraction of *Strongyloides* from dog faeces carries fewer ethical considerations. However, there are still the associated biohazard risks of dealing with the extracted *Strongyloides* in the laboratory.

1.1.6. Conclusion

Currently we have limited knowledge about environmental factors that affect *Strongyloides*. The assumption that the disease is restricted to tropical areas is in question and the reason for its restriction to certain geographical areas is not well understood. On a local scale, we are not sure where in the soil environment the reservoirs that harbour *Strongyloides* exist. Currently used diagnostic methods are unreliable and emerging more reliable techniques are not yet in common use. In many areas clinicians lack awareness of the infection so it is not tested for, which combine to result in inaccurate estimates of infection rates. We are not sure of the role that dog faeces might play as a reservoir and in human transmission of *Strongyloides*. We do not know what environmental factors might control *Strongyloides* distribution (such as moisture and nutrients) and we do not know of any chemical or biological control agents that might be applicable to its control in the soil. Additionally, we do not yet have the ability to maintain a culture of *Strongyloides* in the laboratory for an extended period of time, which hinders laboratory-based experimental research.

For too long we have taken a purely clinical approach to treating *Strongyloides* infection in humans, and ignored both the reinfection rates and the potential for development of anthelmintic resistance. There is an urgent need to address these knowledge gaps if we are to approach control of *Strongyloides* through environmental measures rather than relying solely on clinical intervention.

1.1.7. References

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1.2. Biocontrol for environmental control of the human parasite, *Strongyloides stercoralis*: A necessary next step

The following section is a published journal article authored by, Tara A Garrard, Kirstin E Ross and Michael J Taylor in *Biological Control*, published September 2016, Volume 100, Pages 25—28. (Garrard et al., 2016)

The previous article (Taylor et al, 2014) has a strong emphasis on strongyloidiasis in the Northern regions of Australia. In this setting failed infrastructure and the close relationship that humans and dogs have were identified as potential factors in the transmission process. These factors in combination with footwear not commonly being worn create some environmental reservoirs that are the focus of this research. The next article outlines how these environmental reservoirs provide an opportunity for novel research investigating the use of nematophagous fungi and their derivatives as an additional tool for the control of strongyloidiasis.

1.2.1. Abstract

Strongyloides stercoralis is a human parasitic nematode that infects 100-370 million people globally; a prevalence comparable to malaria. Currently the primary treatment for strongyloidiasis is the anthelminthic drug, ivermectin. The ruminant variant of *S. stercoralis* (*S. papillosus*) has been shown to be resistant to ivermectin. Efforts to control *S. stercoralis* therefore must extend beyond clinical treatment. A similar approach to that taken by integrated pest management systems should be

taken with this disease, including biological control. Malaria is an example of integrated pest management and multiple biocontrol approaches.

The use of nematophagous fungi is widespread in agricultural control of nematodes. A review of the literature demonstrates that nematophagous fungi to control *Strongyloides stercoralis* could be an effective approach. Here we argue that developing biocontrol methods to control *S. stercoralis* is important as multiple approaches to complicated diseases create a more robust approach to disease control.

1.2.2. Introduction

1.2.2.1. Biocontrol

Biological control or biocontrol is a pest or disease management system that uses one organism to control another. It is often used in an agricultural setting where a crop disease is controlled by a natural predator. Biocontrol is becoming an increasingly popular approach to managing livestock disease, particularly against parasitic nematodes (Larsen, 1999). The veterinary literature provides abundant reports of resistance to anthelmentics including ivermectin (Waller and Larsen, 1993) meaning other control measures, particularly those using an environmental approach as opposed to a clinical one, are gaining recognition. Biocontrol of human diseases has been investigated and must be included in disease management programs. *Strongyloides stercoralis* is a parasitic nematode that infects humans and is currently treated only by anthelminthic drugs. This sole treatment option is of concern as the ruminant variant of *Strongyloides* (*S. papillosus*) has been shown to

be resistant to ivermectin (Maroto et al., 2011). A similar approach to that taken by integrated pest management systems should be taken to this disease, which should include biological control.

1.2.2.2. Nematodes

Free living nematodes play an important role in soil health, nutrient recycling and adding to the biodiversity of the environments in which they inhabit (Neher, 2010). Notwithstanding, the spectrum of problems that can be caused by nematodes in the environment is broad, including agricultural pest nematodes and human parasitic nematodes which impact upon agricultural and human health respectively (Nicol et al., 2011, Hotez, 2008). Nematodes cause a range of crop diseases such as root rot disease, cereal cyst disease, root lesion, stem nematode disease and seed gall nematode disease. These nematodes impact on a broad range of crops including cereal crops, vegetable, pastures and grasses. Agriculturally significant pest nematodes also include veterinary nematode diseases. Some examples are *Strongyloides papillosus* which infects cattle, sheep and goats (Chandrawathani et al., 1998), *S. westeri* which infects equine species (Araujo et al., 2010), *Dictyocaulus viviparous* which infects cattle (Pezzementi et al., 2012), *Muellerius capillaris* which infects sheep (Viña et al., 2013) and *Metastrongylus* spp. which infect pigs (Alvarez et al., 2013).

Human health is also affected by nematodes with many parasitic species endemic particularly in tropical regions of the world. Some common human parasitic nematode diseases include Ascariasis (*Ascaris lumbricoides*), Trichuriasis (*Trichuris*

trichiura) also known as whipworm, and Strongyloides (*Strongyloides stercoralis*) (Bethony et al., 2006, Hanan Z. Rayan, 2012). Currently control of these parasites relies almost entirely upon clinical intervention once infection has already occurred.

1.2.2.3. Nematophagous fungi

There is now a large body of research assessing new biocontrol organisms and their modes of action (Morton et al., 2004, Lopez-Llorca et al., 2008). It is expected that there will be significant growth in the bio-pesticide market in years to come (Vos et al., 2014). One important biocontrol technique is the use of nematophagous fungi. Nematophagous fungi reside in moist soil environments and are capable of trapping and digesting nematodes. Predated nematodes are used to supplement low nutrients in some soil environments; nematodes predominantly provide an additional source of nitrogen (Nordbring-Hertz et al., 2006). There are multiple types of nematophagous fungi and they affect different parts of a nematode's lifecycle. The fungi can parasitise the eggs, the reproductive tract of an adult nematode or physically trap larvae or adult worms through constricting rings, adhesive nets, adhesive knobs or adhesive spores along with many other methods (Nordbring-Hertz et al., 2006). These multiple modes of action, together with little or no evidence of developed resistance, makes nematophagous fungi an obvious choice for biocontrol to supplement control using anthelmintics.

If resistance to human anthelminthics develops as has been seen in agricultural settings, environmental control might be one of the few tools left in our arsenal against nematodes.

1.2.3. Nematophagus fungi and biocontrol in agricultural crops

Chemical nematicides are toxic by design. Methyl bromide is a nematicide that is now recognised as an ozone depleting agent and has been phased out by most countries (Margolis et al., 2013). Carbofuran is a nematicide that is highly toxic to humans and other organisms (Gupta, 1994) in addition to the target pests (Otieno et al., 2010). It has been banned or restricted in several countries including Canada and the U.S. (Otieno et al., 2010). With few safe, biodegradable nematicidal options available, there is a need for a better alternative. The number and spread of nematode diseases has been increasing, with a concomitant impact on the agricultural industry (Nicol et al., 2011). Nematodes affect a broad range of crops resulting in effects on the production cost and quality of a large number of food products (Nicol et al., 2011). This reduces crop yields and increases the costs of measures that must take place to reduce nematode disease. Plant parasitic nematodes have been estimated to cost up to USD$358 billion per year worldwide due to yield losses (Askary, 2015).

Nematophagous fungi have been investigated as a safer and less environmentally destructive alternative to these chemicals. Nematophagous fungi also have the ability to persist and grow in soils unlike chemicals requiring repeated applications. There has been success in this research field with several fungal species available commercially such as *Paecilomyces lilacinus* (Melocon® or Bio Act®). These have efficacy comparable to chemical nematicides (Schenck, 2003). *P. lilacinus* was shown

to be up to 98.7% effective at parasitizing root rot nematode *Meloidogyne incognita* eggs in vitro (Aminuzzaman et al., 2012). *P. lilacinus* has also shown to be effective on *Meloidogyne javanica* in tomatoes (Kiewnick and Sikora, 2006).

The application of this nematophagous fungus is one of many examples of biological control successes, and serves to increase momentum for research into other areas of agriculture, conservation and human health.

1.2.4. Human health and biocontrol

Biological control has successfully been used with various human parasitic diseases. Notably, biological control has targeted vectors involved in the transmission process. Examples of this are mosquito transmitted diseases such as malaria, tsetse fly transmitted diseases such as human trypanosomiasis and tick transmitted disease such as Lyme disease. With malaria, biological control mechanisms disrupt the mosquito phase of the malaria transmission cycle. Mermithid nematodes are aquatic nematodes that parasitise arthropods, including mosquitos (Platzer, 2007). The juvenile nematode enters the host via penetration of the external cuticle, obtaining nutrients and killing the host on exit (Platzer, 2007). Entomopathogenic fungi infect adult mosquitoes via direct contact with the external cuticle (ingestion is not required) (Kamareddine, 2012) (Mnyone et al., 2012). The fungus kills the mosquito after infection. The kill rate is slower than that of insecticides (Kamareddine, 2012), however, the lower resistance rate of mosquitos to biocontrols compared with insecticides makes it advantageous. Entomopathogenic fungi are being assessed for their ability for control of malaria carrying mosquitos. In contrast, *Bacillus* toxins are

used to kill mosquitoes at the larval stage. The bacillis spores produce protoxin crystals which are toxic by ingestion (Porter et al., 1993) destroying the gut of the larvae. The bacteria are capable of colonising the reproductive system of males and the eggs of female mosquitoes meaning the next generation are killed as well (Kamareddine, 2012).

The success of biocontrol as part of mosquito management illustrates the value of focussing on intervention points external to the disease causing agent. This approach targets prevention reducing reliance on clinical intervention.

1.2.5. Strongyloides stercoralis

Strongyloides is a genus containing species that affects a number of animal hosts. There are 50 species infecting a range of mammals, birds, reptiles and amphibians (Viney and Lok, 2007). Efforts to reduce infections in *Strongyloides* species are increasing due to the rise in anthelmintic resistance in veterinary use (Gonzalez Cruz et al., 1998). Human strongyloidasis is caused by a parasitic nematode (*Strongyloides stercoralis*) with a lifecycle containing both host and free living stages. It is prevalent in many areas of the world with an estimate of 100-370 million cases worldwide (Taylor et al., 2014). Initial infection occurs when infective larvae penetrate the skin and then migrate through the body to the gastrointestinal tract, where the nematodes then reside and reproduce. *Strongyloides stercoralis* are autoinfective meaning that the infection is usually persistent until treatment occurs. A suppressed immune system can induce hyperinfection which usually leads to dissemination through other tissues and death. Anthelmentic treatment is usually with ivermectin

or albendazole. Ivermectin has the greater efficacy compared with albendazole, which is consequently less commonly used (Datry et al., 1994).

The presence of free living larvae in soil and faeces means that contact with the larvae can occur after treatment, causing infection. The likelihood of this is furthermore increased when infected individuals are living in communities where there is a close association with dogs and footwear is not commonly worn. The strain which infects dogs is morphologically indistinguishable from the primarily human infective strain (Braga et al., 2010). The route of infection is often from soil or faeces through the skin of the foot. In remote communities, where infection rates are high (Adams M et al., 2003), mass drug administration is often used as a treatment method, however reinfection is possible after treatment. Repeated use of ivermectin and similar anthelminthics may give rise to drug resistance as has occurred with other antibiotics. Of particular concern is the rise of anthelmintic resistance reports in the veterinary literature (Prichard, 1994). Importantly, *S. papillosus* has been shown to be resistant to ivermectin (Maroto et al., 2011). As noted above, the main route of transmission is from soil and faeces containing *S. stercoralis,* therefore options that focus on this stage of the lifecycle should be explored. The management of this disease should focus on environmental control in addition to the use of ivermectin. The use of ivermectin should be limited in the long run to help reduce the risk of resistance forming and encourage the use of integrated management strategies.

1.2.6. Use of biocontrol and *Strongyloides* species

Biological control in the area of nematophagous fungi is now being studied in further detail for various *Strongyloides* species due to the free living soil phase of the lifecycle.

Table 1.1 presents a summary of species of nematophagous fungi tested on *Strongyloides* spp. Work by Araujo et al. (2010) describes the effectiveness of three nematophagous fungal species predatory abilities on *Strongyloides westeri,* a species that infects equines. An in vitro assay of the predatory activities found that the decrease in L3 *Strongyloides* larvae (infectious larvae) compared to the control absent from fungi was 80% for *Duddingtonia flagrans*, 68% *Monacrosporium thaumasium*, and 73% for *Arthrobotrys robusta*. This study then also assessed the capabilities of *D. flagrans* and *M. thaumasium* to remain viable after passing through equine gastrointestinal tracts. Both fungal species remained viable and were capable of nematophagous predatory properties after passing through the GI. The results were comparable to the in vitro results.

Strongyloides papillosus is a parasitic nematode that infects ruminants and due to increasing anthelmintic resistance has been the subject of biocontrol studies using various nematophagous fungi. Chandrawathani et al. (1998) described the high replication rates of *S. papillosus* larvae in the bedding of calves and the risk of infection following this. Their study tested the effectiveness of the addition of *Arthrobotrys oligospora* conidia to bovine faeces containing *S. papillosus.* An addition of 2000 conidia/g faeces almost completely removed the infective larvae with >99% less larvae than in the control.

A study by Gonzalez Cruz et al. (1998) compared the trapping abilities of *A. robusta* and *Monacrosporium gephyropagum* on *S. papillosus.* Trapping abilities were assessed by the proportion of infective larvae that were trapped by the respective fungi after a 7 day incubation period. The results showed that 93% of larvae were trapped by *M. gephyropagum* and that 32% of larvae were trapped by *A. robusta*.

Strongyloides venezuelensis has also been used in nematophagous fungi biocontrol studies. *S. venezuelensis* infects rodents and has been used as a model organism for human strongyloidiasis due to a similar lifecycle. Braga et al. (2011) assessed the trapping abilities of *D. flagrans*, *A. robusta* and *Monacrosporium sinense* on *S. venezuelensis* L3 larvae. The percentage reductions of L3 larvae were as follows for each of the nematophagous fungi; *D. flagrans* 93%, *A. robusta* 77% and *M. sinense* 65%.

There has been one study which has explored the use of nematophagous fungi in controlling the human genus of *Strongyloides*. Braga et al. (2010) compared the predatory effects of *D. flagrans*, *M. thaumasium*, and *A. robusta* on L3 *Strongyloides stercoralis* infective larvae. The in vitro assay found that L3 larvae were reduced by 84% *D. flagrans*, 76% *M. thaumasium* and 73% for *A. robusta*. No field studies have been reported.

The results of these studies on various *Strongyloides* species demonstrates that multiple species of nematophagous fungi are capable of predating the nematodes.

Table 1.1: Species of nematophagous fungus tested on Strongyloides species

Nematophagous Fungi	**Nematode species tested**	**Host**	**References**
Duddingtonia flagrans	*Strongyloides westeri*	Equines	(Araujo et al., 2010, Araujo et al., 2012)
	Strongyloides stercoralis	Humans	(Braga et al., 2010)
	Strongyloides venezuelensis	Rodents	(Braga et al., 2011)
Monacrosporium thaumasium	*Strongyloides westeri*	Equines	(Araujo et al., 2010, Araujo et al., 2012)
	Strongyloides stercoralis	Humans	(Braga et al., 2010)
Arthrobotrys robusta	*Strongyloides westeri*	Equines	(Araujo et al., 2010)
	Strongyloides stercoralis	Humans	(Braga et al., 2010)
	Strongyloides venezuelensis	Rodents	(Braga et al., 2011)
	Strongyloides papillosus	Ruminants	(Gonzalez Cruz et al., 1998)
Monacrosporium gephyropagum	*Strongyloides papillosus*	Ruminants	(Gonzalez Cruz et al., 1998)
Arthrobotrys oligospora	*Strongyloides papillosus*	Ruminants	(Chandrawathani et al., 1998)
Monacrosporium sinense	*Strongyloides venezuelensis*	Rodents	(Braga et al., 2011)

Strongyloidiasis is a potentially fatal disease with a comparable prevalence to malaria, and only one highly effective drug available, and yet we continue to neglect broader research targets about the parasites' lifecycle or methods to reduce the number of infections rather than clinical interventions. Biocontrol must be further explored as a method of control to reduce this organism in its environment and lessen our reliance on anthelminthic treatment as a first and only line of defence.

1.2.7. Conclusion

Efforts to control *S. stercoralis* must extend beyond clinical treatment. A review of the literature demonstrates that the use of nematophagous fungus to control *Strongyloides* spp. has been effective. It therefore seems likely that nematophagous fungi will provide an effective method of control of *S. stercoralis* and this is important because multiple approaches to complicated diseases creates a more robust approach to disease control.

1.2.8. Keywords

Nematophagous fungi, Nematophagous fungi in agriculture, Strongyloides detection methods, Strongyloides ratti as a model organism

1.2.9. References

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1.3. Research aims and directions

This study's major research aims can be summarised as follows:

- To investigate molecular methods as a tool for detection of *Strongyloides stercoralis* in environmental samples
- To isolate nematophagous fungi from wet environments from Northern Territory soils in Australia
- To test the efficacy of nematophagous fungi in high nutrient substrates such as faeces
- To test the efficacy of nematotoxins of nematophagous basidiomycetes on the *Strongyloides* model nematode *S. ratti*

Environmental reservoirs of *S. stercoralis* play a key role in transmission of the disease, control of active larvae in these areas as part of an integrated management approach could help to reduce numbers of new infections. Canine faeces, soil and leaking septic tanks are the environmental reservoirs of focus for this research. This study takes on a novel approach using integrated disease management and concepts of biological control from the agricultural industry to complement the clinical approach to control a human disease. With the veterinary industry leading the way in this area, investigation into its use in human disease is overdue.

Environmental samples were collected from the Northern Territory in Australia including dog faecal and soil samples and real time PCR was used to detect *Strongyloides*, to confirm the presence of larvae in these environmental reservoirs. Soil samples collected from the Northern Territory were then used to try and isolate

native nematophagous fungi. This would potentially isolate a species of fungus that could be amplified in a population rather than introduced if it was successful in reducing the *Strongyloides* larval population.

To investigate the capacity of nematophagous fungi to trap nematodes in nutrient rich environments such as canine faeces *Arthrobotrys oligospora* was used as a model species of nematophagous fungi along with *Caenorhabditis elegans* a model species of nematode to test the efficacy of nematode trapping in different substrate types and varying nutrient levels.

Metabolites from nematophagous basidiomyectes were extracted and tested for nematotoxicity against *C. elegans* and the *Strongyloides* model *S. ratti* as a pilot study to determine if the toxins are worthy of further investigation for real world application.

2. Chapter Two: Use of real-time PCR for detection of *Strongyloides stercoralis* in environmental samples

2.2. Introduction

2.2.1. Environmental samples

To help with understanding how transmission occurs in strongyloidasis an aim was to detect the pathogen in environmental samples. This would give an indication of environmental reservoirs that contain the pathogen. This information plays a key role in controlling this disease and reducing infection rates in the future. An additional aim of collecting this data was to develop distribution maps of *S. stercoralis* in environmental samples. To achieve these aims the free-living phase of the *S. stercoralis* lifecycle that occurs in the environment was targeted.

At the commencement of this project a fellow PhD student was working on a related project with *S. stercoralis* whilst working in remote Indigenous communities as an environmental health officer. The project conducted by Francis O'Donahoo (Frank) focused on engaging with Indigenous communities and considering environmental reasons for the persistence of *Strongyloides*. The relationships that had been built between Frank and these communities provided a key component of this project by engaging the communities in the research and allowing them to have involvement

and a degree of agency on the outcomes, providing understanding and access to progress and results. This is an important part of working in Indigenous communities and engaging with them with a mutual understanding helps to ensure respect that has been abused in the past. Through Frank's links with communities, arrangements were made for collection of environmental samples potentially containing *S. stercoralis.* Potential sources had been identified as soil from areas prone to faecal contamination such as near leaking septic tanks and canine faeces. Ethics advice was sought through Flinders University for the collection of dog faecal samples from Indigenous communities to be used in PCR detection of *S. stercoralis*. The Animal Ethics Committee deemed the collection of faeces would comply with the Flinders University Animal Welfare Committees Register of Projects Deemed Not to Require Animal Ethics Approval if collection was conducted under some specific guidelines. These guidelines included having no interactions with the animals we were collecting faeces from, including observing the animal deposit the sample and collections could only occur from public property. Sample collection from private property would have involved human ethics applications as well, including the collection of soil samples.

Under these conditions it was arranged that Frank would collect samples from the Northern Territory and send them back to the Flinders University laboratory for PCR processing.

Additionally, an arrangement was developed with AMRRIC (Animal Management in Rural and Remote Indigenous Communities) an organisation providing veterinary care in rural and remote indigenous communities. They kindly agreed to send canine faecal samples to the Flinders University laboratory.

2.3. Real-time PCR analysis method

PCR methods for detection were adapted from (Sultana et al., 2013).

2.3.1. Sample storage

Samples were transported at room temperature. Once samples were received by Flinders University they were stored at 4˚C until DNA was extracted (>24 hours).

2.3.2. DNA extraction

DNA extraction was completed using the FastDNA™ SPIN Kit for Soil (MP Biomedicals) following the kit protocol. Post DNA extraction extracts were stored at -20 ˚C.

2.3.3. Real-time PCR analysis of soil and canine faecal samples from the Northern Territory

2.3.3.1. Primers

Primers were chosen based on the real-time PCR assay developed by Sultana et al. (2013).

Stro18S-1530F 5’-GAATTCCAAGTAAACGTAAGTCAT TAGC-3’

Stro18S-1630R 5’-TGCCTCTGGATATTGCTCA GTTC-3’.

Primers were diluted in nuclease free water (Qiagen) to a 1 µM solution and stored at -20˚C.

2.3.3.2. Master mix and reagents

The HotStarTaq Master Mix Kit (Qiagen) was used for reagents in real-time PCR analysis. Master mix was stored at -20 ˚C.

Negative controls were generated from DNA extracted from canine faeces from a domestic dog in South Australia which had been recently wormed and had no known exposure to *S. stercoralis*. Negative controls were stored at -20 ˚C.

Positive controls were generated from the *Strongyloides stercoralis* 18S small subunit ribosomal RNA gene, partial sequence; GenBank: AF279916.2

Strong Pos F 5'-

ACGAGGAATTCCAAGTAAACGTAAGTCATTAGCTTACATTGATTACGTCCCTGCCCTTTGTA CACACCGGCCGTCGCTGCCCGGAACTGAGCAATATCCAGAGGCAGGAA-3'

Strong Pos R 5'-

TTCCTGCCTCTGGATATTGCTCAGTTCCGGGCAGCGACGGCCGGTGTGTACAAAGGGCAG GGACGTAATCAATGTAAGCTAATGACTTACGTTTACTTGGAATTCCTCGT-3'

Positive controls were diluted to 0.001 µM individually then mixed and stored at -20 ˚C.

PCR was conducted in 25 µL strip tubes which contained:

12.5 µL HotStarTaq Master Mix (Qiagen)

2.5 µL forward primer Stro18S-1530F

2.5 µL reverse primer Stro18S-1630R

5 µL target DNA

2.5 µL nuclease free water (Qiagen)

Each PCR run contained all samples in duplicate as well as positive and negative controls and a no template (DNA) control in duplicate.

2.3.3.3. PCR conditions

Table 2.1: real-time PCR cycling conditions for detection of *S. stercoralis* in environmental samples

Process	Temperature	Time	Replication
Hold	95˚C	5 minutes	1 x
Cycling	95˚C	5 seconds	30 x
	60 ˚C	10 seconds	
Melt	60 → 90 ˚C @ 1 ˚C/step		1 x

2.3.4. Samples tested

Table 2.2: Environmental samples collected in Australia's Northern Territory for real-time PCR detection of *S. stercoralis*

Nearest town	Community or region	Number of samples collected	Source	Date received
Tennant Creek	Unknown	3	Frank	21/1/13
Tennant Creek	Unknown	5	Frank	6/6/13
Darwin	Nightcliff	3	AMRRIC	6/11/13
Darwin	Unknown	7	AMRRIC	8/11/13
Laramba	Unknown	3	Frank	18/11/13
Elliot	Unknown	8	Frank	6/6/13
Elliot	Unknown	16	Frank	26/3/14
Darwin	Minnarama	1	Frank	14/4/14
Darwin	Bagot	1	Frank	14/4/14
Kalumbulu	Unknown	13	AMRRIC	13/10/14

2.4. Results

All real-time PCR results for canine faecal and soil samples from all towns and regions were negative for *S. stercoralis*.

Real-time PCR results from Tennant Creek (6/6/13) have been included in Figure 2.1 and Figure 2.2 as an example of the PCR outputs which showed no detection of *S. stercoralis* in all samples. Figure 2.1 displays amplification of the positive controls and no amplification of no-template controls, negative controls or samples 1-5. Figure 2.2 displays the melt curve analysis from the PCR run with a clear peak at 83.8˚C in the positive controls and no clear peaks for no-template controls, negative controls or samples 1-5. Combined, these results confirm that there was no detectable *S.*

stercoralis in all samples collected, a result which was consistent across all sampling locations.

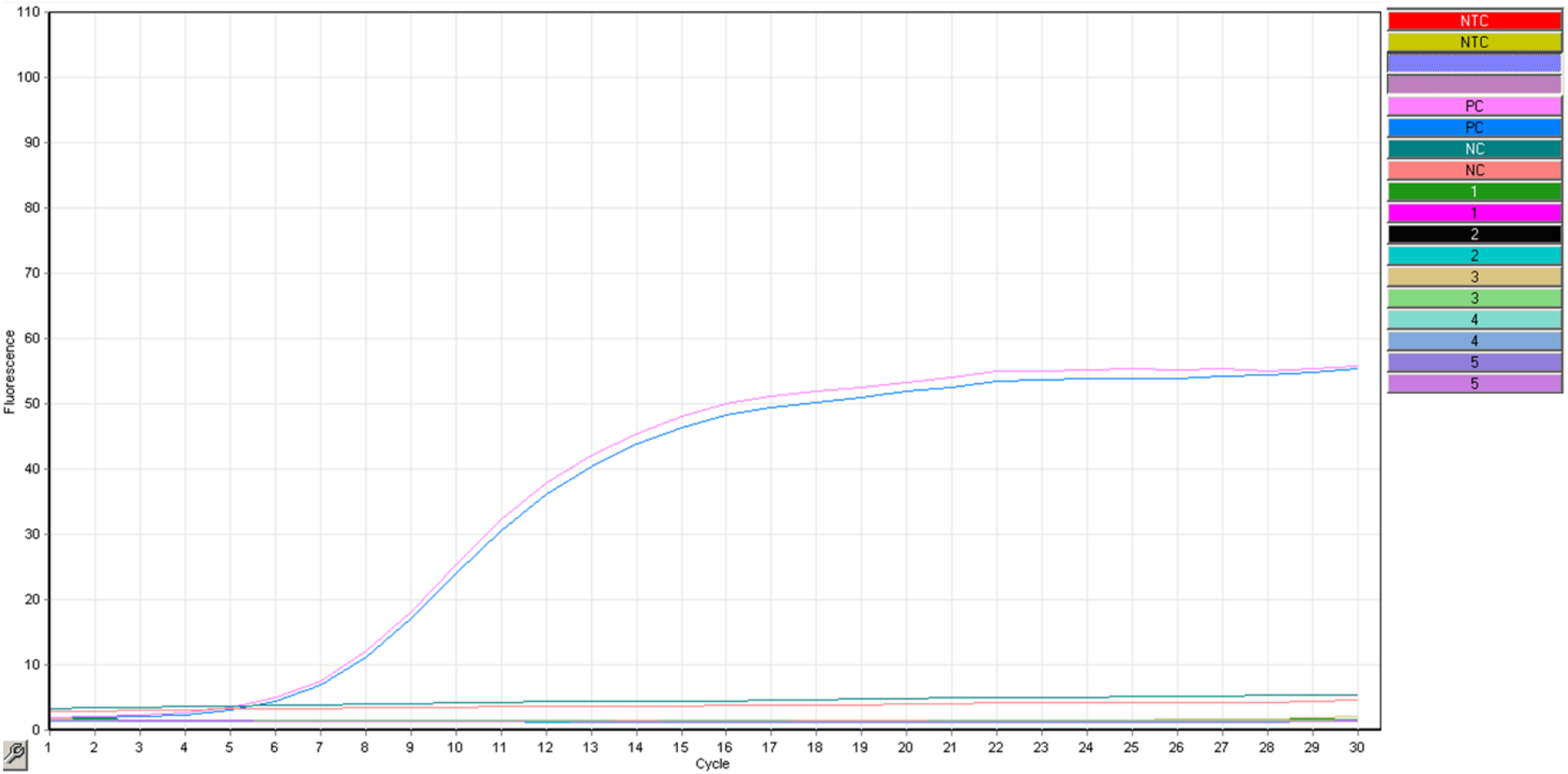

Figure 2.1: Real-time PCR cycling results for canine faecal extracts from Tennant Creek (6/6/13), positive controls (PC) displayed fluorescence whereas negative (NC) and no-template controls (NTC) and all samples (1-5) did not

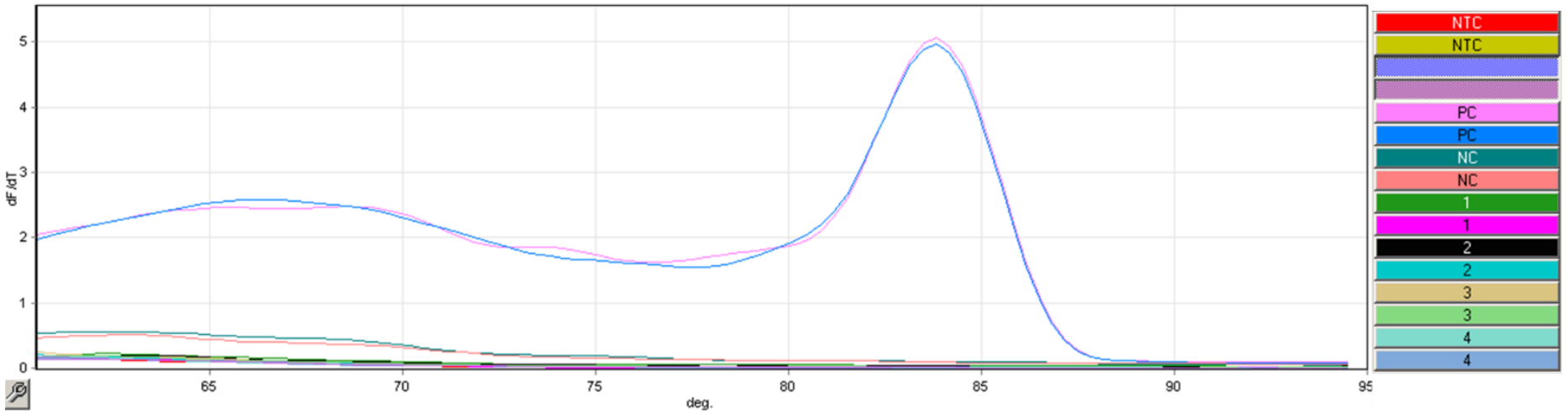

Figure 2.2: Real-time PCR melt curve analysis results for canine faecal extracts from Tennant Creek (6/6/13), positive controls (PC) displayed a clear peak at 83.8˚C whereas negative (NC) and no template controls (NTC) and all samples (1-5) did not display peaks at this temperature

2.5. Issues and pathways forward

The interval between sample collection and arrival of samples at the laboratory varied greatly and was dependent on the collaborators collecting the samples. Unfortunately, this was often longer than 1 week and up to 5 weeks, with the storage conditions before postage also varying dependent on access to refrigeration. Ultimately it was concluded that this was having a large detrimental impact on the results and could be contributing to the lack of detection.

2.5.1. Degradation of DNA in environmental samples

It was not possible to tell if the samples were true negatives or if there was DNA degradation in the samples. Through the *National Strongyloides Working Group* we were aware that other members of the group from the Centre for Infectious Diseases and Microbiology at the Institute of Clinical Pathology and Medical Research at Westmead Hospital were investigating the use of DESS (dimethyl sulfoxide, disodium EDTA, and saturated sodium chloride) as a means of preservation for faecal samples containing *S. stercoralis*. This work had not yet been published and had investigated long term cold storage of faecal samples. The Flinders laboratory decided to continue this work to investigate DESS as a means of sample preservation at room temperature for transport by commencing another research project undertaken by honours student Shelby Millsteed.

2.5.2. Sample collection issues

After several negative results in samples from AMRRIC, further consideration was given to sample collection process. It was concluded that the vets taking the samples were likely collecting them multiple days after the dogs had received worming treatment. This could have caused there to be no *S. stercoralis* larvae in the faeces. Faecal samples collected after this point were to be collected pre-worming treatment. However, with the additional DNA degradation issues sampling was put on hold.

Unfortunately, just over 12 months into the project Frank became unwell and sample collection was suspended until his health improved enough for him to return to work. The intention was for the real-time PCR detection of *S. stercoralis* from environmental samples to resume with an improved sample transport method, AMRRIC samples collected pre-worming and with Frank being able to return to sample collection. After 12 months of focussing on other aspects of the project Shelby Millsteed had collected enough preliminary data to suggest a DESS method for preservation and transport of samples was viable. The most successful method as determined by this work was the use of a 3:1 ratio of DESS to sample, extensive homogenisation of the sample in DESS and limited air space in the sample tube (Millsteed, 2015).

Unfortunately, Frank's health did not improve and in early 2016 he sadly passed away. With Frank's links in the Northern Territory providing the primary source of sample collection it was decided that this avenue of the project would be concluded and aspects of environmental control would become the primary focus of the work.

2.5.3. Future Pathways

Whilst the combination of these issues made it unsuitable to continue this line of research within this project, the work held the potential to deliver useful information to the *National Strongyloides Working Group* and could help to inform transmission of strongyloidiasis. The development of the DESS method and the enthusiasm of the AMRRIC collaborators allowed this project to be continued by another Flinders University PhD student, Mira Beknazarova. Mira has been building relationships with environmental health officers in the Northern Territory and investigating pathways to continue sample collection.

It was ultimately decided that this project would focus on developing potential environmental control methods using fungi and fungal extracts. The basis for this can be divided into the attempt to isolate and characterise native nematophagous fungi to the Northern Territory, Australia, identification of substrates and their nutrient profiles that nematophagous fungi are active in, and the extraction of nematotoxic compounds from nematophagous fungi and their activity on *C. elegans* and *S. ratti*.

2.6. References

MILLSTEED, S. 2015. *DESS as a preservative of Caenorhabditis elegans DNA in a dog faecal matrix for detection by Real-Time Polymerase Chain Reaction.* Hons, Flinders University.

SULTANA, Y., JEOFFREYS, N., WATTS, M. R., GILBERT, G. L. & LEE, R. 2013. Real-time polymerase chain reaction for detection of *Strongyloides stercoralis* in stool. *American Journal of Tropical Medicine and Hygiene,* 88**,** 1048-1051.

3. Chapter Three: Isolation and characterisation of nematophagous fungi from Northern Territory soils

3.1. Introduction

The aims were to isolate nematophagous fungi from Northern Territory soils with the intention of assessing them as potential biological control agents for *Strongyloides stercoralis*. The aim of isolating native nematophagous fungi from NT soils was to adapt an augmentation biocontrol approach utilising fungi that were already native to the region. This ideally would have involved sampling from areas around leaking septic tanks to isolate nematophagous fungi which would be tested against *Strongyloides* spp. However, there were a series of limitations surrounding this and the methods which could be used. These will be discussed throughout the chapter.

Nematophagous fungi are predatory and specialized at trapping and digesting nematodes (Nordbring-Hertz et al., 2006). There are numerous methods of trapping and immobilizing nematodes such as; adhesive branches or knobs, adhesive spores, constricting rings, and egg penetration (Nordbring-Hertz et al., 2006). These fungi then have different mechanisms for digesting the nematode depending on the trapping or immobilization method (Ahrén et al., 1998). These predatory fungi can dwell in soil, leaf litter or animal faeces and are most commonly found in the top 30

cm of soil (Pendse et al., 2013, Smith and Jaffee, 2009). Nematodes are abundant in our environments with over 26,000 free-living and parasitic species currently known and 42% of described species being terrestrial (Stirling, 2014). Nematode communities are typically segregated into plant parasites, fungal feeders, bacterial feeders, substrate degraders (organic matter), predatory nematodes, omnivores and animal parasites (Stirling, 2014).

3.1.1. Biological control methods

Biocontrol can be implemented via various methods depending on the source and application of the agent. Each discipline has adopted different terminology for biocontrol agents such as antagonists and competitors, predators and parasitoids, mass release or inoculative augmentation (Eilenberg et al., 2001). Eilenberg identifies the four main types of biocontrol as: Classical biocontrol, Inoculation biocontrol, Inundation biocontrol and Conservation biocontrol. Definitions of each term by Eilenberg et al (2001) are:

Classical biocontrol: "The intentional introduction of an exotic, usually co-evolved, biological control agent for permanent establishment and long-term pest control."

Inoculation biocontrol: "The intentional release of a living organism as a biological control agent with the expectation that it will multiply and control the pest for an extended period, but not permanently"

Inundation biocontrol: "The use of living organisms to control pests when control is achieved exclusively by the released organisms themselves"

Conservation biocontrol: "Modification of the environment or existing practices to protect and enhance specific natural enemies or other organisms to reduce the effect of pests"

This research initially aimed to isolate nematophagous fungi from habitats native to where the biocontrol would take place. Classical biocontrol involves introduction of an exotic species and is therefore not included in the initial approach to *Strongyloides* control. Shah and Pell describe augmentation biocontrol as augmenting the natural enemies of a pest in an environment (Shah and Pell, 2003); this can be done via inundation or inoculation. They describe inundation as applying the biocontrol agent in large quantities for short term control, and inoculation as introduction of a small amount of the agent which would then establish itself and repeatedly cycle (Shah and Pell, 2003, Weiser et al., 1976). The fate of the biocontrol agent in the environmental reservoir of *Strongyloides stercoralis*, eg. leaking septic tanks, is unknown. However, the aim would be to augment a native species by isolating it from this environment, increasing the population in the lab and reintroducing the fungus in large quantities. Whether the fungus would return to natural levels in the environment or establish itself with a more active role in the environment is unknown. Therefore we will refer to the biocontrol method as augmentation rather than attempting to classify as inoculation or inundation.

3.1.2. Parasitic nematodes and nematophagous fungi

Many species of nematode are parasitic to livestock and have a significant impact on the health of the animals. This also affects the farmers by increasing treatment and

management costs; and negatively impacts the total production of meat and animal products (McLeod, 1995). Gastrointestinal parasitic nematodes usually infect the animal by entering through the skin (usually the feet through the soil or bedding) or by ingestion and then migrate through the body to the lungs via the venous system. They then cross the barrier from the venous system into the alveoli of the lungs where they are coughed up and swallowed. They then breed and reside in the gut of the animal (Chandrawathani et al., 1998). Nematode species such as *Strongyloides papillosus* which infects cattle, sheep and goats follow this lifecycle and infect via skin penetration (Chandrawathani et al., 1998).

Human health is also affected by nematodes with many parasitic species endemic particularly in tropical regions of the world. Some common human parasitic nematode diseases include Ascariasis (*Ascaris lumbricoides*), Trichuriasis (*Trichuris trichiura*) also known as whipworm, Hookworms (*Necator americanus* or *Ancylostoma duodenale*) and Strongyloides (*Strongyloides stercoralis*) (Bethony et al., 2006, Hanan Z. Rayan, 2012). *Strongyloides stercoralis* is estimated to have up to 300 million current cases worldwide and hookworm 500 million (Taylor et al., 2014, Bartsch et al., 2016). The economic and health burden of hookworm has been estimated at up to US $138.9 billion worldwide (Bartsch et al., 2016).

Reliance on chemical nematicides is reducing due to their detrimental impacts on human and environmental health and a need for safer control options such as biocontrol has developed (Khan and Kim, 2007, Stirling, 2014). The use of

nematophagous fungi as a measure for the bio-control of pest nematodes has been considered as an option in a shift to more organic farming methods (Falbo et al., 2013). There is also a possibility for nematophagous fungi to be used as a bio-control measure in human and animal parasitic nematodes which include a free-living soil dwelling stage of their life cycles (Garrard et al., 2016). It is therefore important to continue isolating and characterizing fungal strains which may possess these capabilities (Falbo et al., 2013).

Paecilomyces lilacinus has been tested as a biological control measure on tomatoes against the root knot nematode *Meloidogyne incognita* (Kiewnick and Sikora, 2006). This species is also known to parasitize eggs, juveniles within eggs and adult females of root knot nematodes *Meloidogyne* spp. and the eggs and adults of cyst nematodes *Globodera* and *Heterodera* (Mitchell et al., 1987). It has been previously used as a bio-control agent and many commercial products are currently available, demonstrating the practical capability of these organisms to be applied on an agricultural scale.

Preliminary research has been conducted investigating the use of nematophagous fungi as biological control agents of free-living stages of *Strongyloides* spp. (Araujo et al., 2010, Araujo et al., 2012, Braga et al., 2011, Braga et al., 2010, Chandrawathani et al., 1998, Gonzalez Cruz et al., 1998). These studies were summarised in the introductory chapter and have shown potential in vitro.

3.2. Isolation or identification of nematophagous fungi from soil methods

Detection of nematophagous fungi is traditionally done via culture methods, however more recently molecular methods are becoming available (Smith and Jaffee, 2009). Commonly soil, leaf litter, decaying wood, dung, compost and moss samples are used for the isolation of nematophagous fungi (Smith and Jaffee, 2009, Juniper, 1957, Duddington, 1951, Dixon, 1952). Culture and molecular methods are described below.

3.2.1. Culture methods

There are several methods which can be used to isolate nematophagous fungi from soil. The sprinkle plate technique and the Baermann funnel technique are common techniques used for the isolation of nematophagous fungi (Park et al., 2002). The Baermann funnel method works on the assumption that in natural populations of nematodes a percentage of them will be infected with nematophagous fungi, this is targeted at endoparasitc nematophagous fungi, whereas the sprinkle plate technique is targeted at *Orbililales* (Park et al., 2002, Gray, 1984). The sprinkle plate technique involves adding model nematodes such as *Caenorhabditis elegans* as bait to soil, faeces or other substrates (Park et al., 2002). Nematophagous fungi within these samples are stimulated by the nematodes and then capture them. This can be viewed under a dissecting microscope and individual hyphal strands involved in trapping can then be subcultured and isolated.

The sprinkle plate method involves using 2% water agar (WA) in a petri dish, sometimes with the addition of antibiotics (Gray, 1984, Park et al., 2002). A small amount (0.5-2 g) of the substrate sample (soil, leaf litter etc.) is sprinkled into the centre of the agar plate and 1000-5000 washed, free-living nematodes such as *Caenorhabditis elegans* or *Panagrellus redivivus* are added to the plate (Park et al., 2002, Gray, 1984). Plates are incubated at 20˚C and observed daily under a dissecting microscope for trapped nematodes. Fungal hyphae from trapped nematodes are subcultured onto potato dextrose agar (PDA) or other suitable fungal medium. Gray highlights that whilst very effective this method is very time consuming and it is difficult to test multiple samples at once (Gray, 1984). Plate observation can last from weeks to months (Persmark and Jansson, 1997, Santos et al., 1991).

The Baermann funnel technique extracts the nematodes from the substrate sample by using approximately 30 g of substrate layered in tissue and placed in standard Baermann apparatus (Gray, 1984, Peters, 1955). The nematodes are then collected, concentrated and added to 2% WA, incubated at room temperature and then observed and examined, like the sprinkle plate technique, under a dissecting microscope for nematophagous fungi (Gray, 1984).

The methods described above are suitable for nematode trapping fungi but are not suitable for other nematophagous fungi types such as egg penetrative fungi. *Paecilomyces lilacinus* a species of egg penetrative fungi can be isolated from soils and similar substrates with the aid of a selective medium (Mitchell et al., 1987). The medium slows down the growth of other fast growing species of mould and inhibits the growth of bacteria. The media has a high salt content and contains

pentachlorinitrobenzene which is a fungicide that *Paecilomyces lilacinus* is resistant to (Mitchell et al., 1987). The media allows *Paecilomyces lilacinus* to grow as it can tolerate the harsh chemical composition of the media that many other faster growing fungi cannot. The colonies having a distinctive pigmentation and texture are obvious when on this media allowing for subcultures to be easily taken (Mitchell et al., 1987).

Paecilomyces lilacinus colonies have distinctive morphology on standard fungal agar such as PDA. The colonies are pigmented with a lilac colour and have a suede like texture (Gordon and Norton, 1985). These characteristics make isolation of this species from soil or faeces much easier. The species is however relatively slow growing and is often overtaken with other faster growing species, frequently of the genus *Aspergillus* or *Penicillium*.

3.2.2. Molecular methods

Nematophagous fungi stem from a broad array of taxonomic classifications. The broad range of trapping mechanisms divide them up into different taxonomic divisions. These include *Ascomycota, Basidiomycota, Oomycota, Zygomycota* and *Chytridiomycota* with a range of different families within these (Nordbring-Hertz et al., 2001). This broad taxonomic array makes molecular identification of nematophagous fungi challenging, particularly as many of these divisions are frequently reclassified. However, particular trap types cluster together in terms of taxonomy. For example nematophagous species forming adhesive nets, branches and knobs and constricting rings for the most part are Ascomycetes in the order *Orbiliales* (Nordbring-Hertz et al., 2001). Therefore nematophagous fungi described

as orbilaceous are fungi that specifically form traps as their nematophagous mechanism (Park et al., 2002). Fungi using other trapping mechanisms are more diverse in their taxonomy making the *Orbiliales* a key target for molecular detection and identification. Ahrén et al. conducted a phylogenetic analysis of nematophagous fungi including nematophagous *Orbiliales* (Ahrén et al., 1998). Sequences of the small subunit (SSU) ribosomal DNA (18S rDNA) were used to examine the relationships between these species, the study found that there were three separate linages which could be classified as: species forming constricting ring, non-parasitic species and species forming adhesive structures (Ahrén et al., 1998). Smith and Jaffee developed *Orbiliales* specific PCR primers targeting the ITS and 28s regions and used this technique to compare with the sprinkle plate technique (Smith and Jaffee, 2009). The study isolated eight species of nematophagous fungi with the sprinkle plate technique and only three of those were detected with the molecular assay (Smith and Jaffee, 2009). However, there were an additional 18 species detected by the molecular technique that were not notable in culture, and these were closely related to nematophagous fungi (Smith and Jaffee, 2009). The species detected in the molecular assay could be divided into two clades: *Orbilia* and *Brachyphoris* clade, *Brachyphoris* parasitises nematode eggs (Smith and Jaffee, 2009). Sequences of the *Orbilia* were either exact matches or very closely related to known nematophagous fungi (Smith and Jaffee, 2009). With the potential for these to be slight genetic variants or unknown nematophagous fungi, this method could be used for detection of nematophagous fungi but may be best suited in combination with traditional culture methods.

This study will use the sprinkle plate technique and the *Paecilomyces* selective media, molecular approaches do not isolate target species and still need to be used in conjunction with an isolation technique if this is the aim. The sprinkle plate method was chosen as the nematode trapping is observed before isolation and could reduce the number of steps and sub-culturing involved, this approach is also targeted at orbilacious nematophagous fungi whereas the Baermann funnel method is targeted at endoparasitic fungi (Gray, 1984).

3.2.3. Ethics of augmentation biocontrol method in Indigenous communities

The approach of augmentation biocontrol has been selected for a series of reasons in this scenario, many of which include significant cultural sensitivities. The area chosen for this study was leaking septic tanks in the Northern Territory in Australia with *S. stercoralis* contamination. Many of these are located within Indigenous communities. Many indigenous communities in Australia are closed communities with access to the community granted following extensive consultation and approvals by community elders.

Community engagement is a very important part of conducting research in indigenous communities and is often overlooked by government bodies and research groups. Tindana et al. (2007) describes community engagement as going beyond community participation but as *"working collaboratively with relevant partners who share common goals and interests."* This also involves having members of the community active in every stage of the research process (Tindana et al., 2007). In

addition to these ethics considerations it is also essential to have formal ethics approval from the institution hosting the research.

The concepts of this research project have been discussed extensively with researchers within our own institution but also with Indigenous environmental health officers working in communities and with the *National Strongyloides Working Group* (NSWG) in Australia. The NSWG is comprised of parasitologists, veterinary and molecular scientists, pathologists, environmental health officers, medical practitioners, health promotion and prevention professionals, the group's goal is to eliminate the disease from Australia (Ross et al., 2017). All parties have endorsed the research concepts, and community engagement surrounding *S. stercoralis* research in Australia which is active and ongoing. In close consultation with NSWG we decided to postpone working inside communities until some of the initial pilot research was completed in the laboratory.

The augmentation approach was chosen as increasing the population of a native species is deemed as lower risk than introducing an entirely new species. We inferred that considering the sensitivities around working in indigenous communities the fewer risks and changes that needed to be implemented, likely the greater the level of community acceptance. An option that required as little interference as possible and held the least number of risks was deemed appropriate.

However, considering the level of community engagement required, time, finances and the ethics approval the research group decided to run pilot experiments with soil samples native to the Northern Territory in similar areas to leaking septic tanks but outside of Indigenous communities. If the results of the initial laboratory based work

were promising using this biocontrol approach then the steps required to work inside communities would be taken.

3.3. Aims and Objectives

The aims and objectives of this research were to isolate and characterise orbilacious nematophagous fungi and *Paecilomyces lilacinus* native to the Northern Territory of Australia. The sample collection was to take place outside of indigenous communities in areas which remained consistently wet. The sprinkle plate technique and use of *Paecilomyces* semi selective media were identified as the most relevant isolation methods. Compound and dissecting microscopy were to assess nematophagous fungi in culture.

3.4. Methods

3.4.1. Media and reagents

Paecilomyces semi selective media

- 5 g sodium chloride (Merck Pty. Limited)
- 25 mg pentachloronitrobenzene (Sigma-Aldrich Co.)
- 25 mg benomyl (methyl 1-(butylcarbamoyl)-2-benzimidazolecarbamate) (Sigma-Aldrich Co.)
- 19.5 g potato dextrose agar (Oxoid LTD.)

- 500 mL water (MilliQ)

Post sterilization (aseptically added using 0.2 µm syringe filter):

- 50 mg streptomycin sulfate (Sigma Chemical Co.) added as 1 mL of 0.5 g/10 mL milliQ water
- 25 mg tetracycline hydrochloride (Progen Industries Limited) added as 1 mL of 0.25 g/10 mL milliQ water
- 0.5 mL tergitol NP10 (Sigma Chemical Co.)

Czapek Dox Agar

- 23 g Czapek dox medium (Oxoid LTD.)
- 500 mL water (MilliQ)

Potato dextrose agar

- 19.5 g potato dextrose agar (Oxoid LTD.)
- 500 mL water (MilliQ)

0.25% Malt yeast extract agar

- 10 g technical agar (Oxoid LTD.)
- 3.8 g malt extract (Oxoid LTD.)
- 0.62 g bacto peptone (Difco Laboratories)
- 0.30 g yeast extract (Oxoid LTD.)
- 500 mL water (MilliQ)

2% Water Agar

- 10 g technical agar (Oxoid LTD.)

- 500 mL water (MilliQ)

Nematode growth medium

- 3 g sodium chloride (Merck Pty. Limited)
- 17 g technical agar (Oxoid LTD.)
- 2.5 g bacto peptone (Difco Laboratories)
- 975 mL water (MilliQ)

Post sterilization (aseptically added using 0.2 µm syringe filter):

- 1 mL cholesterol (The British Drug Houses Ltd.) suspended in 5 mL ethanol (Ace Chemical Co.)
- 1 mL 1 M calcium chloride dihydrate (Merek Pty. Limited)
- 1 mL 1 M magnesium sulphate (Merek Pty. Limited)
- 25 mL 1 M potassium dihydrogen orthophosphate (AJAX Chemicals Australia Pty Ltd.)

Nutrient Agar

- 28 g Nutrient agar (Oxoid LTD.)
- 1 L water (MilliQ)

M9 Buffer

- 3 g potassium dihydrogen orthophosphate (AJAX Chemicals Australia Pty Ltd.)
- 6 g disodium hydrogen phosphate (Sigma-Aldrich Co.)
- 5 g sodium chloride (Merck Pty. Limited)
- 1 L water (MilliQ)

Post sterilization (aseptically added using 0.2 µm syringe filter):

- 1 mL 1 M magnesium sulphate (Merek Pty. Limited)

All media were autoclaved at 121˚C for 30 minutes then cooled in a water bath to 50˚C unless otherwise stated.

4% Methylene blue

- 1 g powdered methylene blue (Sigma-Aldrich Co.)
- 25 mL water (MilliQ)

3.4.2. Free-living nematode used in isolation

The free-living nematode *Caenorhabditis elegans* were used to isolate nematophagous fungi from soil. *C. elegans* culture kindly provided by Peter Boag, Monash University.

C. elegans were maintained on nematode growth medium in petri dishes with an *E. coli* OP50 lawn at 20 ˚C (Stiernagle, 2006). *E. coli* OP50 was grown on nutrient agar at 20 ˚C for 7 days, 500 µL of sterile MilliQ water was then aseptically pipetted onto the agar plate manually agitated and the liquid collected in the pipette. 100 µL of this liquid was pipetted onto nematode growth medium and spread aseptically with spreading rod. 20 µL of liquid containing *C. elegans* either from long term culture stock or collected from previous maintained culture was pipetted onto the nematode

growth medium with *E. coli* OP50 lawn. Plates were incubated at 20 ˚C and nematodes transferred to fresh plates every 2-4 weeks.

Nematodes are harvested for assays at 1-2 weeks.

3.4.3. Soil Samples

Soil samples were collected from the Northern Territory in Australia from locations (Table 2.1) that appeared to be consistently damp (nearby creeks and creek beds, under leaf litter, under dripping taps).

Table 3.1: Northern Territory soil sample locations

S 12˚33’548 E 131˚04’341	S 12˚34’553 E 131˚05’891	S 13˚02’811 E 131˚01’723	S 1˚02’463 E 131˚04’863
S 12˚53’688 E 131˚08’425	S 12˚40’403 E 131˚04’745	S 23˚74’781 E 133˚85’992	S 23°41'205 E 133°52'135

Soil samples were placed in sealed bags and stored at room temperature.

3.4.4. Plate Method for the isolation of *Paecilomyces lilacinus* from soils

Media (Mitchell et al., 1987) was prepared to isolate *Paecilomyces lilacinus* from soil samples. 100 µL of each soil sample solution was then aseptically added to a Petri dish containing *Paecilomyces* semi selective agar media (Mitchell et al., 1987) and then spread plated. The agar plates were then incubated at 25˚C for 10 days. The plates were assessed every 2-3 days for fungal colonies resembling *P. lilacinus* (lilac

pigmented, suede textured colonies). Colonies resembling *P. lilacinus* were then aseptically subcultured onto Czapek Dox agar and incubated at 25˚C for 10 days (Gordon and Norton, 1985, Cabanillas et al., 1989).

3.4.5. Sprinkle plate technique

1 g of soil sample was placed in the centre of a petri dish containing 2% water agar + 0.05% chloramphenicol (Sigma-Aldrich Co.). The plates were incubated at 25˚C for 1 week or until fungal mycelium growth appeared on the plate. *C. elegans* were then washed from a nematode growth medium plate using 1 mL of M9 Buffer. The nematodes and buffer were centrifuged in a 1.7 mL microcentrifuge tube for 5 minutes at 80 rcf. Supernatant was removed and the process was repeated four additional times. 1 mL 0.05% chloramphenicol and streptomycin was added to the nematodes and incubated at room temperature for 1h. The nematodes were then centrifuged at 80 rcf for 5 minutes and the supernatant was then removed. 1 mL of M9 buffer was added. Nematodes were then counted using a Koga Slide and the concentration of worms/µL calculated. The solution was diluted as required and 1000 nematodes added to each plate around the soil. The plates were then incubated at 20˚C. Plates were viewed under dissecting microscope every 2-3 days for trapped nematodes. Trapped nematodes were aseptically transferred to potato dextrose agar to isolate the fungus and incubated at 25 ˚C. The fungal isolates were subcultured until clean if necessary.

3.4.6. Method positive control

This method was repeated using a nematophagous fungus to spike the soil. Soil collected from Flinders University campus was autoclaved at 121˚C for 30 minutes and allowed to cool. Soil was then spread into two clean petri dishes and inoculated with *Arthrobortys oligospora* agar plugs, plates were incubated at 25˚C. Once fungal growth reached the edge of the petri dish the sprinkle plate technique and assessment of cultures isolated from sprinkle plate technique (above) were repeated using this soil.

3.4.7. Assessment of cultures isolated from sprinkle plate technique

Isolates from the sprinkle plate technique were then assessed for nematophagous capabilities. Cultures were transferred from potato dextrose agar to 0.25% malt yeast extract agar or 2% water agar.

Cultures were incubated at 25˚C until growth reached edges of the petri dish. Approximately 1000 *C. elegans* were transferred to each plate following the washing and counting steps described above. Plates were then assessed using the dissecting microscope and the compound microscope. Flag preparations were used to create slides for the compound microscope. A 2.5 cm section of clear tape was placed directly onto the culture and lightly pressed against the mycelium. 5 µL 4 % methylene blue or lactophenol blue (Sigma-Aldrich Co.) were transferred to a clean microscope slide and the tape lift placed on top (Harris, 2000). Excess stain is wiped

away. Microscopic assessment of the culture is used to morphologically identify the genus of fungus if possible and assess nematophagous properties.

3.4.8. Viability stains

Lactophenol blue was used for fungal identification and 4% methylene blue was used as a nematode viability stain. After some experimentation it was observed that the methylene blue at a higher concentration only penetrated the nematode if there was damage to the sheath. This stain was then used for assessing the fungal nematode interactions.

3.5. Results

3.5.1. Northern Territory soils

3.5.1.1. Sprinkle plate technique

Several potential nematophagous fungi were isolated from the Northern Territory soils and the genus of each isolate was identified where possible. Determination of the isolate's nematophagous capabilities proved to be difficult and will be expanded upon in further detail in the discussion. Identification to genus level showed that none of the isolates were strictly nematophagous, however, the characteristics of the fungi in the presence of nematodes showed that some interactions were occurring between the organisms. The characteristics are listed for each genus and discussed in more detail in the discussion.

Fungal isolates taken from the Northern Territory were identified as either *Trichoderma, Fusarium* or *Cunninghamella*. These three genera were consistently isolated from all soil samples, species identification was not completed due to lack of nematophagous capabilities and were therefore not target isolates.

3.5.1.2. Sprinkle Plates

Despite yielding no nematophagous isolates the sprinkle plate technique was an effective method for viewing fungal nematode interactions from the soil fungi. Figure 3.1 below shows the sprinkle plate set up in the Petri dish before incubation. Figure 3.2 and Figure 3.3 display the hyphal network of the fungi and examples of nematodes being colonised by them, additionally un-trapped nematodes are displayed in

Figure 3.7 and Figure 3.8. These nematodes being colonised by the fungal hyphae are examples of nematodes that were picked and sub-cultured. Figure 3.4 shows the diversity of isolates from this technique. These isolates could predominantly be classified as *Trichoderma, Fusarium* or *Cunninghamella*, descriptions used to classify these genera are described below.

Figure 3.1*:* Sprinkle plate technique with soil sample and *C. elegans* as bait

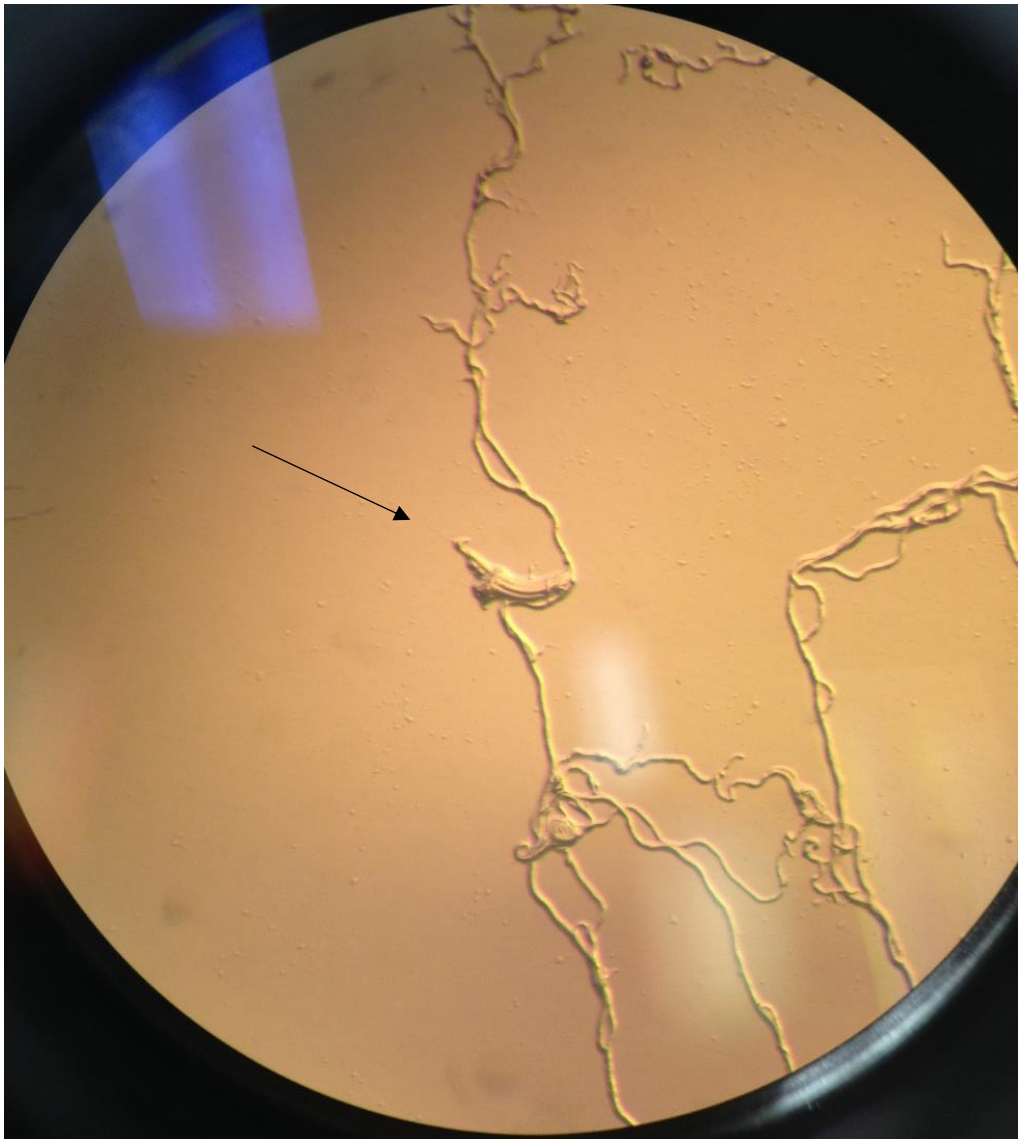

Figure 3.2: Example of *C. elegans* appearing trapped from sprinkle plate

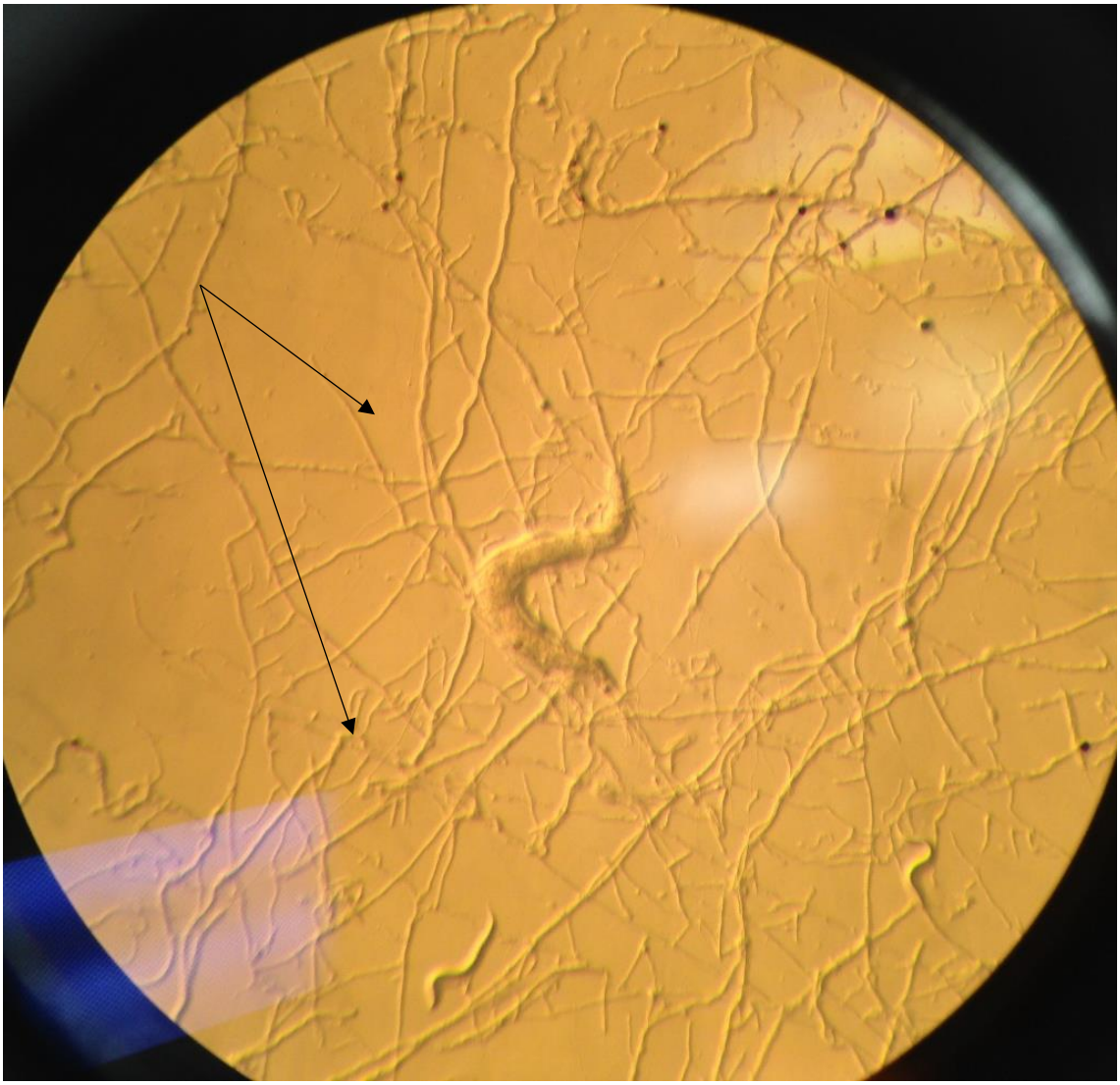

Figure 3.3*:* Example of *C. elegans* potentially trapped and being digested by fungal hyphae on sprinkle plate as well as free nematodes

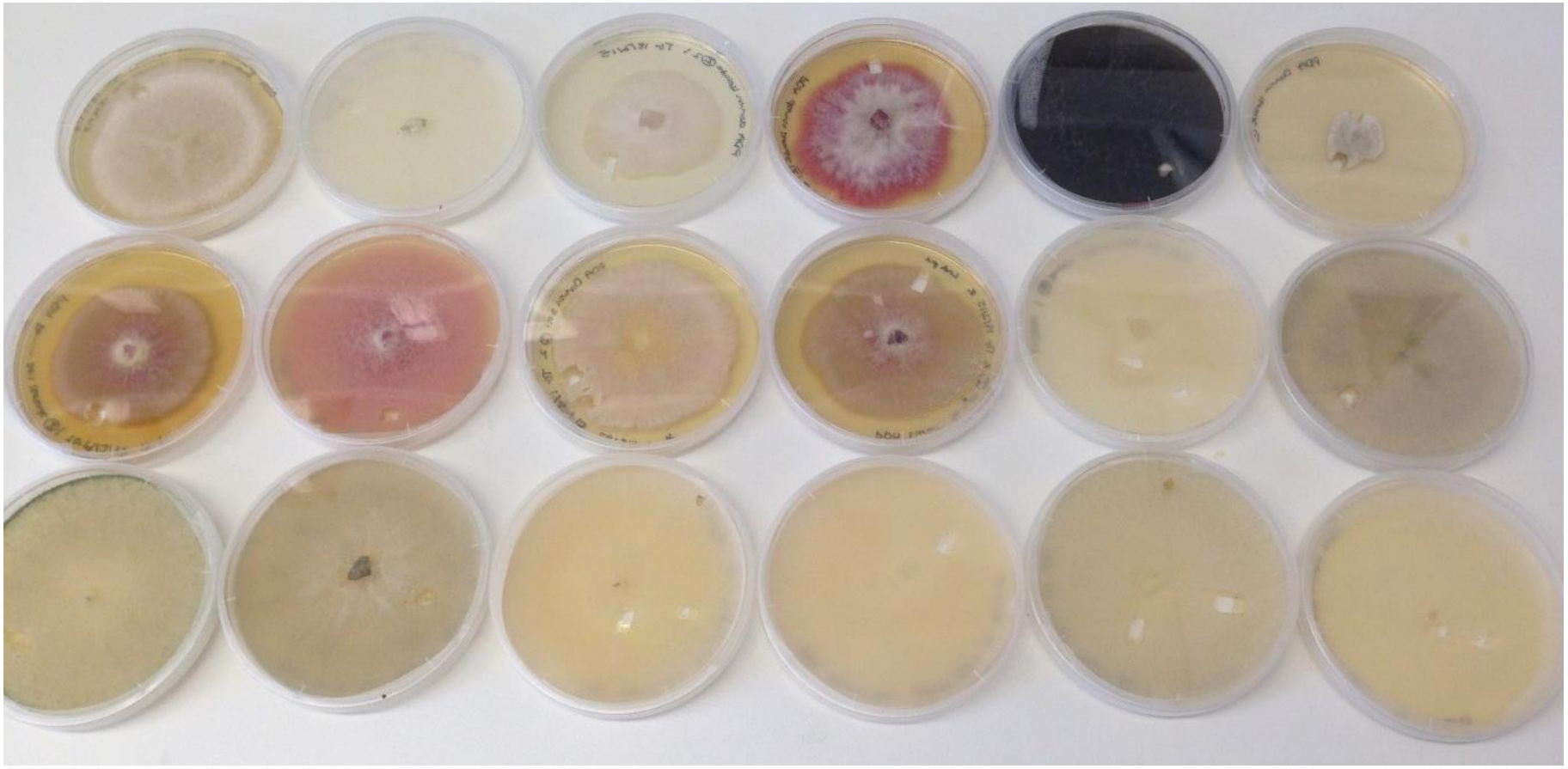

Figure 3.4*:* Isolates from sprinkle plate technique at various stages of growth, pigmentation and sporulatio*n*

3.5.1.3. Assessment of cultures isolated from the sprinkle plate technique

Assessment of cultures for nematophagous capabilities required viewing the fungal nematode interactions in more detail. Creating slides for the compound microscope enabled these interactions to be seen in greater detail.

Isolates on 2% WA produce limited aerial mycelium, this enabled clear viewing of hyphae and nematodes under the dissecting microscope. However, details of interactions could not be seen at this level of magnification. The limited aerial mycelium created difficulties with preparing flag preparations as the hyphae growing on the direct surface of the agar were well attached and did not lift onto the tape. The nematodes also displayed shallow burrowing into the agar on this medium. When flag preparations were created from these plates there was not enough fungal hyphae or nematodes on the slides to assess the interactions.

The ¼ strength malt yeast extract agar produced more aerial mycelium than the 2% WA and denser overall growth. Whilst denser growth was observed on this agar it was sparser than growth on PDA or other full strength fungal medium. This allowed for flag preparations to be easily prepared with a denser aerial mycelial network. Viewing the fungal nematode interactions was easier with this method, however, the number of nematodes picked up by this technique was minimal and many slides had to be made to view the interactions.

3.5.1.3.1. *Trichoderma sp*. and assessment of nematophagous activity

Trichoderma: colonies fast growing and dark green pigmentation post sporulation on agar. Microscopic morphology - Septate hyphae forming phialides. Phialides typically branched in groups of three producing single celled globose conidia (Watanabe, 2010).

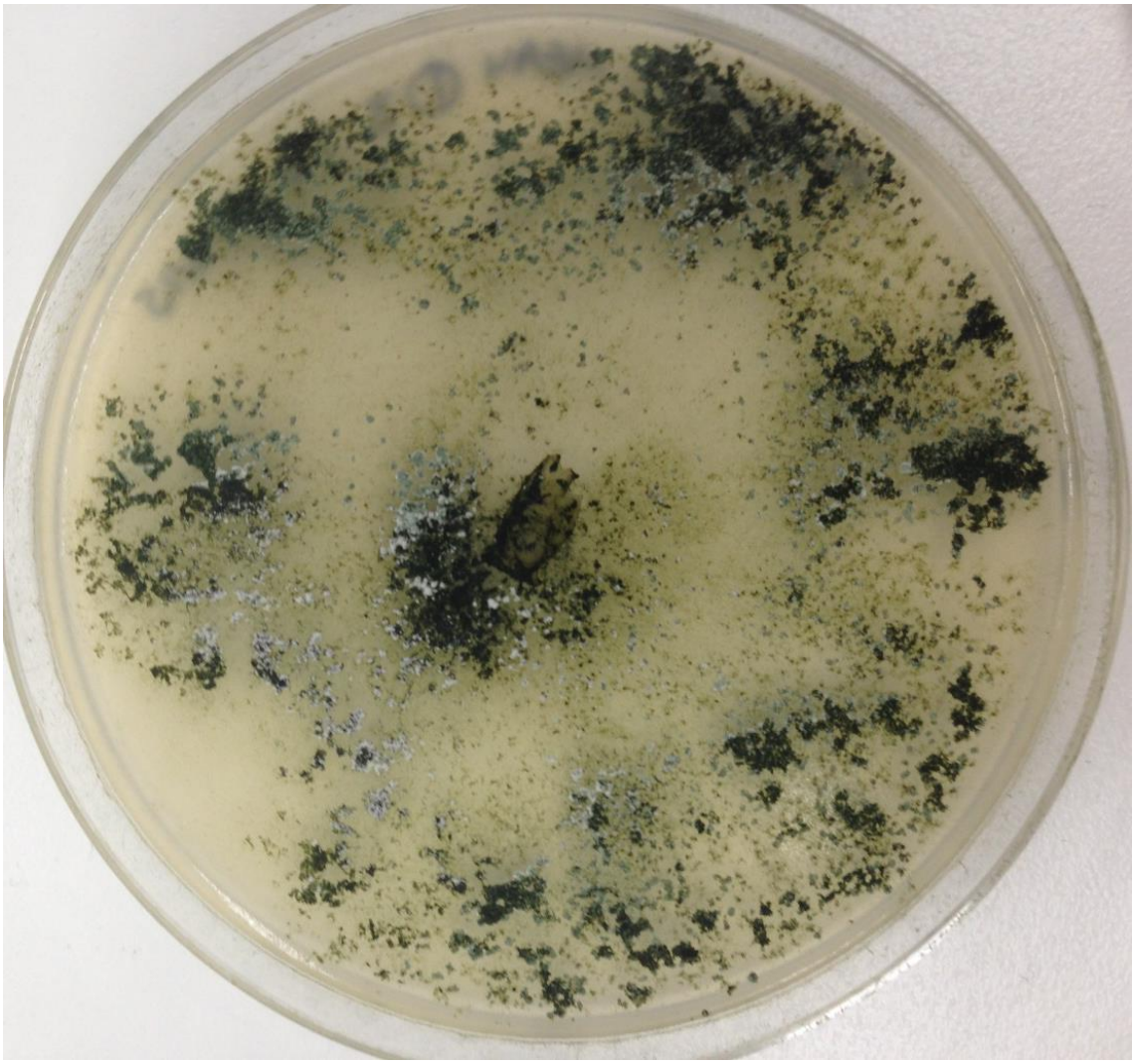

Figure 3.5: *Trichoderma* sp. on PDA displaying characteristic green pigmentation post sporulation

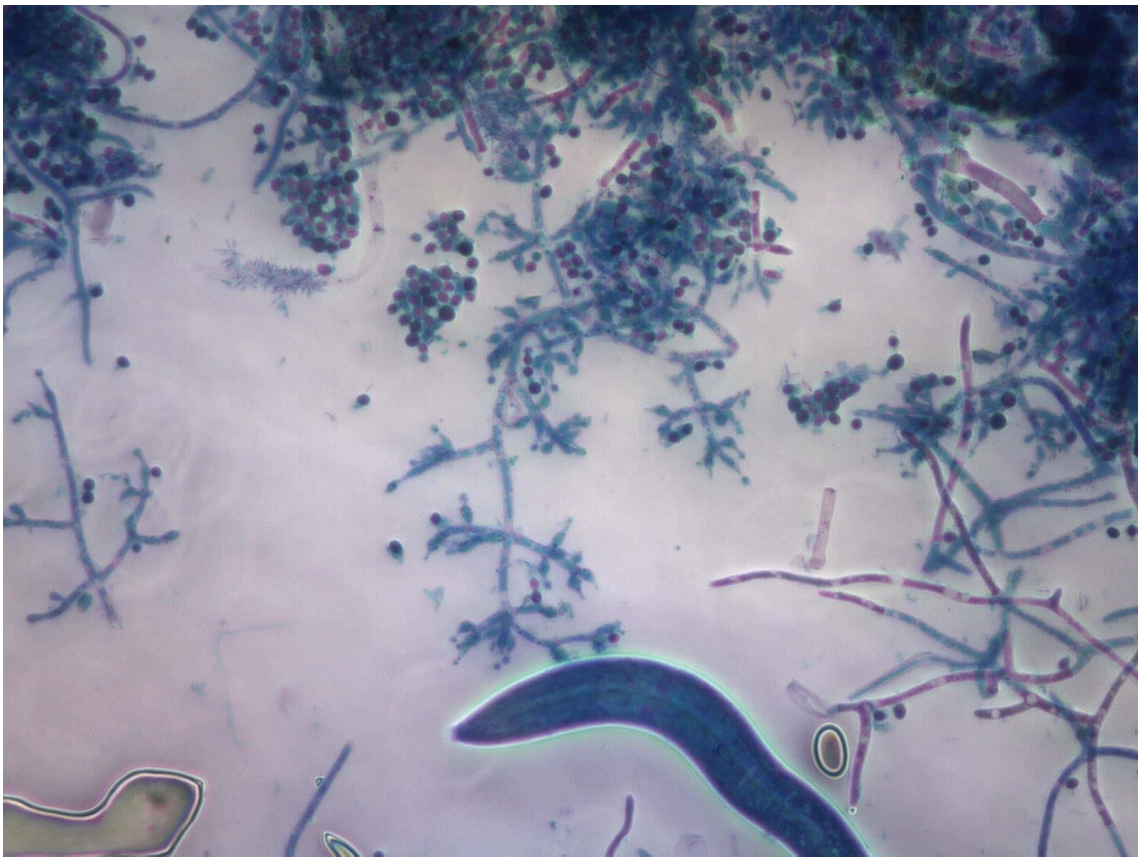

Figure 3.6: *Trichoderma* sp. and *C. elegans* stained with Methylene blue, *Trichoderma* sp. displaying characteristic single celled globose conidia from branched phialides.

Trichoderma displayed some properties similar to nematophagous fungi including the following: nematicidal effects, reduction in nematode numbers, apparent trapping on sprinkle plates.

However, when isolated the nematicidal effects and reduction in nematode numbers persisted to some extent but no further trapping was observed. Closer observation under the compound microscope showed the nematodes moving freely through the fungal matrix. It appeared not to have an immediate nematicidal effect and the nematode decline was occurring gradually.

Figure 3.7 and Figure 3.8 show *C. elegans* moving through hyphae and spores of *Trichoderma* sp. unaffected by the fungus.

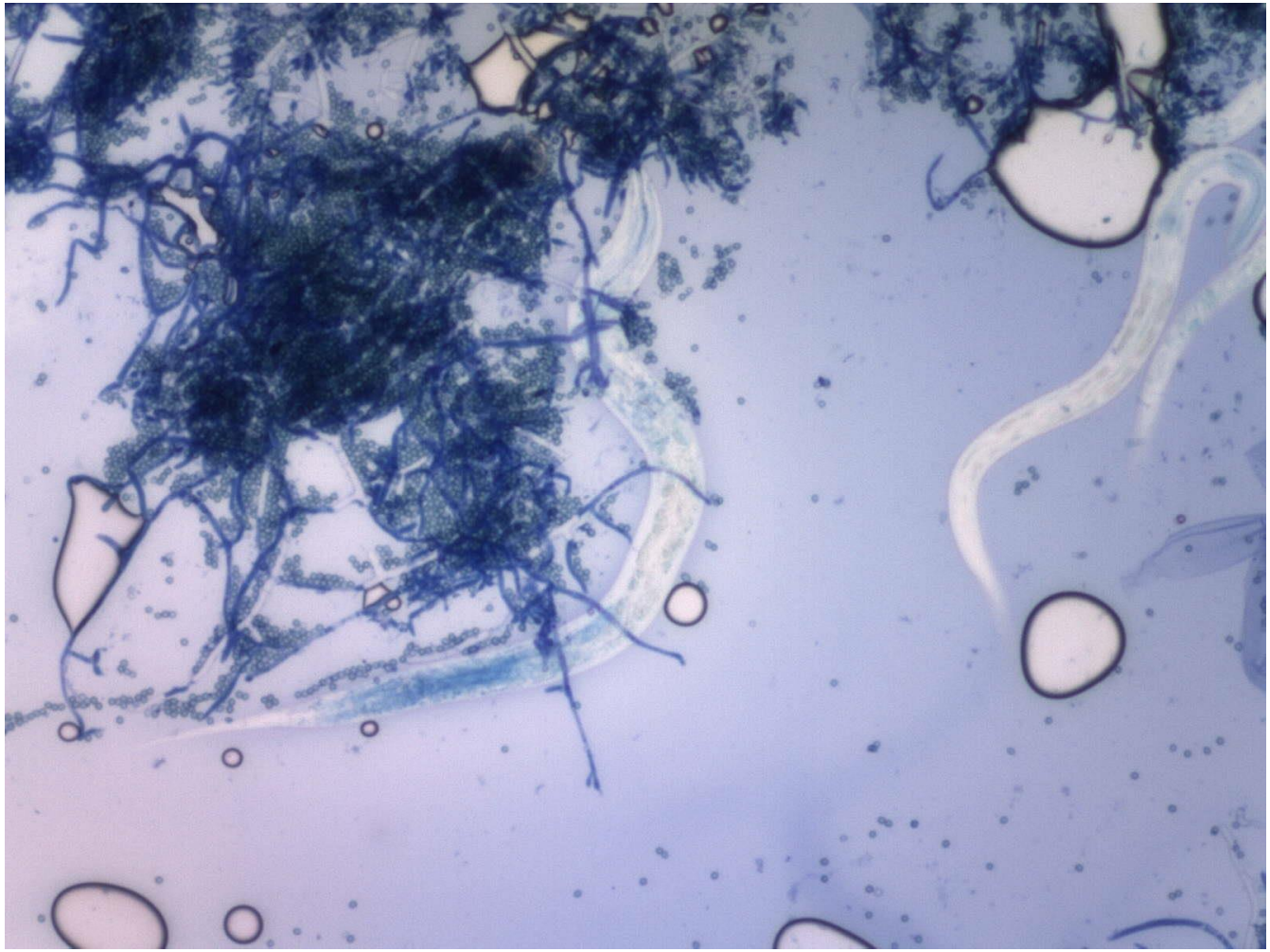

Figure 3.7: *Trichoderma* hyphae and conidia and untrapped *C. elegans*

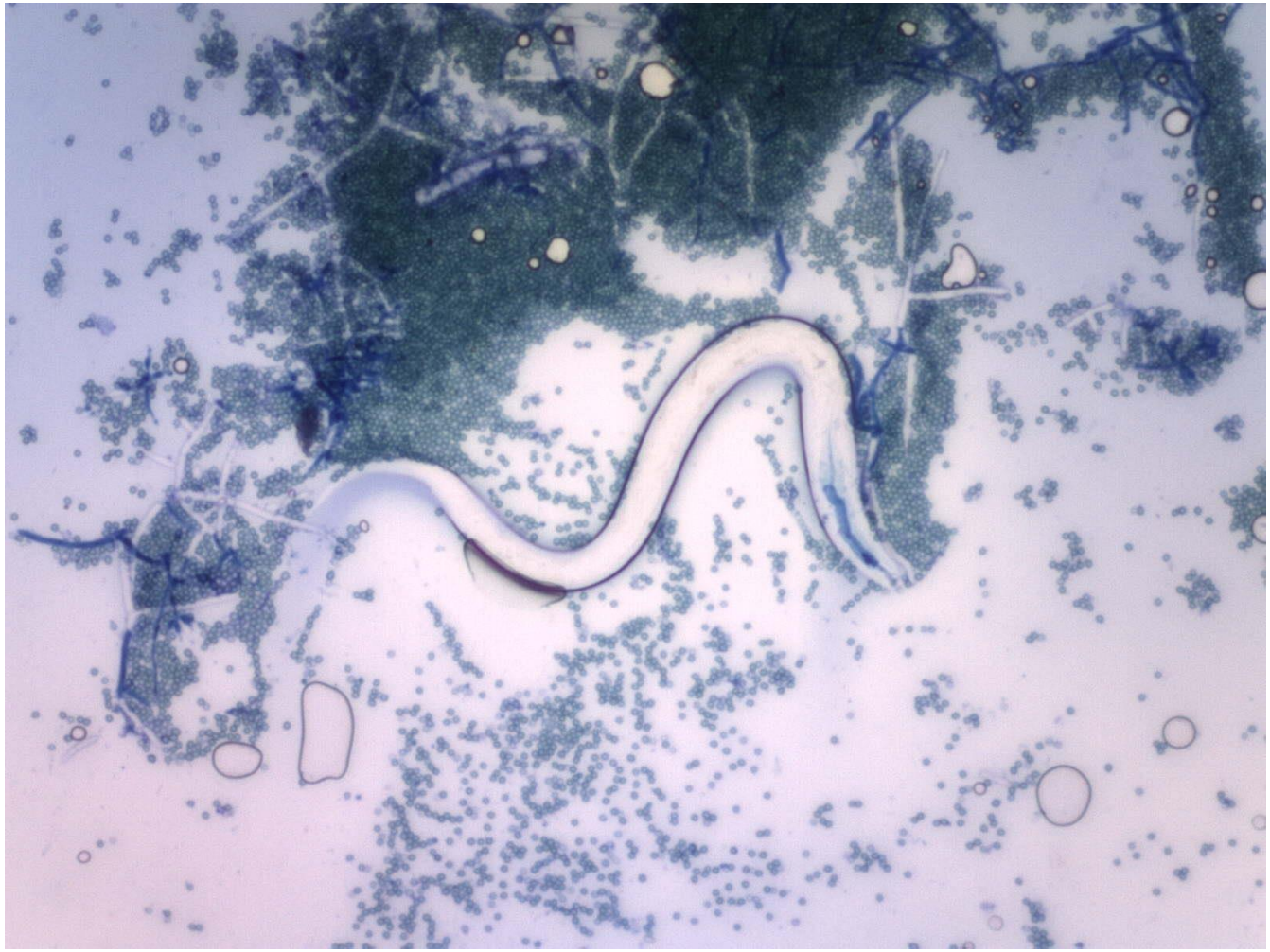

Figure 3.8: *Trichoderma* hyphae and conidia and untrapped *C. elegans*

3.5.1.3.2. *Cunninghamella* sp. and assessment of nematophagous activity

Cunninghamella: colonies initially white turning to grey post sporulation on agar. Microscopic morphology - aseptate hyphae branching at right angles, terminating in sporangiophores. Sporangiospore formation from sporangiophores, globose with spines (Watanabe, 2010).

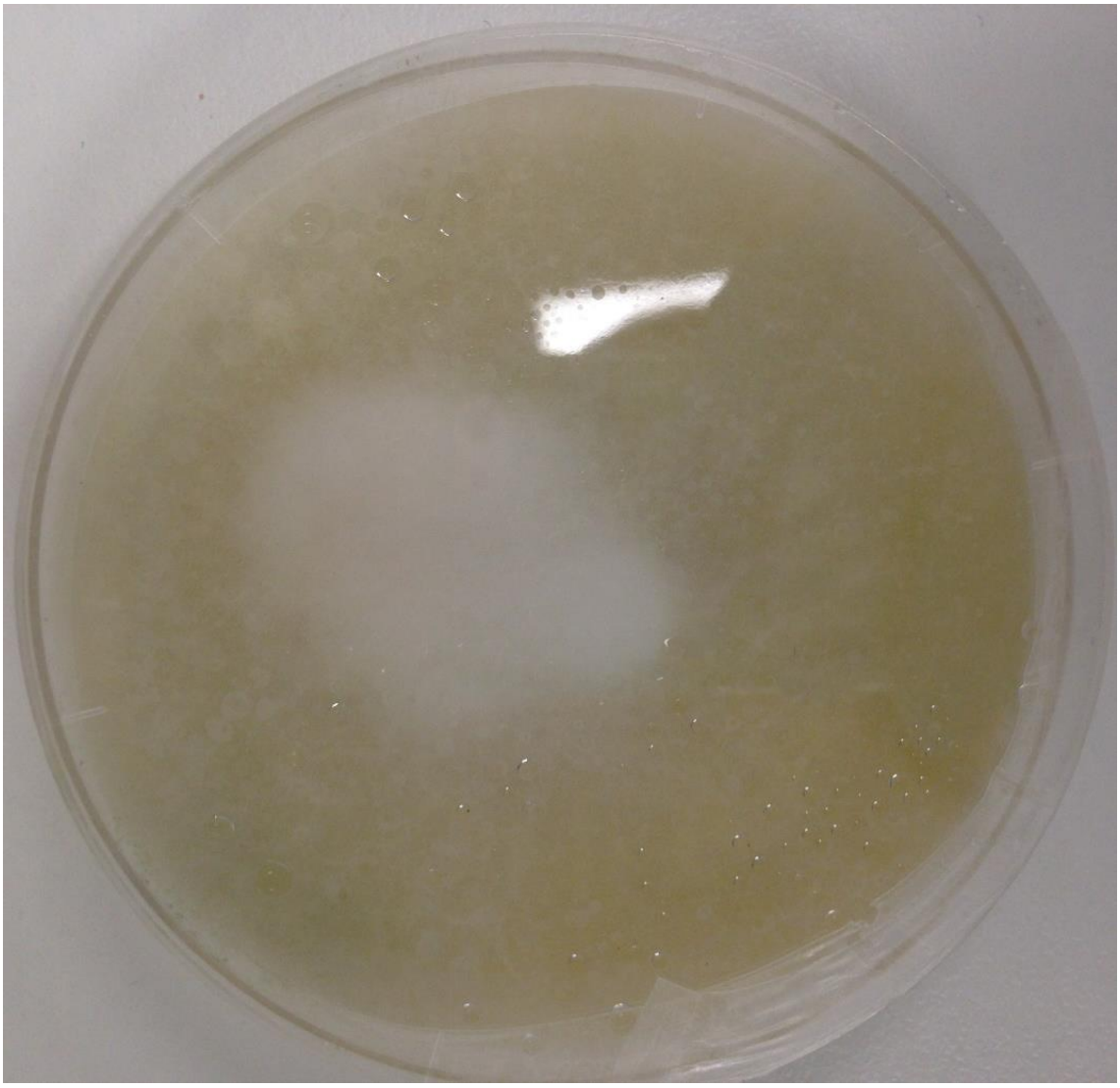

Figure 3.9: *Cunninghamella* sp. on PDA before pigmentation and sporulation

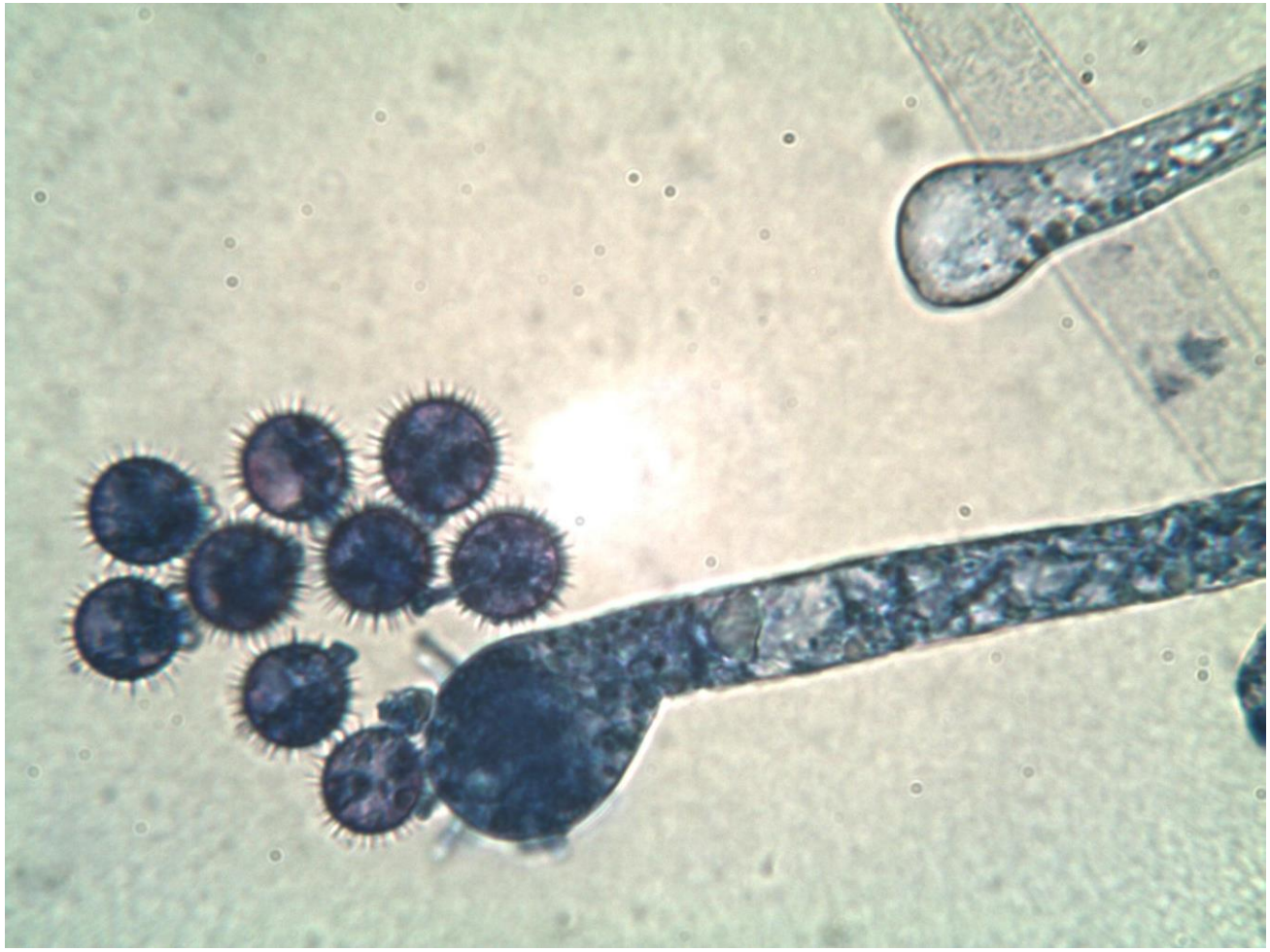

Figure 3.10: *Cunninghamella* sp. stained with Lactophenol blue, displaying characteristic sporangiophores and globose spined sporangia

Cunninghamella sp. displayed mild nematicidal properties and appeared to trap nematodes on sprinkle plates. No strong nematicidal effects were observed and once isolated no more evidence of nematode trapping was observed.

3.5.1.3.3. Fusarium sp. and assessment of nematophagous activity

Fusarium: colonies white with pink to orange pigmentation after 7 days, sporulation developing after 2 weeks. Microscopic identification – septate hyphae with intercalary chlamydospore formation and production of 4 -6 celled lunate macroconidia (Watanabe, 2010). Chlamydospores bumpy and borne in chains of 2-3.

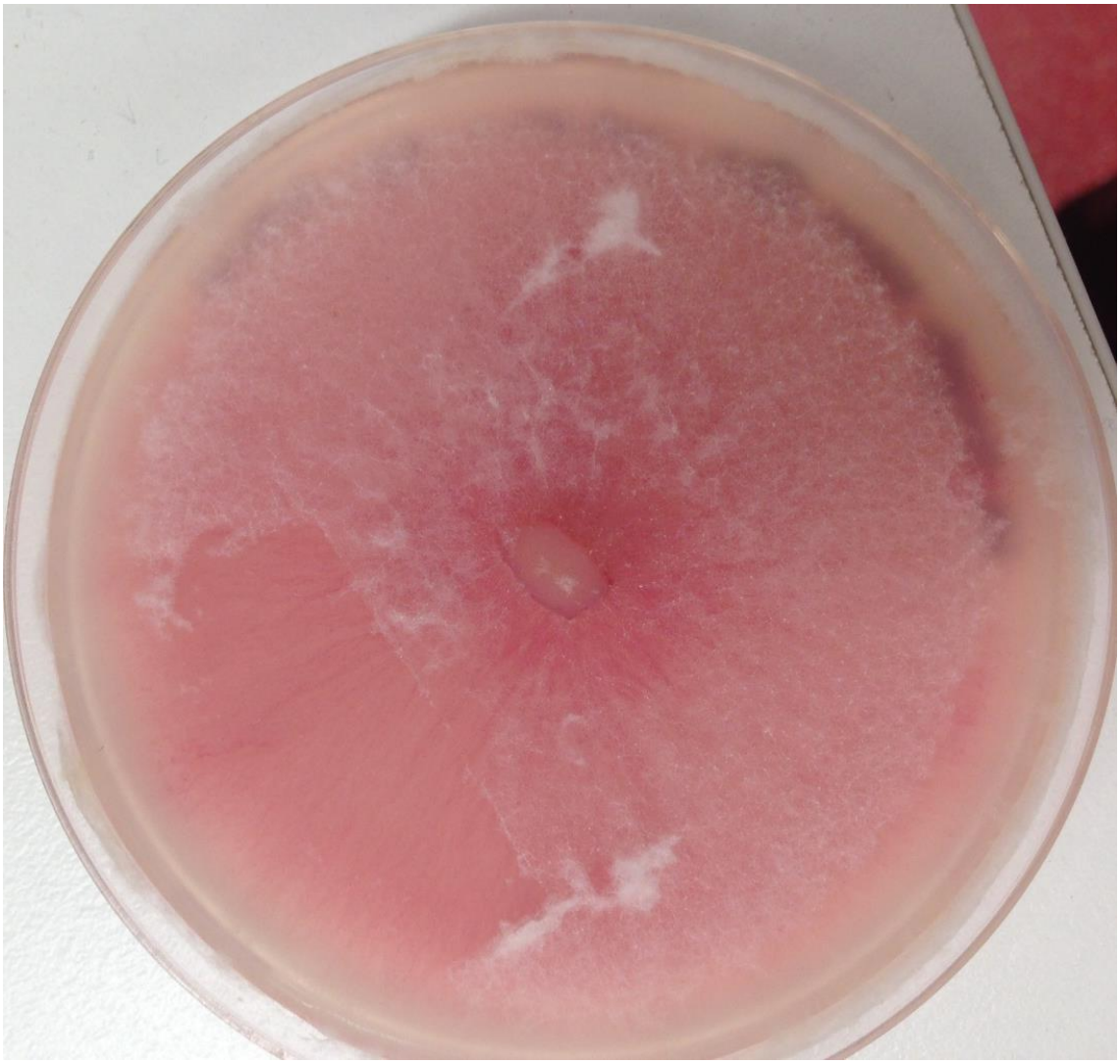

Figure 3.11: *Fusarium* sp. on PDA displaying characteristic pink pigmentation

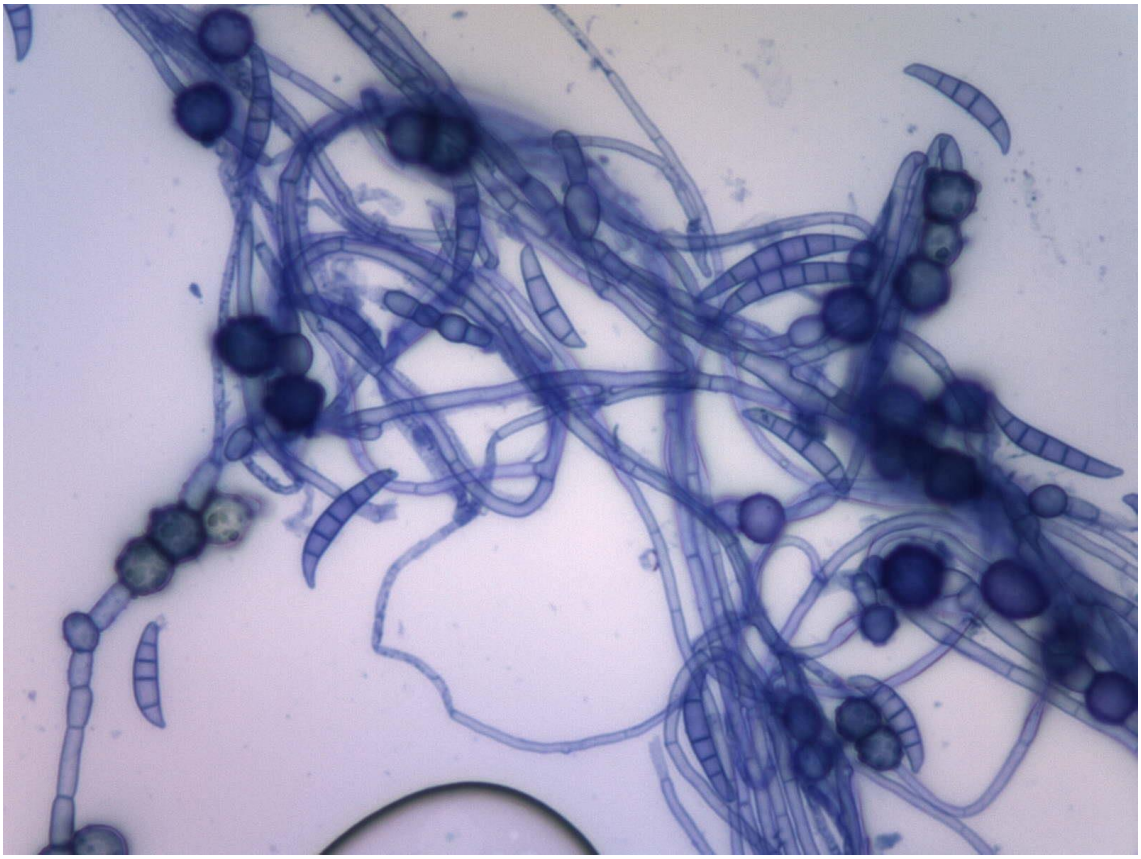

Figure 3.12: *Fusarium* sp. stained with Methlyene blue, displaying characteristic chlamydospores and 4-6 celled lunar macroconidia.

Fusarium sp. displayed traits similar to nematophagous fungi appearing to trap nematodes on sprinkle plates, mild nematicidal effects, structures giving the impression of trapping rings, structures giving the impression of adhesive knobs. After isolation and assessment no significant nematicidal effects or trapped nematodes were observed. Trapping rings or loops appeared to have no trapping properties and adhesive knob structures developed into chlamydospores.

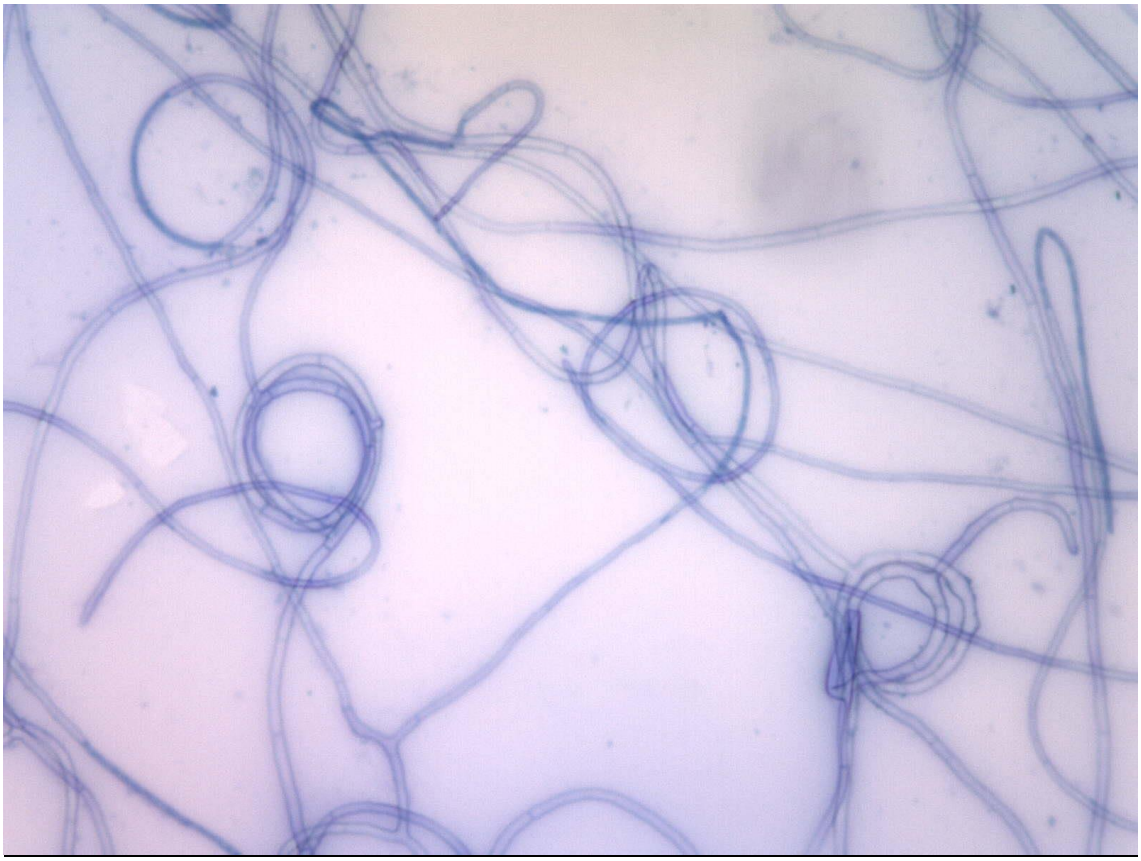

Figure 3.13: *Fusarium* sp. displaying hyphal looping

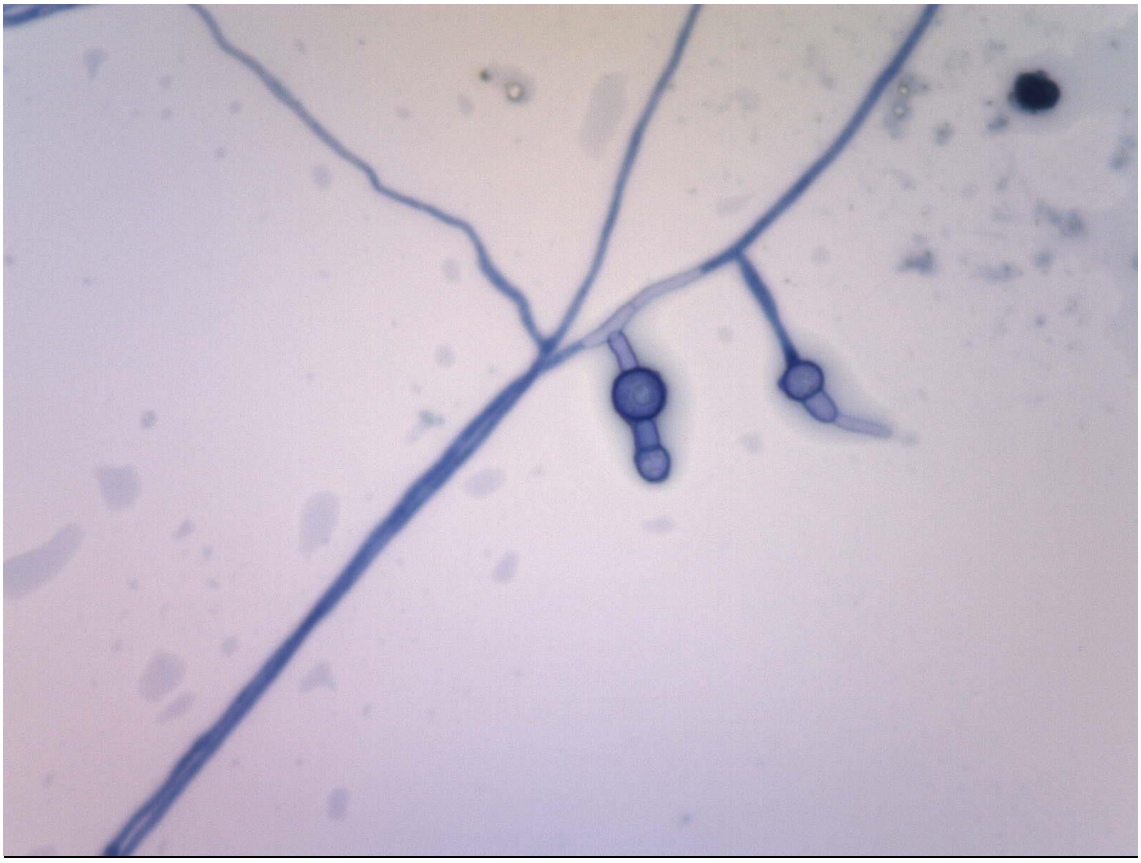

Figure 3.14: Chlamydospore formation on *Fusarium* sp.

3.5.2. *Paecilomyces lilacinus* selective media

No colonies with consistent colony morphology to *P. lilacinus* were observed on selective medium from Northern Territory soil samples.

However, a colony with consistent colony morphology to *P. lilacinus* appeared as a contaminant on another researchers PDA plate. The culture was isolated onto Czapeck Dox agar as per Gordon and Nortons methods (Gordon and Norton, 1985). The isolate displayed slow growth, the colony appeared white with a cotton wool like texture for first the 7 days before pigmenting pink/lilac with a suede like texture. The isolate was assessed via a flag preparation and displayed characteristic fruiting structures of *Paecilomyces* sp. Hyphae were septate, branching into small clusters of phialides (2-3) producing chains of ovoid conidia (Watanabe, 2010).

Isolates grown on 2% WA with *C. elegans* showed parasitism of eggs as can be seen in Figure 3.17. Live nematodes were not affected.

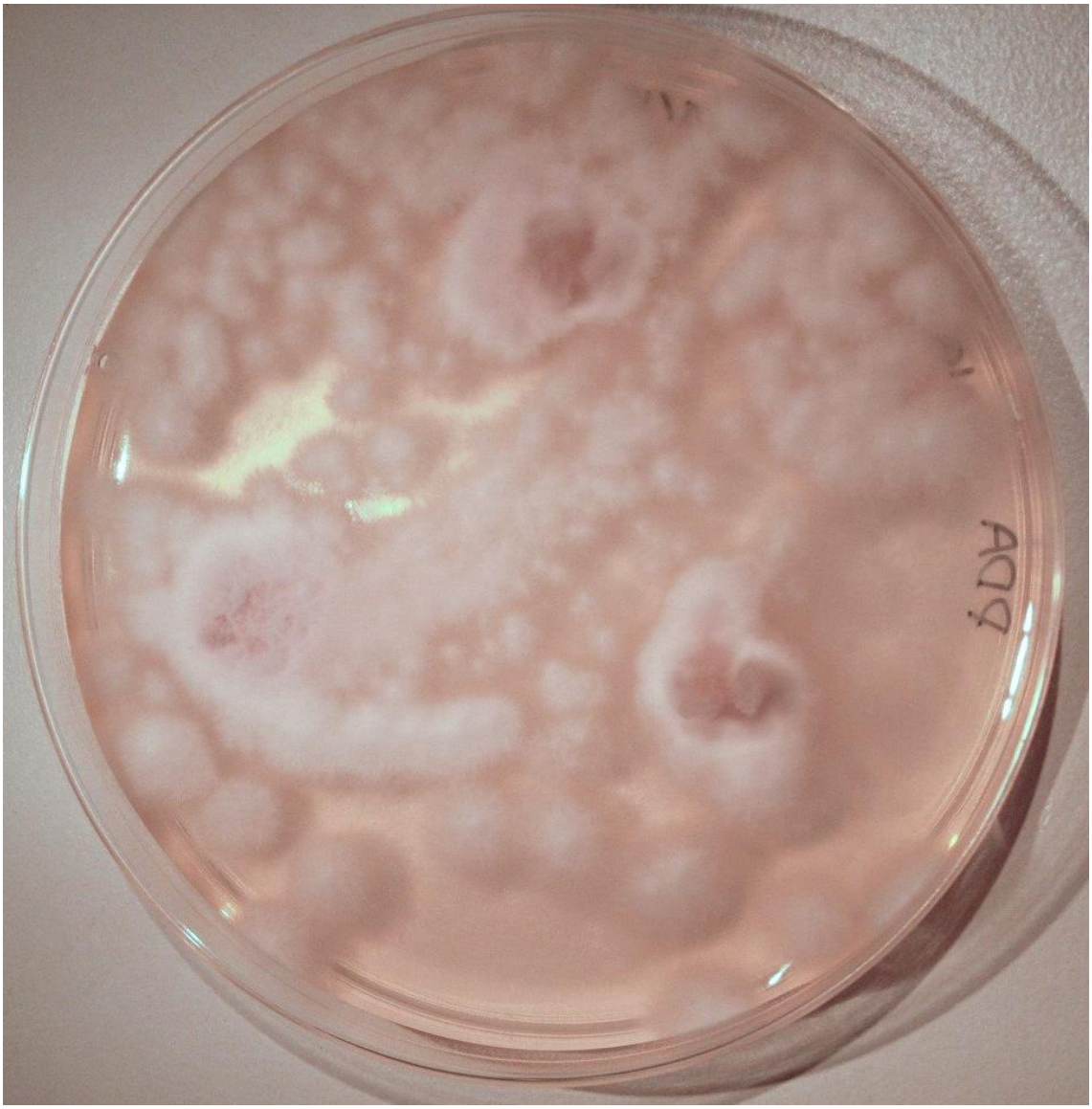

Figure 3.15*: Paecilomyces* sp. on PDA displaying white cotton wool textured colonies that are beginning to turn pink.

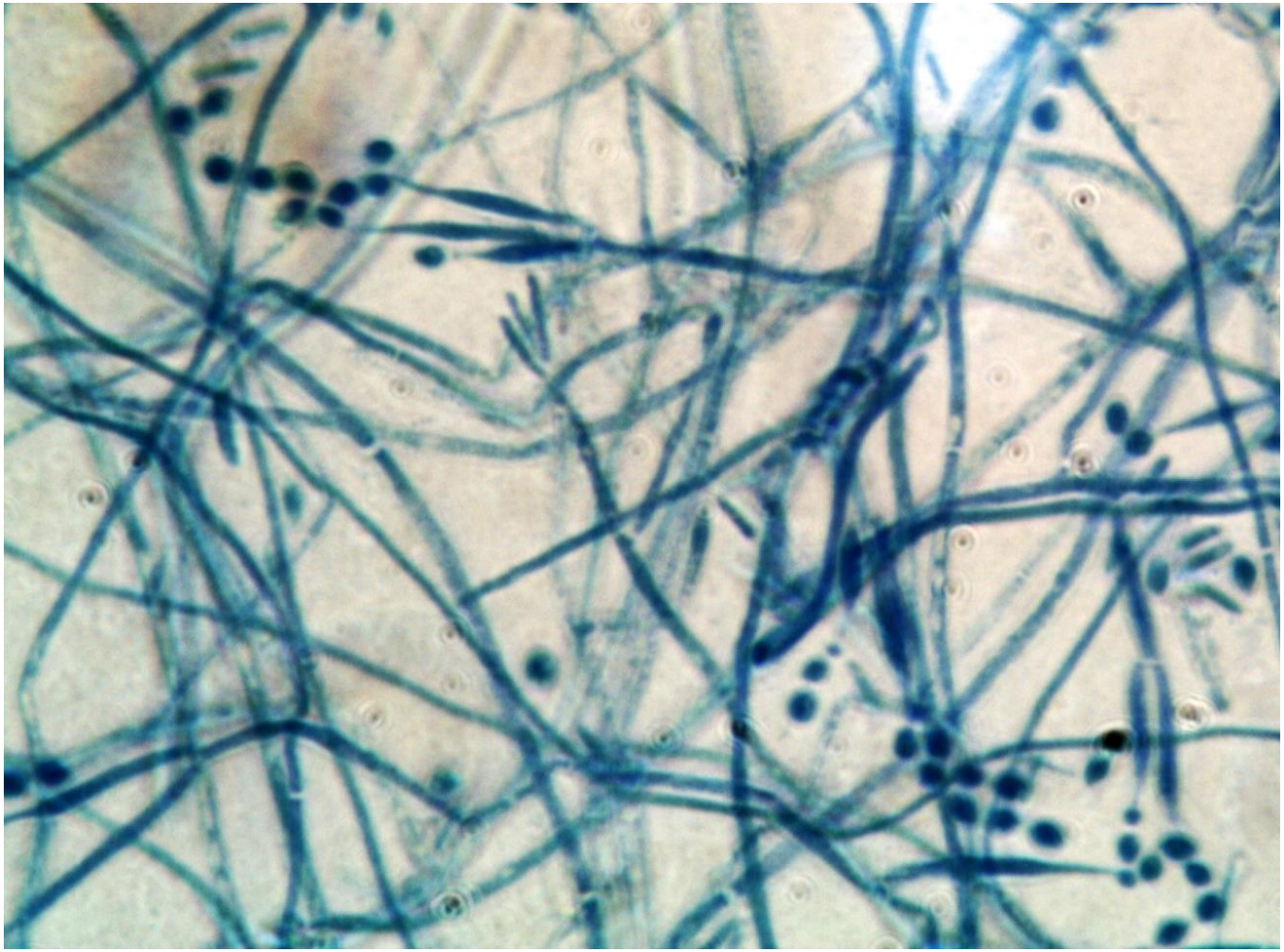

Figure 3.16: *Paecilomyces* sp. stained with lactophenol blue displaying characteristic phialides and ovoid conidial chains

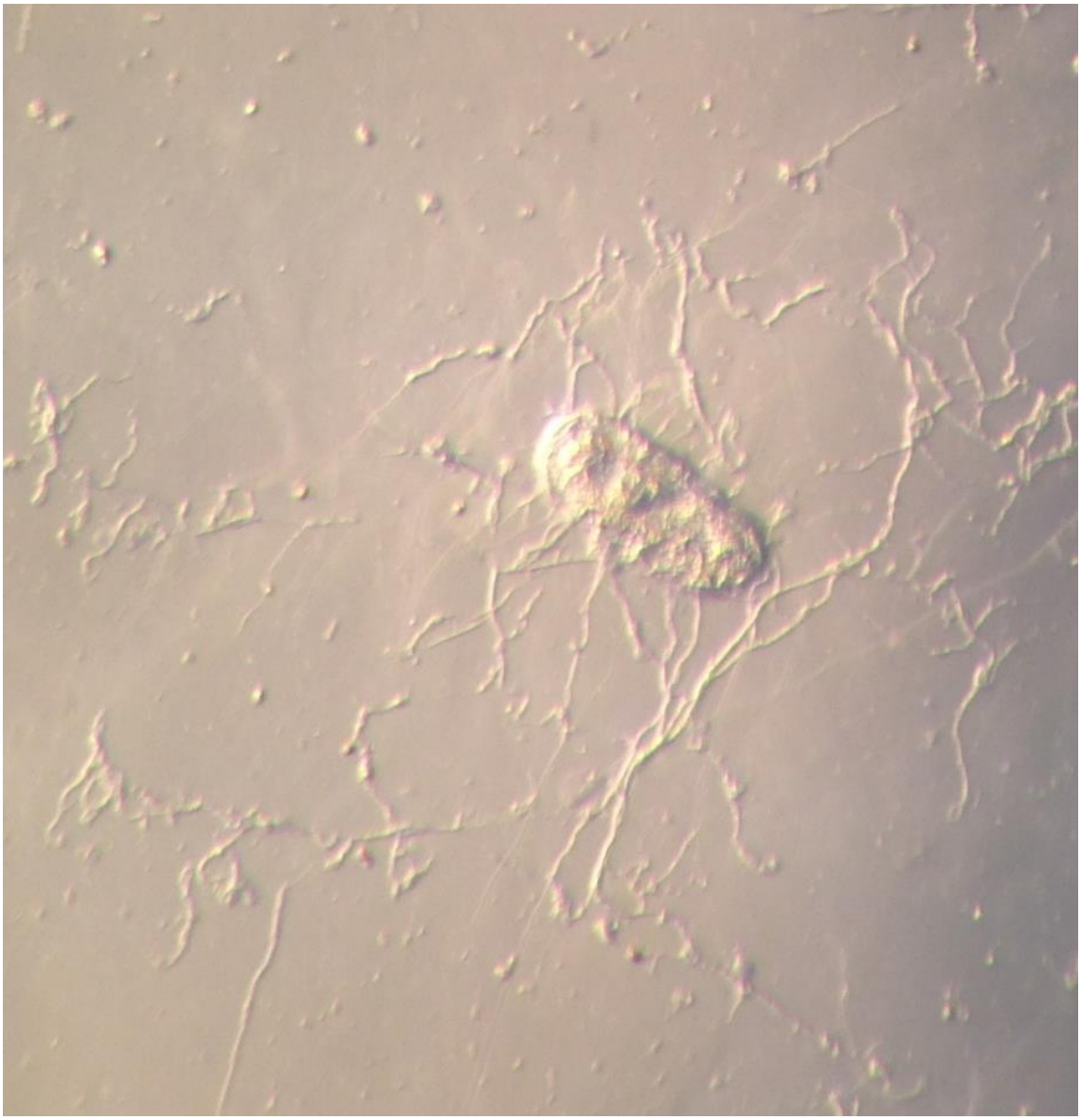

Figure 3.17*: Paecilomyces* sp. hyphae colonising *C. elegans* egg.

3.5.3. Positive control

The positive control was successful and *A. oligospora* was recovered from the sprinkle plates comprising soil inoculated with the fungus, the species was confirmed via morphology and trapping ability. If nematophagous fungi were present in the soil samples from the Northern Territory they would have been able to be isolated using this technique. The isolate produced double celled pear shaped conidia and septate

hyphae typical of this species morphology (Watanabe, 2002). The characteristic adhesive trapping nets of *A. oligospora* were observed in the culture both with and without trapped nematodes, Figures 3.19, 3.20, 3.21 and 3.22, display *C. elegans* trapped by an adhesive net from *A. oligospora*. However, when you compare figures 3.19 and 3.20 against figures 3.26 and 3.27 there is minimal obvious differences between intentional traps formed by *A. oligospora* and nematodes being colonised by non nematophagous species. The *A. oligospora* traps were obvious when the trapped nematodes were viewed whilst still alive, once dead and beginning to be colonised it was very difficult to tell between saprotrophic and parasitic activity.

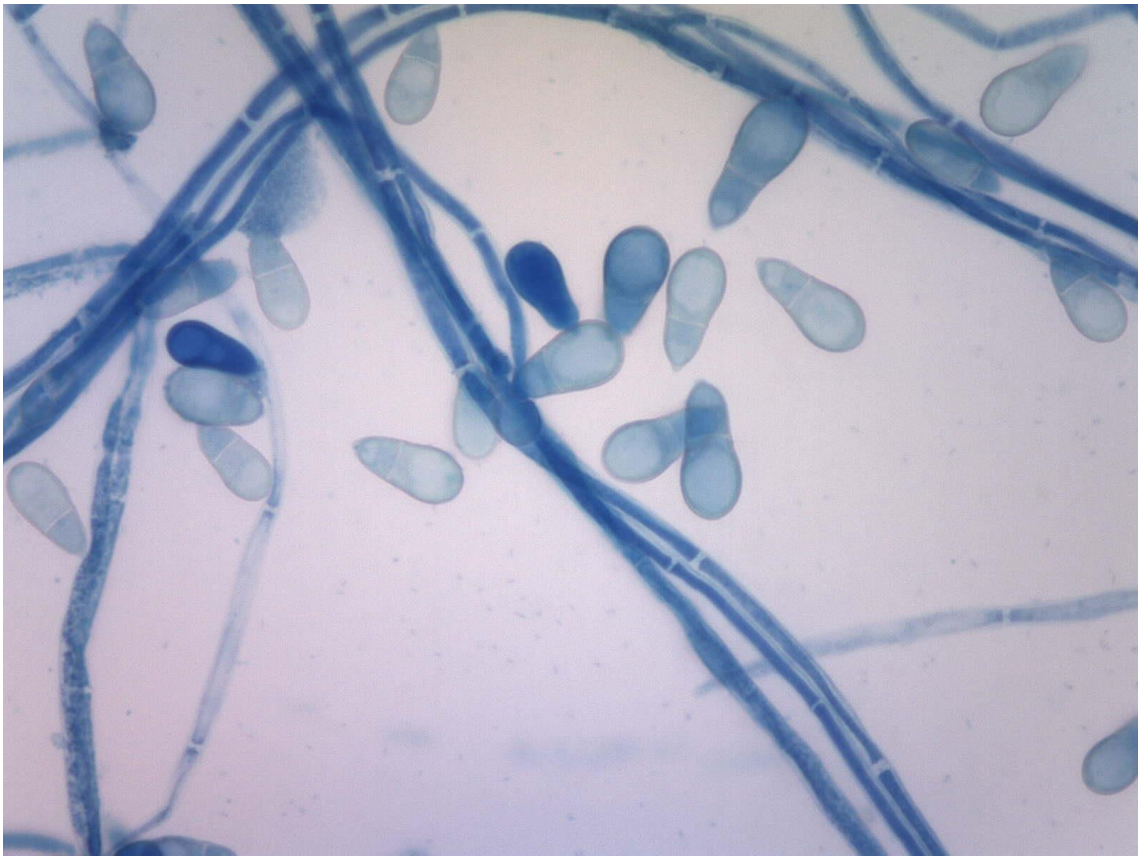

Figure 3.18*:* morphological identification of *A. oligospora* stained with Lactophenol Blue, displaying characteristic septate hyphae and conidia

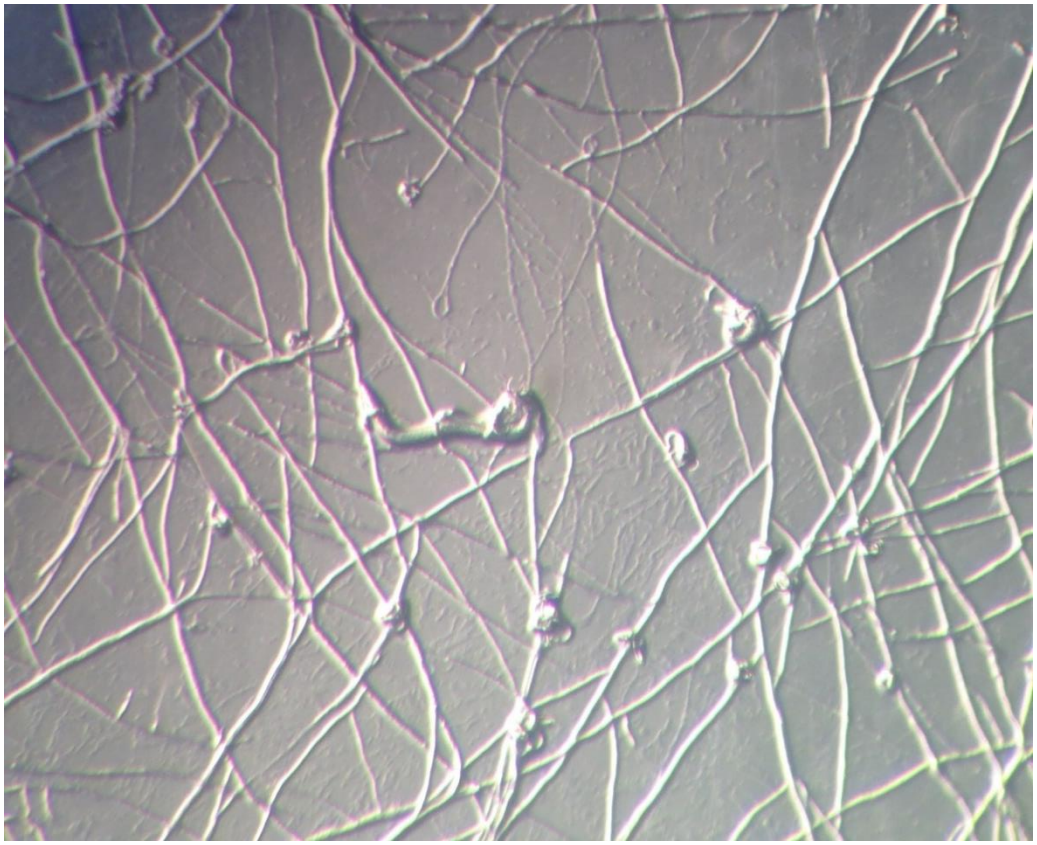

Figure 3.19: *A. oligospora* recovered by the sprinkle plate technique from soils inoculated with the fungus showing adhesive nets trapping *C. elegans*

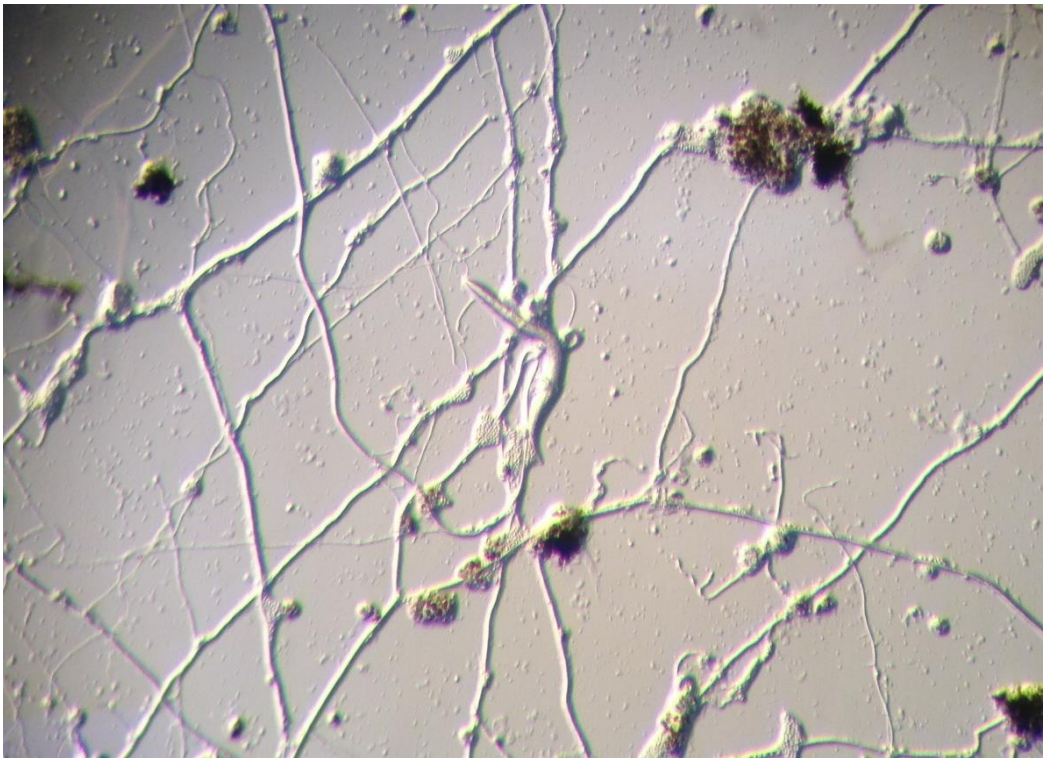

Figure 3.20: *A. oligospora* from the positive control sprinkle plates shown forming adhesive nets and trapping *C. elegans*

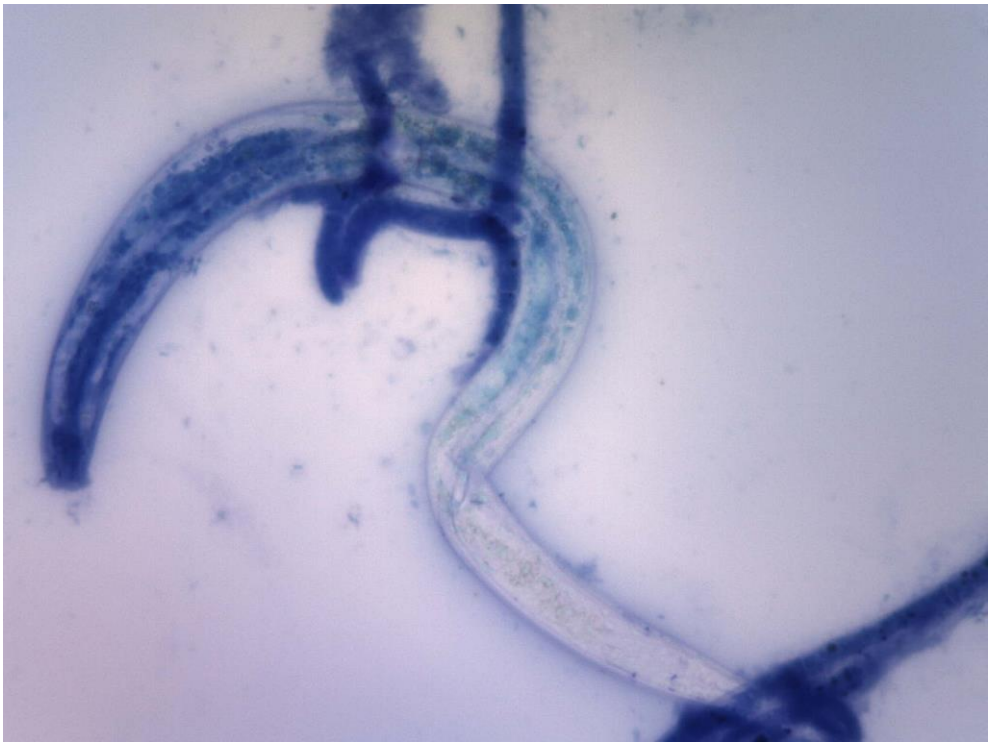

Figure 3.21: *Adhesive net of A. oligospora* trapping *C. elegans* stained with methylene blue at 200x

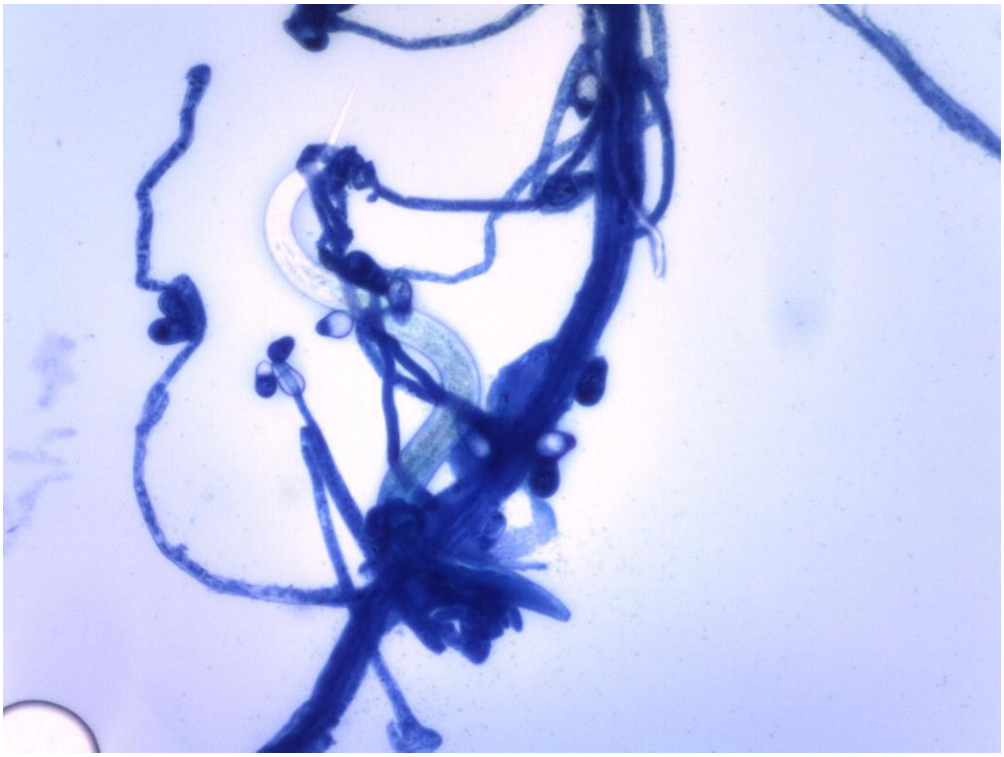

Figure 3.22. *A. oligospora* trapping *C. elegans* stained with methylene blue at 100x, stain indicates penetration of nematode cuticle but this cannot be seen under hyphal mass.

3.6. Discussion

In summary there were no orbilacious nematophagous fungi isolated from Northern Territory soils using the sprinkle plate technique. There were difficulties with the assessment methods used on the isolates and there was a lack of clarity with identifying nematophagous properties. There were potentially no nematophagous fungi present in the samples which could have been confirmed with the use of molecular methods. With a lack of nematophagous fungi appropriate for use as augmentation biological control from these samples none of the isolates will be further investigated for their predatory activity on *S. stercoralis*. These conclusions will be discussed in greater detail below.

3.7. Assessment of fungal cultures isolated from the sprinkle plate technique

The species isolated from the Northern Territory samples are not readily associated with nematophagous fungi capable of physically trapping nematodes.

The positive control using *A. oligospora* highlights the ease in which the sprinkle plate technique can be used for isolation of nematophagous fungi from soils. If the soil samples from the Northern Territory had contained an abundance of nematophagous fungi then this technique would likely have been ideal. However, once isolated nematophagous properties can be difficult to identify even with dedicated trapping structures like *A. oligospora* produced. This was also indicative of the assessments of the fungal isolates from the Northern Territory soils. Clear indicators of nematophagous properties in known nematophagous species such as

A. oligospora were difficult to detect which made the determination in unknown species very subjective.

Identification to genus level was carried out using Watanabe's (Watanabe, 2010) fungal identification key, literature could then be used to help determine if the genus was linked with nematophagous capabilities.

The assessment of the nematophagous capabilities was completed once the culture had been isolated from the sprinkle plate technique using both dissecting and compound microscopy. These were used in combination with media adjustment, flag preparations and viability stains to assess the predatory activity of the fungus.

3.7.1. Microscopy – Dissecting microscope

All of the assays in this chapter were performed in petri dishes, which were viewed under the dissecting microscope. 2% water agar is optimal for viewing under a dissecting microscope as fungal hyphae are sparse and individually viewable. Nematodes may appear to be trapped but due to the limitations of the magnification of a dissecting microscope the specific interaction between the nematode and the fungi was often not clear. The dissecting microscope is more useful for making general observations of the nematode population with regard to how many nematodes appear trapped and how many appear free. The specific interactions between potentially trapped nematodes and the fungi is better viewed at higher magnification.

Alterations in the media used allowed for more optimal viewing of the fungal nematode interactions under different magnifications. The fungal nematode interactions were required to be recreated after isolation from the sprinkle plate

technique. The media used for this determines the rate and density of fungal growth which alters the viewing conditions for microscopy.

3.7.2. Microscopy - Compound microscope

The low level of nutrients in 2% water agar are designed to make fungal hyphae sparse so they are easily viewable, this also causes very minimal aerial mycelium (Watanabe, 2010). The nematodes remain on the surface of the agar when trapped by fungal hyphae. The fungal hyphae in the absence of nutrients also tend to have a greater level of attachment to the surface of the agar and display shallow burrowing into the agar. This decreases the effectiveness of the flag preparation technique when creating slides for the compound microscope assessment. This technique uses transparent tape to lift fungal hyphae off the surface of the agar and placed onto a glass slide, usually with the addition of a stain (Harris, 2000). When the fungal hyphae are well attached to the agar they do not lift onto the tape resulting in an empty slide. The use of agar which is normally used for fungal growth such as potato dextrose agar creates an abundance of growth with a very dense network of mycelium including aerial mycelium. Creating a flag preparation from this is relatively simple but there are limitations to its use in this method. The volume of mycelium is too great to be able to assess easily and the nematodes are not likely to venture into the aerial mycelium. This means a flag preparation made to assess nematophagous capabilities from this kind of agar is impractical. This work found that ¼ strength malt yeast extract agar produces limited aerial mycelium in most species, less burrowing

and a greater mycelial mass than 2% water agar. This agar with many species allows for easier flag preparation slides to be made.

Even with a slide that can be viewed at higher magnification under the compound microscope identifying the relationship between nematode and fungi as nematophagous can be challenging. The nematodes become stationary once fixed in the slide making it difficult to determine if the nematode is trapped or not. Classifying the fungus to genus level can immediately eliminate some candidates; if the genus can be morphologically easily distinguished then a literature search can determine if it has known nematophagous capabilities.

A flag preparation allows a direct transfer of the fungi on the agar plate to the slide with minimal disruption (Harris, 2000). A direct microscopic mount is an alternative fungal slide preparation method which involves removing a portion of agar with a needle, staining on a clean microscope slide and squashing the agar flat with a cover slip. This process is suitable for a fungal culture as it would still allow for distinctive fungal morphology to be viewed. The use of this process would not be suitable for a fungal nematode interaction as the exact placement of the fungal hyphae and the nematode is important. Disrupting the fungal hyphae in this manner would distort the natural arrangement of hyphae and nematodes.

The traits that were assessed are discussed in more detail below:

3.7.2.1. Evidence of trapping rings, nets or adhesive knobs

Part of the difficultly with assessing nematophagous capabilities was the ability to tell if a nematode was trapped by a fungus or just intertwined in the fungal hyphae. Once a nematode dies of natural causes in the presence of fungal hyphae, the fungal

hyphae tend to continue growing around and through the nematode and digest it. Determining the difference between this situation and a situation where the fungi have purposefully formed a trap and trapped and digested the nematode with intention is difficult. The latter classifies as nematophagous capabilities whilst the other does not.

Evidence of trapping structures may indicate that nematodes are being trapped rather than just intertwined, particularly if a trapping structure is present where a nematode is trapped. These structures can appear to occur without the presence of nematophagous capabilities. The following figures are of the nematophagous fungus *Hohenbuehelia clelandii,* which forms adhesive knobs as nematode traps. The figures show the development of the bowling pin shaped structure and then the adhesive secretion forming around the tip. Similar structures were formed in isolates from the Northern Territory soils as can be seen in figures 3.13 and 3.14. However, these structures continued to develop into chlamydospores of *Fusarium* sp. These figures display how difficult it can be to determine nematophagous fungal structures from non-nematophagous fungal structures.

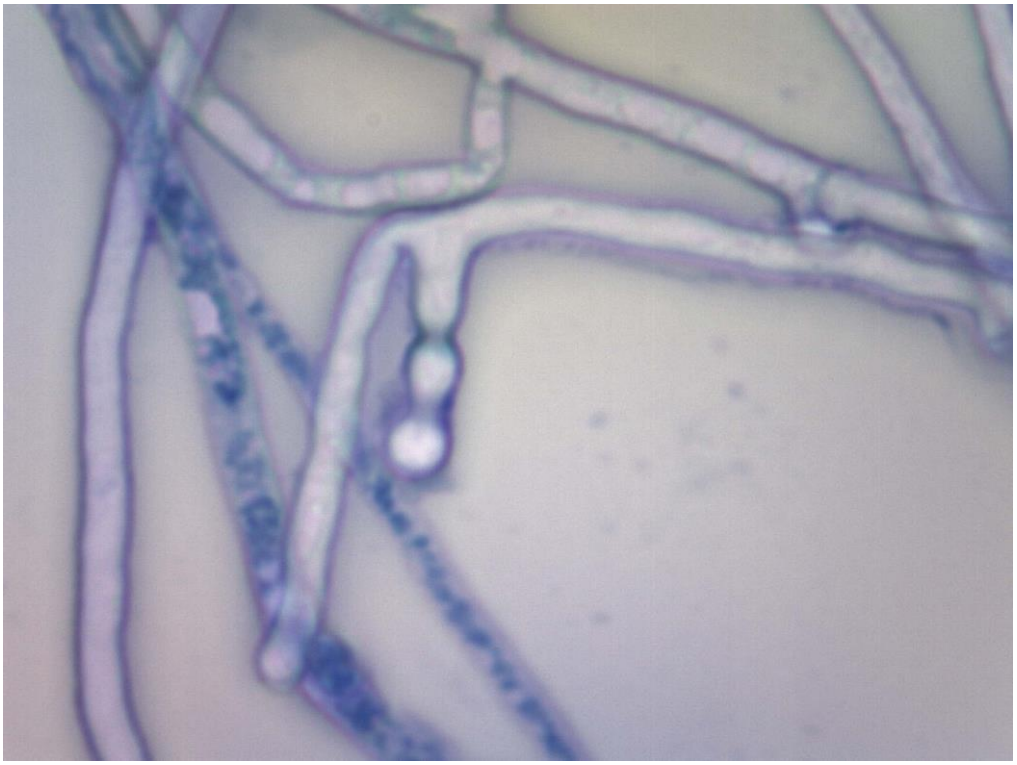

Figure 3.23*: H. clelandii* hyphae developing bowling pin shaped trapping structures

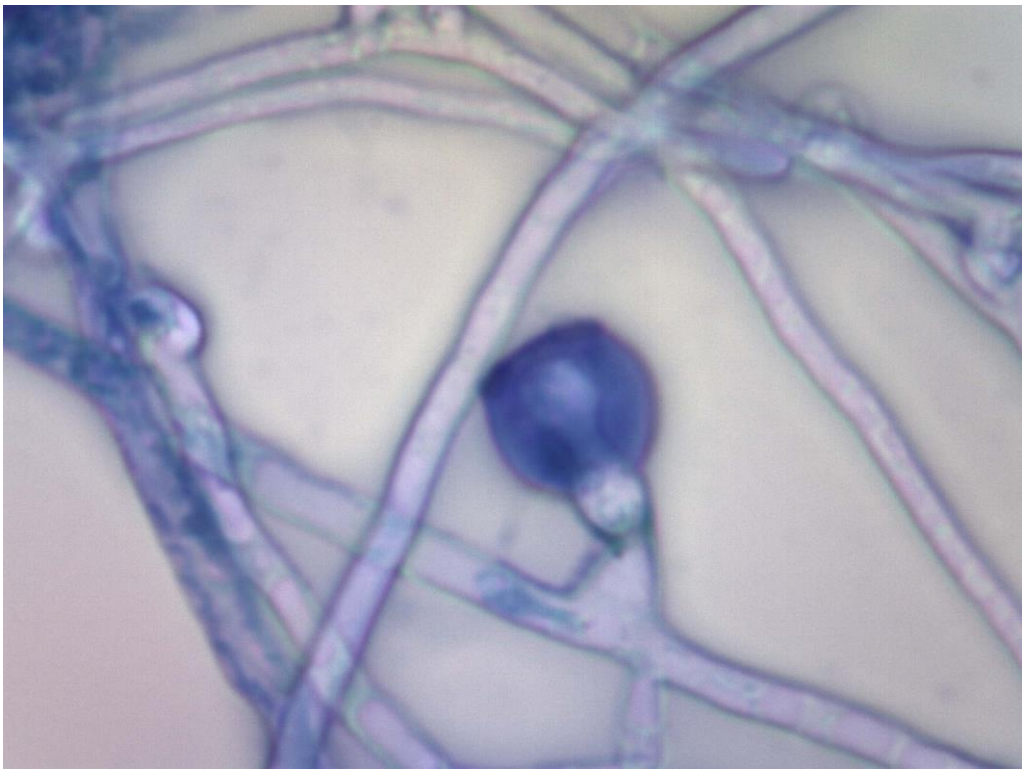

Figure 3.24: *H. clelandii* hyphae developing bowling pin shaped trapping structures with adhesive secretion developing from tip

Constricting rings and adhesive nets are trapping structures used by species such as *Arthrobotrys dactyloides* and *A. oligospora.* Constricting rings can be easier to identify in the situation of a trapped nematode as the fungal hyphae swell constricting the centre of the loop, pinching the nematode and trapping it (Nordbring-Hertz et al., 2006). This structure should be clear with pinching in the nematode and swelling in the hyphae of the trapping structure.

Adhesive nets can also be difficult to distinguish without other traits or structures. An adhesive net from *A. oligospora* is displayed below in Figure 3.25, compared with a looping hyphal structure from *Fusarium* sp. from the Northern Territory soil isolates which is not a specialised trapping structure. Hyphae of many species of fungus can form hyphal loops similar to *Fusarium* sp. making diagnosis of an adhesive net difficult.

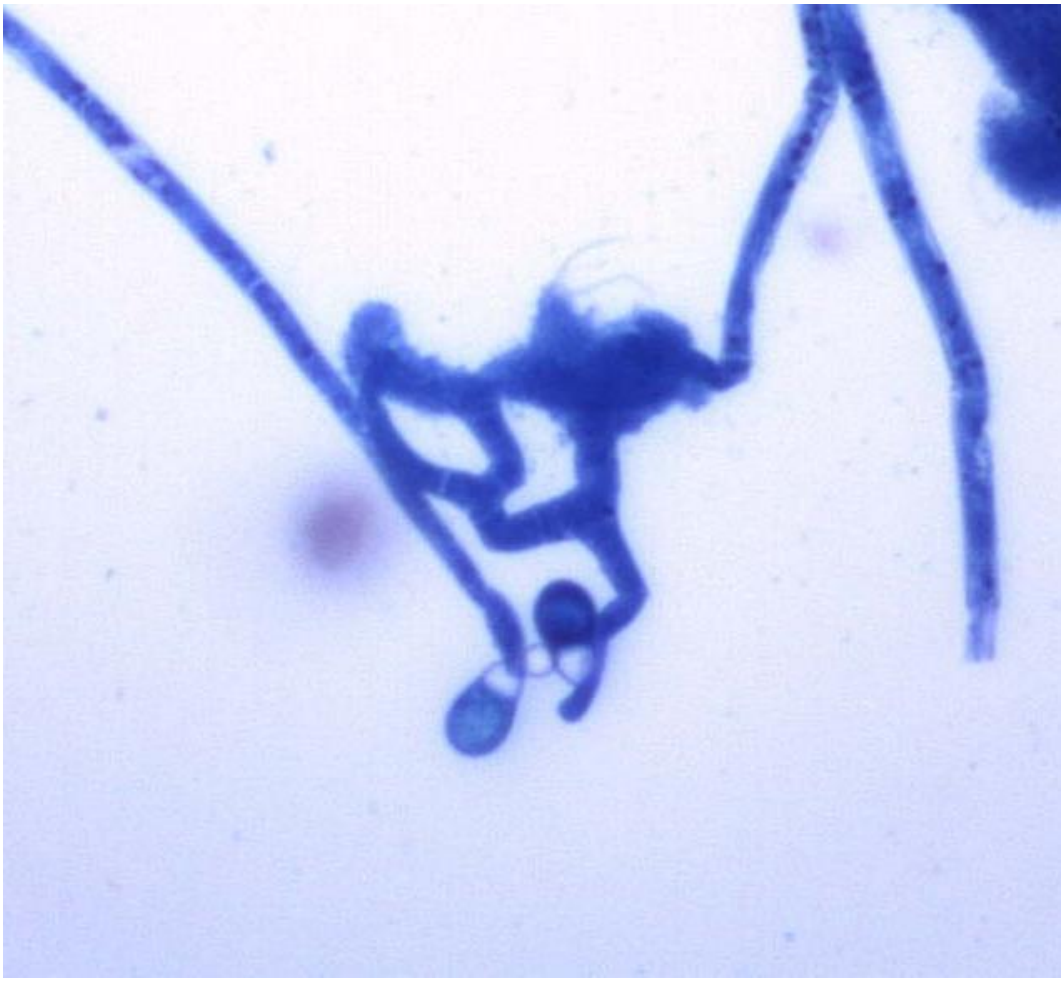

Figure 3.25: Hyphae of *A. oligospora* forming an adhesive net and characteristic *Arthrobotrys* conidia

3.7.2.2. Penetration of nematodes by fungal hyphae and hyphal growth inside the nematode

The end goal of nematophagous fungi is to digest the nematode which usually occurs by hyphal penetration and colonisation of the nematode. Presence of nematodes with fungal growth inside them can be an indication of nematophagous fungi. However, it can be difficult to tell if the nematode was trapped and digested or died of natural causes and was then saprotrophically digested. *Pleurotus ostreatus* produces droplets of nematotoxin which paralyse the nematode so that the fungal

hyphae can penetrate the nematode cuticle and digest the internal contents (Kwok et al., 1992, Satou et al., 2008). Figure 3.26 shows *C. elegans* which has been parasitised by *P. ostreatus* and internally colonised by its hyphae.

Determining hyphal penetration can be aided by the use of a viability stain that can only infiltrate a damaged nematode cuticle. Methylene blue at 10x the standard concentration for microscopy staining was found to do this within a short time period. If slides were not observed within an hour of mounting it was likely the nematode would die initiating the cuticle degradation process allowing the stain to infiltrate. Live nematodes with undamaged cuticles remain unstained.

This staining technique was used as a tool to determine hyphal penetration of nematophagous fungi with the intention of observing the process whilst the nematode was still alive. This eliminates the possibility that the nematode was being degraded saprotrophically and increases the likelihood that the nematode was being predated by the fungus.

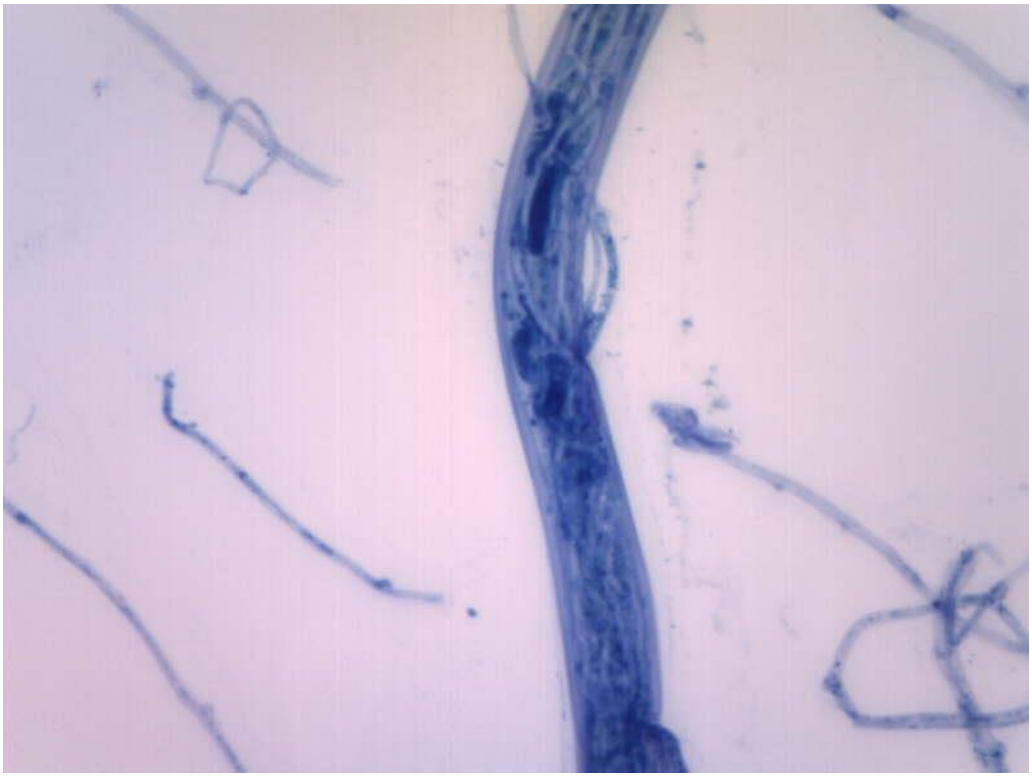

Figure 3.26: *P. osteatus* hyphae colonising *C. elegans* stained with methylene blue

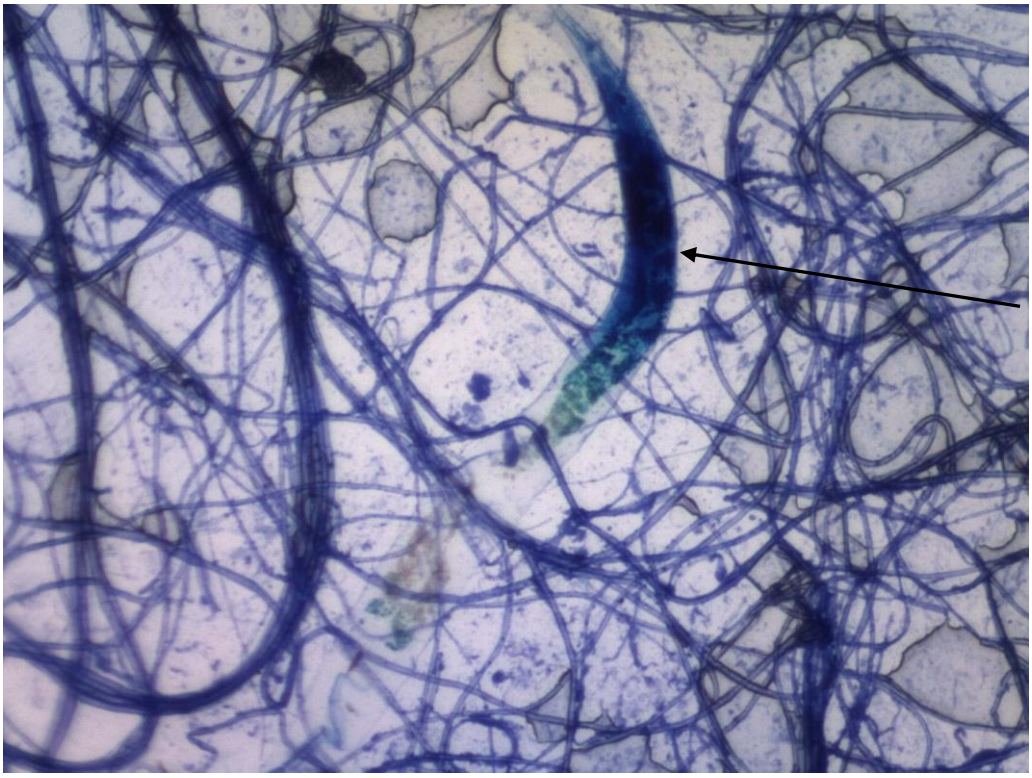

Figure 3.27: *C. elegans* moving through *Fusarium* sp. hyphae with tail end of nematode stained with methylene blue indicating cuticle damage (indicated with arrow)

Figures 3.21, 3.22 and 3.26 showed the capability of methylene blue to stain the nematode after cuticle penetration. It also displays the difficultly in clearly being able to observe the fungal hyphae penetrating the nematode.

In Figure 3.27 a partially stained nematode can be observed indicating cuticle damage to that portion of the nematode. However, there is no clear penetration point or any evidence of trapping structures. Additionally there is no evidence of fungal growth inside the nematode or pinching from trapping rings or nets. This displays the lack of clear difference between evidence of parasitism from known nematophagous fungi compared to isolates whose nematophagous capability is uncertain.

The aid of higher magnification may help to observe penetration points, trapping structures and the specific fungal nematode interactions but is costly and time consuming.

3.7.3. Presence of nematophagous fungi in the sample?

The difficulties associated with this study were due to the significant complexities of determining the nematophagous behaviours of fungi. It is difficult to then determine if this was due to the lack of nematophagous fungi in the samples or due to limitations in the method and clarity of nematophagous behaviours themselves. It is possible that these limitations may have been reduced if there was a higher proportion of orbiliaceous fungi in the soil samples taken. Whilst in the positive control with *A. oligospora* it was still problematic to determine the nature of fungal nematode interactions, a general reduction in nematode numbers and determination of genus significantly aided the understanding of this interaction. If

there was a higher proportion of well described orbilaceous species present, the genus of known nematophagous species may be determined more rapidly. Having little prior knowledge of nematophagous species and lack of a useful taxonomic key for nematophagous fungi made this process arduous. In this study the difficulties can be attributed to lack of orbilaceous fungi in the samples.

3.7.4. Fungal species that are not strictly nematophagous but display similar properties

Many species that are not described as nematophagous have interactions with the nematodes in their environment (Yang et al., 2010, Szabó et al., 2012). Fungal feeding nematodes are known to consume more than 50% of the fungal hyphae in the soil matrix (Anke et al., 1995, Paul and Clark, 1989). It is therefore not surprising that fungi have developed protective mechanisms to prevent this from affecting them.

The three predominant genera that were isolated from this study are not orbilaceous nematophagous fungi, however, they displayed consistent properties that indicated the presence of some fungal nematode interactions. Species from *Trichoderma*, *Fusarium* and *Cunninghamella* have all been studied as biocontrol agents on nematodes despite their lack of orbilaceous properties.

3.7.4.1. Trichoderma

Species from the genus *Trichoderma* are rhizosphere competitors which are plant symbionts (Yang et al., 2010). Many produce secondary metabolites which can have nematicidal and bacteriostatic properties (Yang et al., 2010). As well as nematicidal metabolites *Trichoderma* spp. can produce proteases which breakdown the walls of

nematode eggs allowing the fungus to penetrate and colonise (Szabó et al., 2013). Considering these traits it could be argued that *Trichoderma* sp. are nematophagous although not orbilaceous. *Trichoderma* do however, possess many traits that ward off other microorganisms. It is also known as mycoparasitic and as previously mentioned it produces bacteriostatic metabolites (Ojha and Chatterjee, 2011).

Some of these traits of *Trichoderma* sp. were consistent with observations in this study with activity displayed against *C. elegans*. Whilst there was the appearance of trapped nematodes by *Trichoderma* sp. in the sprinkle plates when in isolated culture with *C. elegans* no trapping occurred, however, nematicidal effects were present and there was a notable reduction in nematode numbers over several days.

3.7.4.2. *Fusarium*

Fusarium sp. are predominantly known as soil saprotrophs and plant parasites such as *F. pseudograminearum* and *F. culmorum* - the pathogens causing crown rot of cereals, and *F. oxysporum f. sp. Lycopersici* which causes *Fusarium* wilt of tomatoes (Williams et al., 2002, Walker, 1971). However, *Fusarium* spp. have been isolated from the nematode *Meloidogyne* spp. eggs and females and evaluated for their potential as biocontrol agents (Aminuzzaman et al., 2012). 55.3% of the isolates from *Meloidogyne* spp. in Aminuzzaman et al's study were from the genus *Fusarium* (Aminuzzaman et al., 2012). This was confirmed with pathogenicity assays showing that the isolates were capable of parasitising eggs and juveniles of *Meloidogyne* spp. (Aminuzzaman et al., 2012). This research indicates that *Fusarium* spp. possess nematophagous capabilities although not traditionally described as nematophagous fungi.

The appearance of nematophagous properties by *Fusarium* sp. from the sprinkle plates was not replicated when isolated into pure culture. There was an observed overall reduction in the number of *C. elegans* on subsequent plates but no active trapping structures.

3.7.4.3. Cunninghamella

Cunninghamella spp. range from soil dwellers to human pathogens (Zhu et al., 2010, Sands et al., 1985). Many species have the capability to degrade polycyclic aromatic hydrocarbons and as a result have been studied extensively for these capabilities (Cerniglia et al., 1989, Cutright, 1995). Although not strictly classified as nematophagous *Cunninghamella elegans* has been studied as a biocontrol agent for the control of plant parasitic nematodes due to its collagenolytic properties (Galper et al., 1991). Many nematodes have a high collagen content in the cuticle including *Caenorhabditis elegans* (Galper et al., 1991). Galper et al. then found that the collagenolytic properties of *Cunninghamella elegans* enabled it to immobilise *M. javanica* larvae in the presence of soil amended with collagen (Galper et al., 1991). Whilst this is likely an indirect relationship between the fungus and the nematode the collagenolytic fungi such as *Cunninghamella elegans* produce collagenases which break down the nematode cuticles (Galper et al., 1991). It is likely that a similar interaction was occurring between the *Caenorhabditis elegans* and the *Cunninghamella* sp. from the sprinkle plate technique rather than nematophagous activity.

The behaviours of the three genera above blur the lines regarding the classification of nematophagous fungi. *Penicillium* spp. is another example of fungi that are not strictly nematophagous, one study found that Penicillium spp. had very high nematicidal activity against *Meloidogyne incognita* and *Radopholus similis* (Molina and Davide, 1992). These examples are indicative of the complexity of the microbial community in the soil. With such a large number of predators and competitors in a small space, adaptations have led to the development of an arsenal of protective mechanisms for survival. Secondary metabolites such as extracellular enzymes that degrade cell walls are produced by many organisms in the rhizosphere including plants as protective mechanisms (Whipps, 2001). Indirect and non-specialised attacks of a fungus on nematodes, where specialised trapping or attacking structures are not formed, are not likely to be classified as strictly nematophagous fungi.

Based on the characteristics of the fungi isolated in this study they would fit the definition of augmentation biological control as they display some properties capable of reducing nematode numbers. However, they would not fit the aims of this study, *S. stercoralis* requires only one infective larvae to infect a host so an orbilacious nematophagous fungi would be more appropriate as they are adapted to specifically trap nematodes it is not a secondary mechanism. This would likely include the speed and efficacy of larval reduction.

3.7.5. Other potential detection methods

Difficulties with the clarity of results and the time consuming nature of culture methods begs for a modern solution to aid isolation of nematophagous fungi. The

use of molecular methods has the potential to aid this process but not replace it. Molecular methods alone cannot isolate a culture from soil and therefore traditional techniques are still required. Despite the uncertainty reported by Smith and Jaffee on the nematophagous properties of species detected by *Orbiliales* specific PCR primers, the technique could be a useful addition to culture methods to reduce the time input required (Smith and Jaffee, 2009). These molecular techniques could be used to screen substrate samples before more time intensive culture methods are used. If no *Orbiliales* are detected then the substrate sample could be omitted from further culture processes. This would have been a useful addition in this study as *Orbiliales* were either not present, not culturable or not nematophagous.

3.7.6. Use of isolates in further work with *Strongyloides* spp.

The isolates from this study displayed some weak nematophagous properties but were not capable of effectively removing *C. elegans* from the agar plates. There are significant barriers to the application of nematophagous fungi to control human exposure to *S. stercoralis.* Infection with *Strongyloides stercoralis* in humans produces L3 infective larvae directly in faeces; and although eggs are also produced, larvae can undergo one sexual lifecycle in the free-living phase in soil or faeces. As there are immediately infective larvae in contaminated faeces and infection can occur with penetration of only one larvae, nematophagous fungi that use egg parasitism or do not have strong nematophagous properties are unlikely to be suitable or effective for further work.

Initially, this study was going to include parasitic nematode species such as *Meloidogyne* spp. and additional forms of nematophagous fungi, such as egg parasitic fungi, the rationale for including *P. lilacinus* in the detection assays.

Due to the difficulties experienced with this method and the ability to determine nematophagous properties, we decided not to proceed with isolation of nematophagous fungi in Australian Indigenous communities. Particularly noting the sensitivities surrounding working in these communities as described in the introduction contributed heavily to this decision. Therefore the decision was made to conduct further work with known nematophagous fungi from purchased and donated strains rather than species that had been natively isolated.

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4. Chapter Four: Nematophagous fungi efficacy in varying substrates and nutrient levels

4.1. Introduction

Nematophagous fungi trap and digest nematodes as an additional nutrient source (Nordbring-Hertz et al., 2006, Lopez-Llorca et al., 2008, Stirling, 2014). However, little is known about the behavior of these fungi under varied nutrient conditions, where base nitrogen and carbon requirements are already met. The areas of focus in this study are canine faeces and leaking septic tanks as outlined in the introduction. The substrates of focus would therefore include canine faeces and soil - faecal matrices. Septic tanks are seated in low nutrient sand, adding an additional substrate to consider. As trap formation requires energetic input, development of trapping structures may be induced by alterations in environmental nutrient conditions. This is particularly important if nematophagous fungi are to be used as a method of biocontrol for *Strongyloides stercoralis*. For this method to be effective nematophagous fungi would need to actively trap nematodes in high nutrient substrates.

The implications of this work would also have impact on other areas where biocontrol is a potential control measure for other parasitic nematodes. Parasitic nematodes in cropping systems and livestock are of particular interest. The nutrient environments for these parasites range extensively from low nutrient substrates to high nutrient

substrates, which could include nutrient additions such as fertilisers or livestock faeces.

The mycelium of nematophagous fungi can act saprotrophically but are capable of transitioning into a nematophagous form via the formation of trapping structures (Nordbring-Hertz, 1973). It has been claimed that low nutrient environments favour morphogenesis into this predatory phase (Nordbring-Hertz, 2004). Nordbring explains the use of low nutrient media to induce trap formation in *Arthrobotrys oligospora*, particularly with the addition of small peptides (Nordbring-Hertz, 2004), such as nemin, a compound thought to be released by nematodes which may induce trap formation in predatory fungi (Anamika, 2015, Pendse et al., 2013). The low nutrient conditions helping to induce trap formation could be attributed to the nematophagous fungi using nematodes as an additional nutrient source, particularly nitrogen. Several species of nematode trapping ligninolytic degraders have been described as using nematodes as a supplementary source of nitrogen (Kwok et al., 1992, Thorn and Barron, 1984). However, it has been suggested that the formation of traps may be a response to competition for nutrients with other microorganisms (Zhang et al., 2014, Persmark and Jansson, 1997). This shows there are theories suggesting that trap formation could be due to nutrient availability or competition with other microorganisms.

The behaviour of nematophagous fungi in higher nutrient conditions requires further elucidation as they may not require additional nutrients and therefore not spend additional energy on forming traps. If nematophagous fungi are unlikely to trap nematodes in the presence of adequate available nutrients then this has implications

for the use of the fungi in biological control of parasitic nematodes, as many parasitic nematodes are present in high nutrient environments.

A. oligospora has been demonstrated to have the ability to trap nematodes with cells that were destined to become traps before trap formation has commenced (Nordbring-Hertz, 2004). This paper also suggests that growth conditions and environmental factors strongly influence the direction of morphogenesis in this system (Nordbring-Hertz, 2004). Scholler and Rubner (1994) found that *A. oligospora* traps and consumes nematodes for both C and N, they conducted experiments to measure predacious activity by altering the agar substrate. The findings showed that the levels of C and N present in the substrate altered whether or not trap formation occurred, concentrations below 0.12 M C and 0.05 M N caused trap formation to occur (Scholler and Rubner, 1994).

Zhang et al (2014) suggest that the nutrients required by nematophagous fungi to switch from saprotrophic to parasitic differ between species and genera (Zhang et al., 2014). A study by (Mo et al., 2005) explored the nutritional requirements for growth of various nematophagous fungi, this research analysed the effects of carbon nitrogen ratios (C:N) and pH on growth and sporulation of *Pochonia chlamydosporia* (Mo et al., 2005). A recent study by (Anamika, 2015) explored optimal growth conditions of *Arthrobotrys oligospora* and *Dactylaria eudermata* in investigating carbon and nitrogen sources and temperature. The optimal nitrogen sources were potassium nitrate and sodium nitrate and the optimal carbon source was glucose with 25° C reported as the optimum temperature for trap formation. This study used radial growth as an indicator and did not assess trapping efficacy or trap formation.

Another study evaluated the effects of carbon and nitrogen sources, carbon-to-nitrogen ratio (C:N) and initial pH value on the growth and sporulation of the nematophagous fungus *Pochonia chlamydosporia* (Mo et al., 2005). This study found the optimal C:N ratio was 10:1 at pH 3.7, the most optimal sources were sweet potato and L-tyrosine.

These papers predominately measured radial growth of the fungi. Regardless of growth, sporulation or trap formation, trapping efficacy may differ dependent upon environmental conditions, with trap formation having less to do with trapping capacity than originally thought. Cells that are destined to form traps in *A. oligospora* are capable of trapping nematodes long before the trap itself has developed (Nordbring-Hertz, 2004). Optimal concentrations of carbon and nitrogen for trap formation have been studied in *Duddingtonia flagrans* (Anan'ko and Teplyakova, 2011), finding that optimal concentrations and sources of carbon and nitrogen were: sucrose (0.4%), ammonium ions (0.2%), and tryptone (0.2%) to promote trap formation, however higher concentrations were inhibitory (Anan'ko and Teplyakova, 2011). These findings were not consistent with the ratios or substrate types found in the studies discussed above.

This research reported here was undertaken to determine the effect of available nutrients in various substrates on nematophagous efficacy and therefore the morphogenesis to trap formation. To do this the fungus *A. oligospora* was used as a model nematophagous fungi and its nematophagous efficacy was assessed with *Caenorhabditis elegans* in five different substrates with differing nutrient profiles. *Neurospora crassa* is a model fungus that was used as a non-nematophagous control

in this experiment (Tian et al., 2009). This was only used in the canine faeces treatment to reduce the amount of substrates required and time spent counting nematodes as an indication as to whether the presence of fungi has an impact on nematode survivability in substrate.

4.2. Methods

Three primary substrate types were chosen to represent low, medium and high nutrient conditions (sand, organic soil (sandy loam) and canine faeces respectively). These were then mixed in a 1:1 ratio of sand: organic soil and organic soil: canine faeces (Table 4.1).

Sand and organic soil were obtained from domestic gardens and the canine faeces were obtained from a domestic dog. Canine faeces were collected over the course of a week whilst the dog maintained a consistent diet, collected faeces were stored at 4˚C during collection period and homogenised before use in assay.

Table 4.1: Substrate types and blends used in nematophagous fungi assays with *A. oligospora* and *C. elegans*

Substrate Types				
100% Sand	50% Sand 50% Organic Soil	100% Organic Soil (sandy loam)	50% Organic Soil 50% Canine Faeces	100% Canine Faeces

4.2.1. Soil Nutrient analysis

Nutrient analysis was performed in triplicate on the three main substrate types sand, organic soil and canine faeces, substrate blends were not analysed.

4.2.1.1. pH

Methods were adapted from Rayment and Lyons using a 1:5 soil/water suspension (Rayment and Lyons, 2011). 5 g of sand, organic soil, or canine faeces were added to 25 mL deionised water and mixed by tube rotator(RSM7DC, Ratek Instruments Pty, Ltd, Australia) for 1 hour and pH of these solutions was recorded (pH700, Eutech instruments Pty, Ltd).

4.2.1.2. Phosphorus

The sodium bicarbonate method, also known as the Olsen method, was used for extraction of phosphorous from soil (Olsen, 1954). The phosphorus content was measured using the FOSS PO_4 analyser following the phosphorus Stannous Chloride Method as described in test 4500-P D (Greenberg and Clesceri, 1992). The substrate samples were air dried and sieved through a 1.5 mm sieve. A 2 g sample was mixed with 40 mL, 0.5 M $NaHCO_3$ and mixed by tube rotator (RSM7DC, Ratek Instruments Pty, Ltd, Australia) for 30 min. Samples were gravimetrically filtered through 90 mm Whatman no.1 filter paper (11 µm). Filtrates were stored at 4°C before analysis. Filtrates were acidified to neutral by adding 1.2 mL of 10 % sulphuric acid (Merek) to each sample of approximately 5 mL, stored overnight at 20˚C and then sonicated (895 Ultrasonic Cleaner, Cooper Vision Surgical Inc., 35W) until effervescence ceased.

4.2.1.3. Nitrogen and Carbon

Triplicate, 2 g samples of substrate (sand, organic soil or faeces) were suspended in 18 mL deionised water in 50 mL tubes and placed on a tube rotator (RSM7DC, Ratek Instruments Pty, Ltd, Australia) for 1 h. The solutions were then filtered through 90 mm 1.2µm glass fibre filter paper (FisherBrand). The filtrates were analysed for total

carbon, total organic carbon, inorganic carbon and total nitrogen using Shimadzu TOC-LSCH analyser (Shimadzu Ltd) with autosampler.

4.2.1.4. Lipids

Lipids were extracted using 1g of substrate in triplicate following Bligh and Dyer's chloroform: methanol method (Bligh and Dyer, 1959). Extracts were dried and weighed to determine total lipids.

4.2.2. Nematophagous efficacy

Substrates were sterilised via autoclave (121˚C for 30 minutes), wetted with sterile MilliQ water to moisture holding capacity and 5 g (wet weight) added to 60 mm Petri dishes to which was added a 5 mm agar plug from a 7 day old *A. oligospora* culture to the treatment plates and omitted for controls. *A. oligospora* culture was purchased from ATCC.

This procedure was repeated with canine faeces and *N. crassa* as a non-nematophagous fungal control. *N. crassa* culture was kindly provided by David Catcheside, Flinders University.

Plates were incubated at 25 C° for 1 week to allow the *A. oligospora* or *N. crassa* to colonise the substrate, whilst controls were stored at 4 C° to reduce growth of other microorganisms.

C. elegans (culture kindly provided by Peter Boag, Monash University) were maintained on Nematode Growth Medium with an *Escherichia coli* OP50 lawn as described in Chapter 3. The plates were washed with sterile deionised water and contents collected into a 2 mL tube. Nematodes were then washed and sterilised,

before nematode concentration in solution was determined via counting via Stiernagle's methods described in Chapter 3 (Stiernagle, 2006).

The total volume (µL) containing 1000 nematodes was then calculated and 1000 nematodes were pipetted to each 60 mm Petri dish containing either the substrates inoculated with *A. oligospora* or *N. crassa* or the respective substrate in the absence of fungal inoculum. These were then incubated at 25 C° for 7 days.

After 1 week the Baermann technique (Khieu et al., 2013) was performed on substrates from each of the Petri dishes for 5 hours to extract the nematodes. The filtrate from the Baermann technique (approximately 10 mL) was collected and nematodes counted.

4.2.3. Statistical analysis

Statistical analyses were performed using IMB SPSS Statistics.

Data was assessed for normality using the Shapiro-Wilk test. Q-Q plots were generated and assessed for abnormal distribution. Unless otherwise stated all data was normally distributed. Means were tested using a one way ANOVA with Tukey's post hoc test. Significant differences are reported where $p<0.05$.

4.3. Results

4.3.1. Nematode survival and nematophagous efficacy

Predatory activity by nematophagous fungi was indicated by lower nematode numbers in the extracts from substrates with the *A. oligospora* treatment compared to the respective controls with no nematophagous fungi. Despite inoculation and incubation conditions remaining consistent for *A. oligospora* growth on all substrate

types there were visible differences in mycelium density. Sand displayed sparse mycelial density and the density increased in substrates containing organic soil and more so in substrates containing canine faeces. This was likely due to available nutrients, however, enumeration of the fungal density without sample destruction was a limiting factor so this remained as an observation.

As controls were run for every treatment nematophagous efficacy could be measured in every substrate type. Nematophagous properties were displayed in canine faeces, 50% canine faeces/ 50% organic soil blend and organic soil. Canine faeces yielded high numbers (mean 1309) of nematodes in the control and low numbers (mean 0.33) in the *A. oligospora* treatment as displayed in Figure 4.1. This can also be expressed as 99.99% nematode removal in the *A. oligospora* treatment in comparison to the control. The number of nematodes recovered from the control were higher than the number of nematodes inoculated into the assay, indicating they multiplied in the canine faeces substrate, this did not occur in any other substrates. In all substrate types that did not contain sand significantly less nematodes were extracted from *A. oligospora* treatment compared to controls. Statistically there were significantly fewer nematodes enumerated from the following substrates treated with *A. oiligospora* compared to the controls; organic soil (p= 0.013), 50 % organic soil/ 50% canine faeces blend (p= 0.015) and canine faeces (p= 0.001)

However, nematophagous activity was not detected in any of the substrates containing sand. These substrates yielded very low numbers of live nematodes in controls and *A. oligospora* treatments. This indicates that nematode survival was poor in these substrate types.

The non-nematophagous fungi control showed no significant difference between the control and *N. crassa*, indicating this fungus was not effecting nematode survival.

Canine faeces yielded a significantly greater number of nematodes in both controls and treatments than sand or 50% sand/ 50% organic soil with p= 0.030 and 0.031 respectively (Figure 4.2). This indicates that the nematodes had the best survival in substrates containing canine faeces and nematode survival was very poor in substrates containing sand.

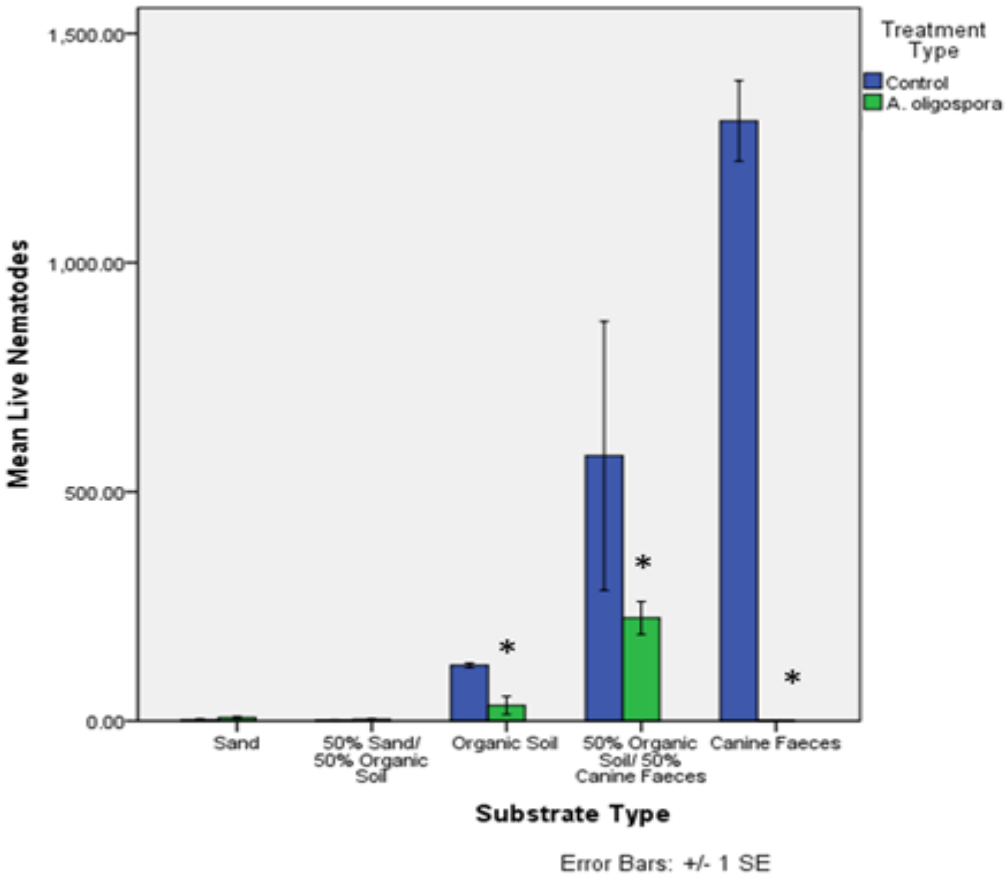

Figure 4.1: Mean number of live nematodes extracted from substrates comparing controls to *Arthrobotrys oligospora* treatments.
* Indicates significance between treatment and control where p<0.05

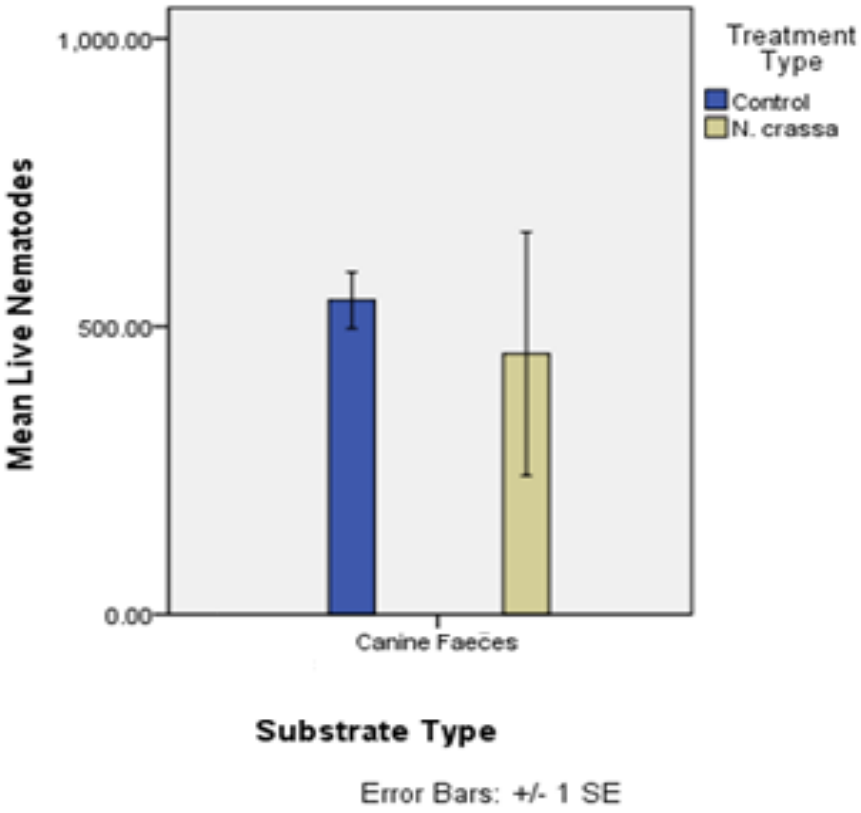

Figure 4.2: Mean number of live nematodes extracted from canine faeces comparing control to *Neurospora crassa* treatment.
* Indicates significance between treatments and controls where p<0.05

4.3.2. Nutrient Analysis

This analysis is a comparison of nutrient type and concentration and survival, these comparisons were derived from the original dataset and analysis. The comparisons graphically represent the nematode survival against nutrient type and concentration.

Nutrient analysis showed that sand consistently contained the lowest levels of all measured nutrient parameters other than inorganic carbon, whilst canine faeces contained the highest measured levels for each parameter (Table 2), with significantly higher levels of total and organic carbon in canine faeces compared to organic soil or sand (p<0.05).

Table 2: Analysis of substrate extracts; total organic carbon (TOC) (calculated from TC-IC), total carbon (TC), inorganic carbon (IC), total nitrogen (TN), phosphorus (P), total lipids (TL) and pH.

	TOC (g/Kg)	TC (g/Kg)	SD	IC (g/Kg)	SD	TN (g/Kg)	SD	P (mg/Kg)	SD	TL (g/Kg)	SD	pH
Sand	0.22	0.26	<0.01	0.04	<0.01	0.03	<0.01	1.02	0.20	2.80	0.12	6.66
Soil	0.75	0.76	<0.01	0.01	0.02	0.08	<0.01	1.62	0.31	5.40	0.44	7.07
Faeces	20.68	21.19	0.36	0.51	<0.01	3.82	0.10	57.61	5.06	6.30	0.46	7.60

Higher amounts of total carbon were associated with higher numbers of surviving nematodes extracted from untreated controls (Figure 4.3). This effect is particularly pronounced in canine faecal samples with the highest amounts of carbon being associated with the greatest number of surviving nematodes. Contrastingly, the low levels of carbon in the sand and organic soil were associated with low levels of nematodes in the corresponding controls. It is also notable that the maximal difference between *A. oligospora* treatment and control is highest in canine faeces (Figure 4.1).

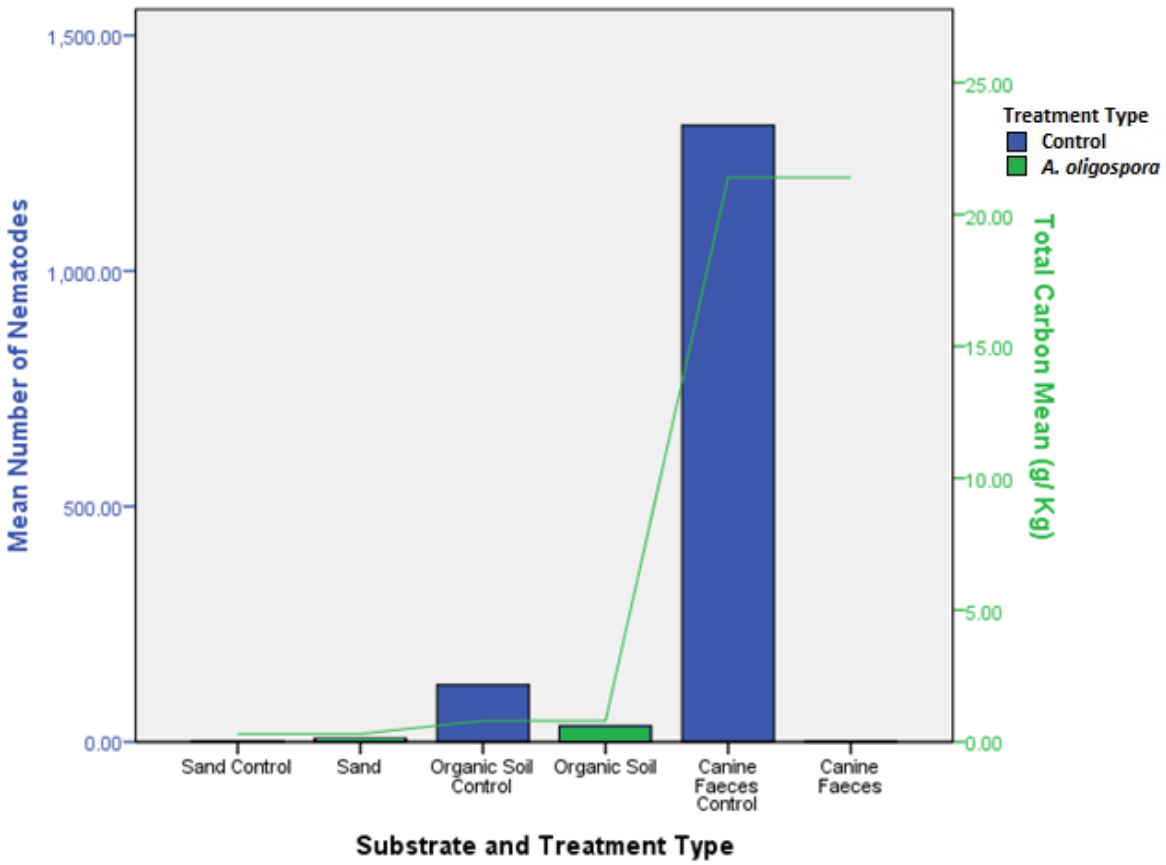

Figure 4.3: Mean number of *C. elegans* extracted from sand, organic soil or canine faeces substrates per treatment type (*A. oligospora* or control), overlayed with mean total carbon for each substrate (g/Kg).

Similarly to carbon, higher amounts of nitrogen in substrates was associated with higher numbers of surviving nematodes extracted from untreated controls (Figure 4.4). The highest levels of nitrogen were present in canine faeces, corresponding with the largest amount of surviving nematodes in these controls, with correspondingly lower numbers of surviving nematodes in organic soil and sand, and decreasing amounts of nitrogen present respectively. Once again, the maximal difference between *A. oligospora* treated samples and their corresponding control was notable in canine faeces (Figure 4.1).

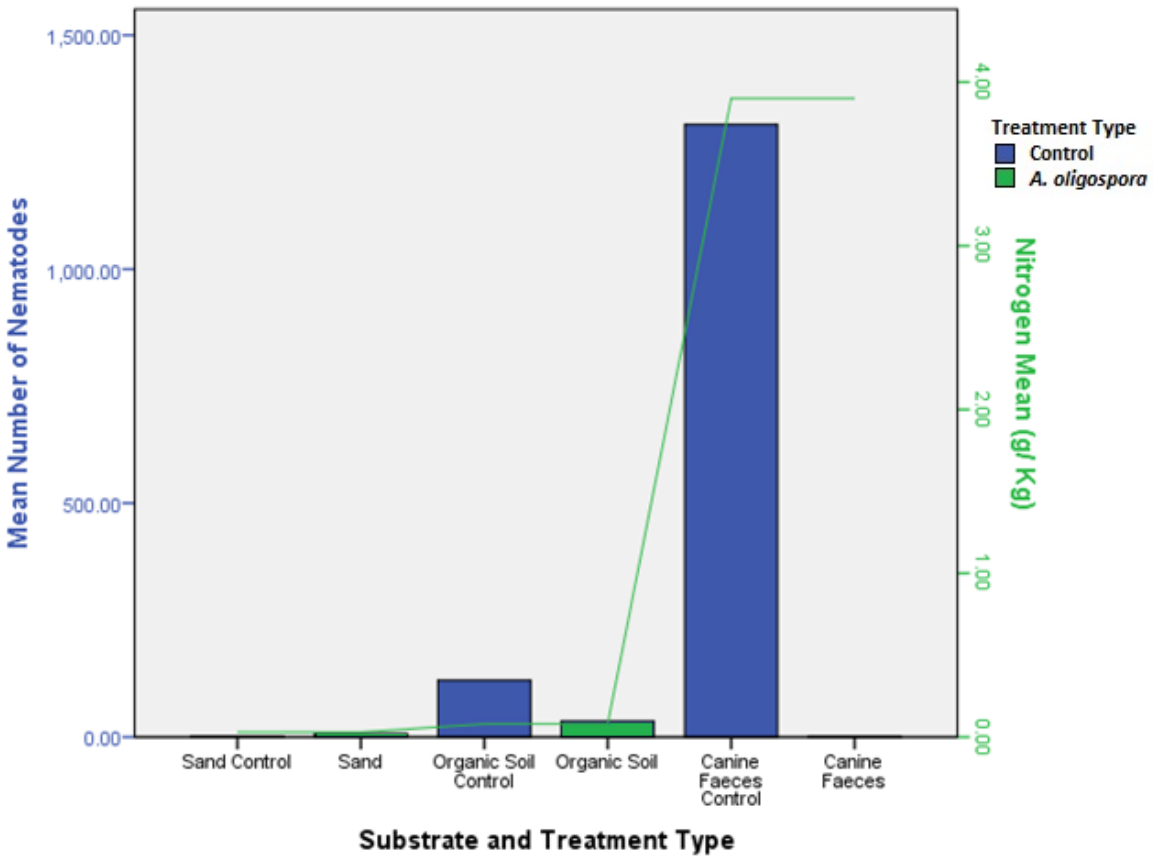

Figure 4.4: Mean number of *C. elegans* extracted from sand, organic soil or canine faeces substrates per treatment type (*A. oligospora* or control), overlayed with mean total nitrogen for each substrate (g/Kg).

In agreement with measurements of carbon and nitrogen, canine faeces contained the highest amount of phosphorus, followed by organic soil and sand respectively (Figure 4.5). The highest number of surviving nematodes extracted from untreated controls was found in canine faeces, followed by organic soil and sand.

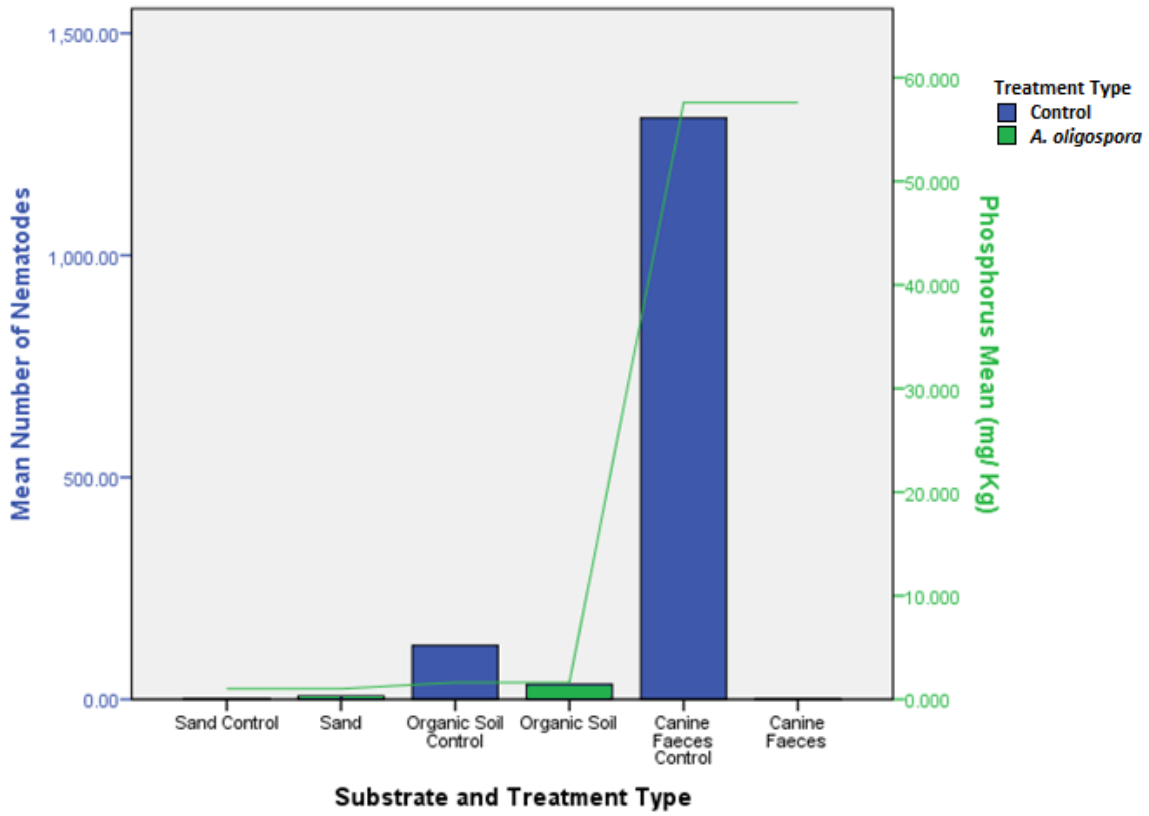

Figure 4.5: Mean number of *C. elegans* extracted from sand, organic soil or canine faeces substrates per treatment type (*A. oligospora* or control), overlayed with mean total phosphorus for each substrate (mg/Kg).

Canine faeces additionally contained the highest amount of total lipids, followed by organic soil and sand respectively (Figure 4.6). The highest number of surviving nematodes extracted from untreated controls was found in canine faeces, followed by organic soil and sand.

Sand and organic soil had a neutral pH rating and canine faeces was slightly or mildly alkaline as per (Rayment and Lyons, 2011) pH rating scale.

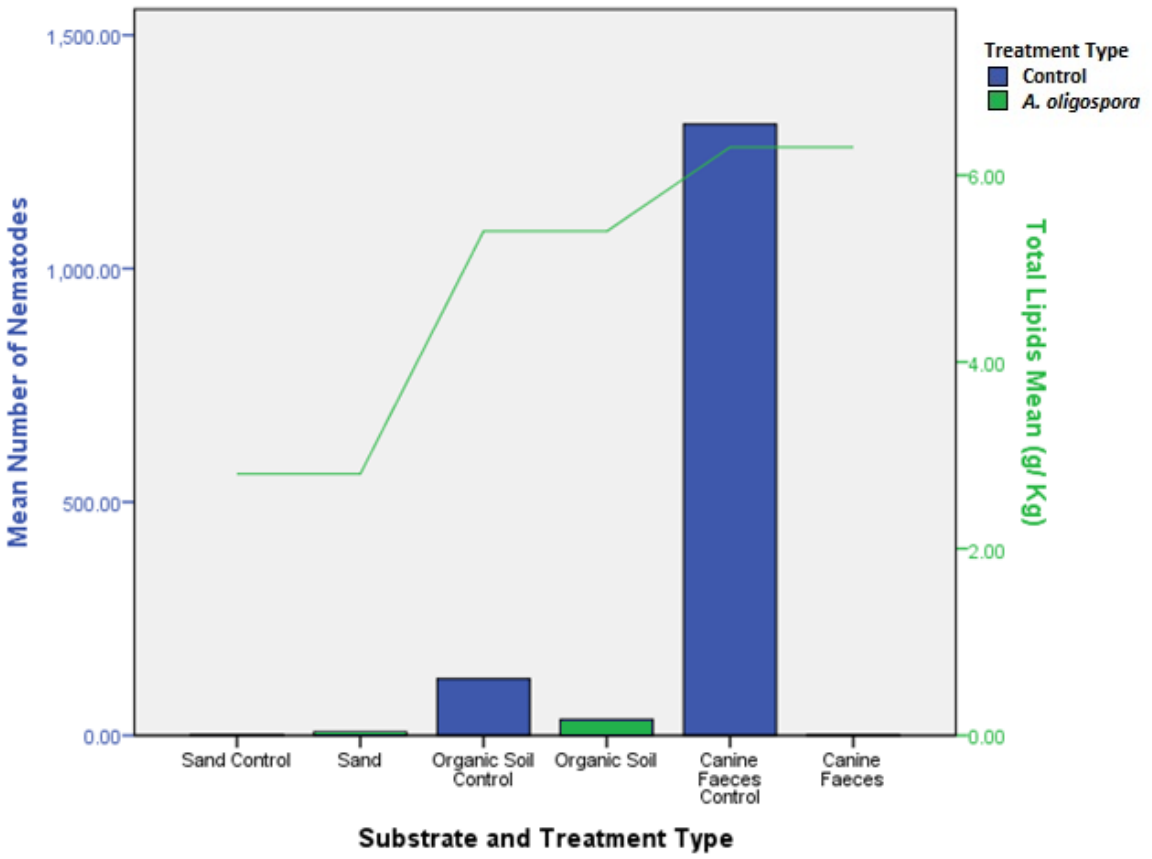

Figure 4.6: Mean number of *C. elegans* extracted from sand, organic soil or canine faeces substrates per treatment type (*A. oligospora* or control), overlayed with mean total lipids for each substrate (g/Kg).

4.4. Discussion

4.4.1. Nematophagous efficacy and nematode survival

The results of this research show that the nematophagous fungus *Arthrobotrys oligospora* displayed greater nematophagous properties in substrates containing higher available nutrients, namely carbon, nitrogen, phosphorus and lipids. This favourable nutrient condition likely allows for better establishment of fungi in the substrate, as well as promoting nematode replication. In canine faeces *A. oligospora*

had 99.99% efficacy of removing *C. elegans* from substrate. It can be concluded that high nutrient environments such as faeces, sufficient nutrients are available for strong growth of nematophagous fungi without inhibiting morphogenesis to nematode trap formation.

Substrates containing sand visually displayed limited fungal establishment as well as limited nematode survival. With very few nematodes capable of surviving in this environment it was difficult for the nematophagous fungi to display effective trapping capabilities, and likely difficult to determine trapping efficacy with environmental conditions exerting a strong influence on nematode survival. These characteristics of limited growth and survival may be attributed to the low nutrient content of sand, however, the lack of nematode survivability may also be attributed to the shape of the sand particles. Sand particles may shear the nematode cuticle when disrupted or when nematodes move through the pore spaces killing the nematode in the process. Whilst there is not strictly evidence of this occurring with sand this kind of damage does occur in other situations. The nematophagous fungus *Coprinus comatus* produces spiny structures on it hyphae which shear the nematodes cuticle with a similar mechanism (Luo et al., 2007).

Our understanding that nematophagous fungi are more abundant in substrates with higher organic matter dates back to the 1930s and has been demonstrated in other studies since (Linford et al., 1938, Pendse et al., 2013). This is consistent with the growth and efficacy of *A. oligospora* in this experiment. Canine faeces contained the highest carbon content and therefore implying the highest organic matter. This was

also consistent with nematode growth and this substrate was the only one which displayed an increase in nematode numbers in the controls.

The experiment was repeated with canine faeces and *Neurospora crassa* a saprotrophic non nematophagous fungi to ensure that the effects of the fungi on the nematodes extracted was due to the nematophagous properties as opposed to the presence of fungi in substrate. There was no significant difference between the number of nematodes extracted from the control and the *N. crassa* indicating that the mere presence of any fungi did not play a role in nematode removal when not nematophagous.

The nematophagous efficacy appeared to increase as nutrient content of the substrate increased. However, the number of nematodes returned in the control also increased as the nutrient content of the substrate increased. This indicates the capability of the nematodes to grow and survive also increased with the nutrient content of the substrate. Additionally the nutrients available to the fungi increased as the nutrients of the substrate increased. It could be hypothesised that the abilities of the nematophagous fungi to form traps increased with the nutrient content of the substrate. Therefore the nematophagous efficacy increased with increased available nutrients in the substrate. This information is contrary to previous reports which suggest nematophagous activity is induced in low nutrient environments. However, this favours the theory that nematophagous activity is induced by the presence of nematodes.

4.4.2. Application in biocontrol

The capacity of nematophagous fungi to produce traps and trap nematodes in high nutrient environments may have applications in a range of scenarios. Nematophagous fungi has been suggested as a biological control measure for parasitic nematodes, this ranges from parasites of crops to parasites of livestock and parasites of humans. This information provides novel insight into the role that nematophagous fungi could play in these scenarios.

4.4.2.1. Cropping systems

In agricultural cropping systems there are many fluctuations in nutrient and moisture levels over the course of the season (Bell, 2015). Often there is a limited period of the year where moisture levels are high enough to sustain plant growth. Nutrient levels fluctuate during the year as the plants use nutrients from the soil. In cereal cropping nutrients have to be added during the growing season and the plants use these resources leaving little remaining for the following season meaning the cycle has to be repeated. Nitrogen, phosphorus and potassium are the most common nutrient additions in cereal production (Sapkota et al., 2016), and are nearly always added at time of sowing. This type of cropping cycle may provide a scenario where high nutrients are required along with use of nematophagous fungi. With parasitic nematodes often causing problems with plant establishment, adding nematophagous fungi with N and P amendments at sowing, such as in a compost complex could allow for nematode removal in a high nutrient complex whilst the plant is establishing. It would be expected that the fungi would not have longevity in the soil and that the native microflora would return to its normal equilibrium. This would allow the beneficial nematophagous fungi to be prominent in the

establishment stage of the crop, reducing chance of infection by plant parasitic nematodes and potentially fungi. The nematophagous amendment would then easily slot into crop management practices and provide benefit of nematophagous fungi acting efficiently in high nutrient environments.

4.4.2.2. Livestock parasites

Parasitic nematodes of livestock and humans function differently and have limited scenarios where they are capable of causing infection. Parasitic nematodes with free-living stages of their lifecycles have been the focus of biocontrol using nematophagous fungi. Livestock usually live and defecate in the same environments, putting them at higher risk of infection and reinfection. The soil-faecal matrix and hay bedding are likely to make up these environments. Chandrawathani et al. (1998) investigated the use of *A. oligospora* as a biocontrol measure for *Strongyloides papillosus* in livestock bedding and found the fungus to be effective at removing *S. papillosus* from bovine faeces. The results reported here supports the evidence that *A. oligospora* displays its nematophagous capabilities in high nutrient environments such as faeces. The longevity of the fungus in these matrices especially when soil is included may be limited making the prospect less viable, but this would need to be tested in more detail.

4.4.2.3. Strongyloides stercoralis

The free-living stage of human parasitic nematode life-cycles such as those of *S. stercoralis* are more difficult to target as the host infection occurs under more specific circumstances. These circumstances can range from host exposure to larvae in water sources or rice paddies to failed infrastructure including leaking septic tanks. Water sources are likely to contain low nutrient levels and are a difficult matrix for

terrestrial nematophagous fungi to be active in. Leaking septic tanks are more applicable to this context as it involves faecal matter leaking into soil. Septic tanks are however seated in low nutrient sand, so any nutrients are likely to come from the leaking septic tank. This would create an unsustainable environment for nematophagous fungi to continuously survive in, once again raising the issue of longevity of the fungus in the environment. Additionally based on the results of this study nematode survival may be limited in sand, however there would likely be variation between *C. elegans* and *S. stercoralis*. Further studies into the efficacy of nematophagous fungi in low nutrient sand wetted with liquid faecal waste would provide insight into the specifics of this interaction. However, for the amount of input required to sustain nematophagous growth in this environment, it would be suggested that efforts would be better spent fixing failed infrastructure.

4.4.3. Future work

Further studies should be conducted that utilise these results and help to explain in greater detail the relationship between nematophagous fungi, substrate type and nutrient content. Considering the doubts about nematode survivability in sand more suitable substrates for the nematode species should be used. The nutrient content of these substrates could then be intentionally altered in order to determine if the effects can be attributed to particle shape or nutrient content of the substrate. Additionally the nutrient content of the organic soil that was chosen was lower than expected and did not provide the linear increase in nutrients as sought. This could be mitigated by intentionally altering the nutrient content of a chosen substrate to produce a more defined gradient in nutritional conditions. In this study however, the

substrates that were chosen were intended to mimic the substrates that occur naturally in the field rather than presenting idealised, artificial conditions.

Fungal enumeration should also be a consideration in future work as the mycelial density appeared to increase with nutrient availability in the substrates. As many methods of fungal enumeration are destructive or not possible in these substrate types such as spore suspension enumeration or optical density, an additional replicate would need to be designated for this purpose. Use of quantitative PCR to determine the amount of specific fungal DNA in a known amount of sample would be an ideal method for this assay.

4.5. Conclusions

Nematode growth and survival is reduced in low nutrient substrates, therefore nematophagous fungi are limited by nematode availability. Sand and 50% sand/ 50% organic soil contained low nutrient levels and had this effect. High nutrient substrates promote high nematode growth and high nematophagous trapping efficacy. The canine faeces contained higher nutrient levels, produced high nematode numbers in the control and displayed excellent nematophagous trapping efficacy of *A. oligospora* to *C. elegans*. The results of this research show that there is potential for use of nematophagous fungi as a biocontrol measure in high nutrient substrates such as faeces.

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5. Chapter Five: Nematotoxins from Nematophagous Fungi

5.1. Introduction

Fungi exist in ecologically and microbially rich environments, this makes them susceptible to predators such as insects, bacteria, nematodes and other fungi (Stirling, 2014). For example nematodes can consume more than half of the fungal mycelium present in soil (Anke et al., 1995). Development of defence mechanisms against these other organisms can help to ensure fungal competitiveness, survival and reproduction. Defence mechanisms include spore distribution, the production of secondary metabolites, these have a range of applications such as: antibiotics, mycotoxins, nematotoxins and phytotoxins (Calvo et al., 2002, Keller et al., 2005, Anke et al., 1995, Yu and Keller, 2005). Secondary metabolites are often produced at sporulation, not solely for the protection of the reproductive phase of the lifecycle but some metabolites aid the process (Calvo et al., 2002).

The discovery of penicillin was instrumental in the investigation of secondary metabolites of fungi (Keller et al., 2005). Penicillin was extracted from *Penicillium notatum* by Alexander Fleming in 1929, this antibiotic prompted the pharmaceutical industry to screen thousands of species for bioactive secondary metabolites (Keller et al., 2005). Despite the industry focus on secondary metabolites there is still much

unknown about them although there are still new biologically active entities discovered each year.

5.1.1. Mycotoxicology

Mycotoxins are secondary metabolites that have a negative impact on human or animal health, causing acute or chronic effects (Hussein and Brasel, 2001). Some examples of mycotoxins of significance are: aflatoxins, ochratoxins, trichothecenes, zearalenone, fumonisins, tremorgenic toxins, and ergot alkaloids (Hussein and Brasel, 2001). These are often found in foods such as peanuts and grains and pose significant human and animal health risks. Production of these toxins can occur when food becomes contaminated with the moulds that produce them such as aflatoxins produced by *Aspergillus flavus* which are carcinogenic and mutagenic and cause cancer in all animal species that have been studied (Chen et al., 2014). Zearalenone which is produced by *Fusarium* sp. which can contaminate food and feeds, this causes reproductive disorders and hyperoestrogenic syndromes in humans and animals that consume contaminated food (Zinedine et al., 2007). Aflatoxins and zearalenone are both produced by relatively common moulds that are common contaminants of stock feed and can also contaminate human food sources making them a risk to both human and animal health.

These toxins are often produced in the mycelial phase of the fungus, although generally toxins produced by mushrooms and yeasts are excluded from this definition (Hussein and Brasel, 2001, Bennett, 1987). However, it is important to note that fungal fruiting bodies do produce toxins such as the amatoxin and phallotoxin

produced by the fruiting body of *Amanita phalloides* which is often deadly to humans when consumed (Berger and Guss, 2005).

5.1.2. Nematotoxins

Nematophagous fungi are well known for their nematode trapping structures such as adhesive knobs, adhesive nets and constricting rings, in addition to this many nematophagous fungi also produce nematotoxins (toxins with nematicidal qualities.) These toxins paralyse or kill the nematodes as part of the nematophagous capabilities of the fungus protecting the mycelia or fruiting body of the fungus (Anke et al., 1995). The nematode is then stationary and cannot move away from the hyphal structure of the fungi. The fungus can then degrade the nematode in a saprotrophic manner. The capability to produce nematotoxins has been identified in lignicolous mushrooms (Thorn and Barron, 1984, Kuikarni and Dighe, 2000). There are currently just under 300 nematicidal metabolites that have been isolated from fungi, spanning 280 species (Degenkolb and Vilcinskas, 2016a). These metabolites have been isolated from a broad range of fungal types, not just lignicolous Basidiomycetes, including nematophagous Ascomycetes, nematophagous Basidiomycetes and non-nematophagous fungi (Degenkolb and Vilcinskas, 2016b).

Some extracted compounds have been tested in detail for their nematotoxic capabilities whilst others are yet to be studied in more detail. Where mechanisms for toxicity are known or theorised they are discussed below.

5.1.3. Nematotoxins from Ascomycetes

There are a broad array of nematophagous fungi that are classified as Ascomycetes, a small percentage of these produce nematotoxins in addition to their nematode

trapping mechanisms. The characterisation and capabilities of some of these secondary metabolites have been studied in further detail.

Linoleic acid was initially extracted from nematophagous fungus *Arthrobotrys conoides* but has since been isolated from *Arthrobotrys oligospora, Drechslerella brochopaga* and *Drechslerella dactyloides* (Stadler et al., 1993a, Degenkolb and Vilcinskas, 2016b, Stadler et al., 1993b). The compound was tested against *Caenorhabditis elegans*, the LD_{50} was 5-10 µg/mL which is not as effective when compared to anthelminthic ivermectin at 0.1 µg/mL (Stadler et al., 1993a, Degenkolb and Vilcinskas, 2016b).

A related species *A. oligospora* was found to produce the compounds oligosporon, oligosporol A and oligosporol B. The compounds were tested against *C. elegans* and *Haemonchus contortus* (barber's pole worm, infecting ruminants) with no nematicidal activity against *C. elegans* (Stadler et al., 1993b) and an LD_{50} of 25 µg/mL against *H. contortus* (Anderson et al., 1995, Degenkolb and Vilcinskas, 2016b) . Anderson et al. (1995) did not publish methods for bioassays for LD_{50} determination, however, they also found no nematicidal activity using their methods against *C. elegans*. This suggests there is specificity and variability in the response of nematodes to nematotoxins. Degenkolb notes that many of these nematicidal metabolites such as the unsaturated fatty acids and oligosporon-type metabolites are unstable which could inhibit their effectiveness once extracted (Degenkolb and Vilcinskas, 2016b).

From an Australian perspective Park et al. tested the culture filtrate from *Byssoclamys nivea* isolated from mud in Western Australia on *C. elegans* (Park et al., 2001). The cultures additionally produced guttation droplets which were also tested

on *C. elegans*. Both the filtrate and guttation droplets were found to cause mortality in the *C. elegans* and cuticle disruption of the nematodes. The filtrate also completely inhibited hatching of *C. elegans* eggs. The active compounds were found to be thermostable at 100°C but not at -20°C which disabled the activity of the compounds (Park et al., 2001).

5.1.4. Nematotoxins from Basidiomycetes

Production of fruiting bodies is energetically expensive but is also the only form of sexual reproduction in fungal growth. This makes competition and survival at this stage of growth vital and could explain production of bioactive secondary metabolites at this stage. Little is known about how much is produced by various species or at what growth stage production occurs at the highest rates if at all.

5.1.4.1. Pleurotus ostreatus

Pleurotus ostreatus is a basidiomycete commonly known for its edible fruiting bodies, oyster mushrooms. This fungus is an example of a species which displays predatory behaviours using nematotoxins. Toxin droplets are formed by specialised hyphal cells which immobilise the nematode. The fungal hyphae can then penetrate the nematode cuticle allowing it to access and colonise its internal structures (Kwok et al., 1992, Degenkolb and Vilcinskas, 2016a).

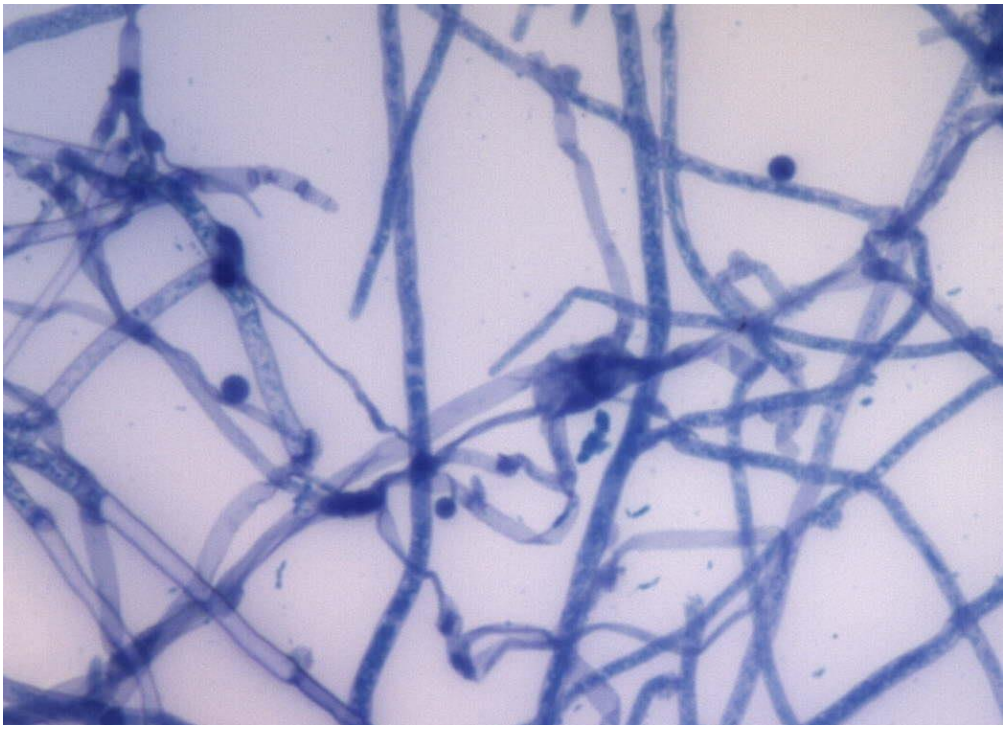

Figure 5.1: toxic droplets appear as small spherical knobs produced among hyphal mass

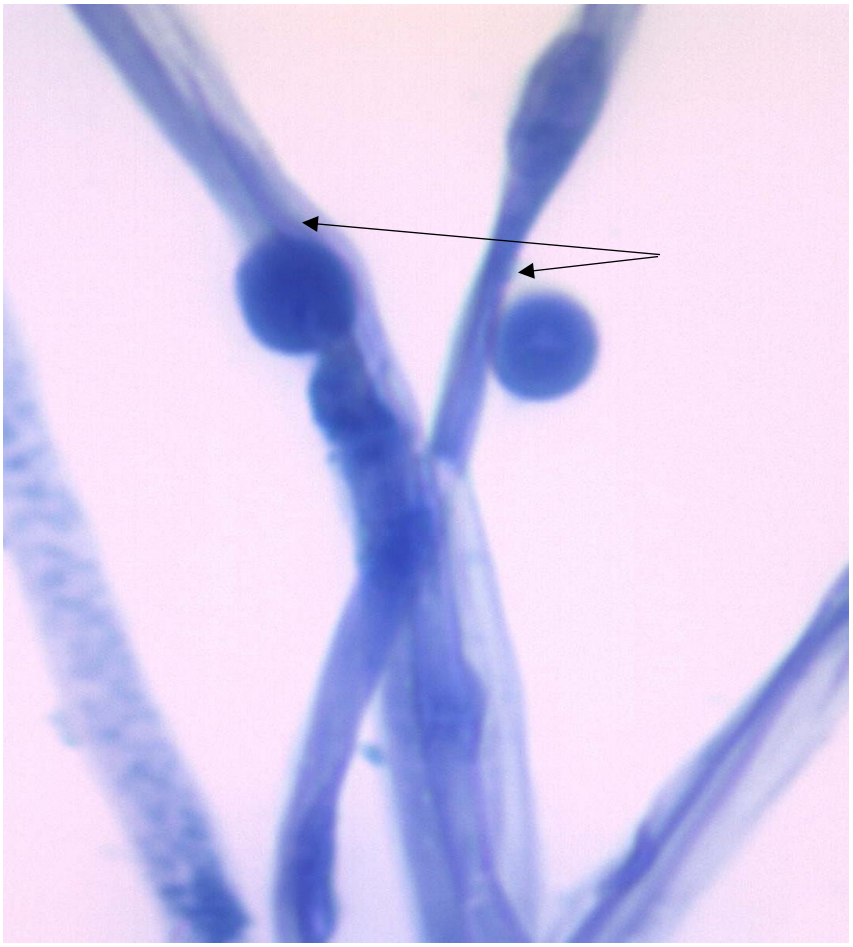

Figure 5.2: toxic droplets produced by *P. ostreatus* hyphae, indicated with arrows.

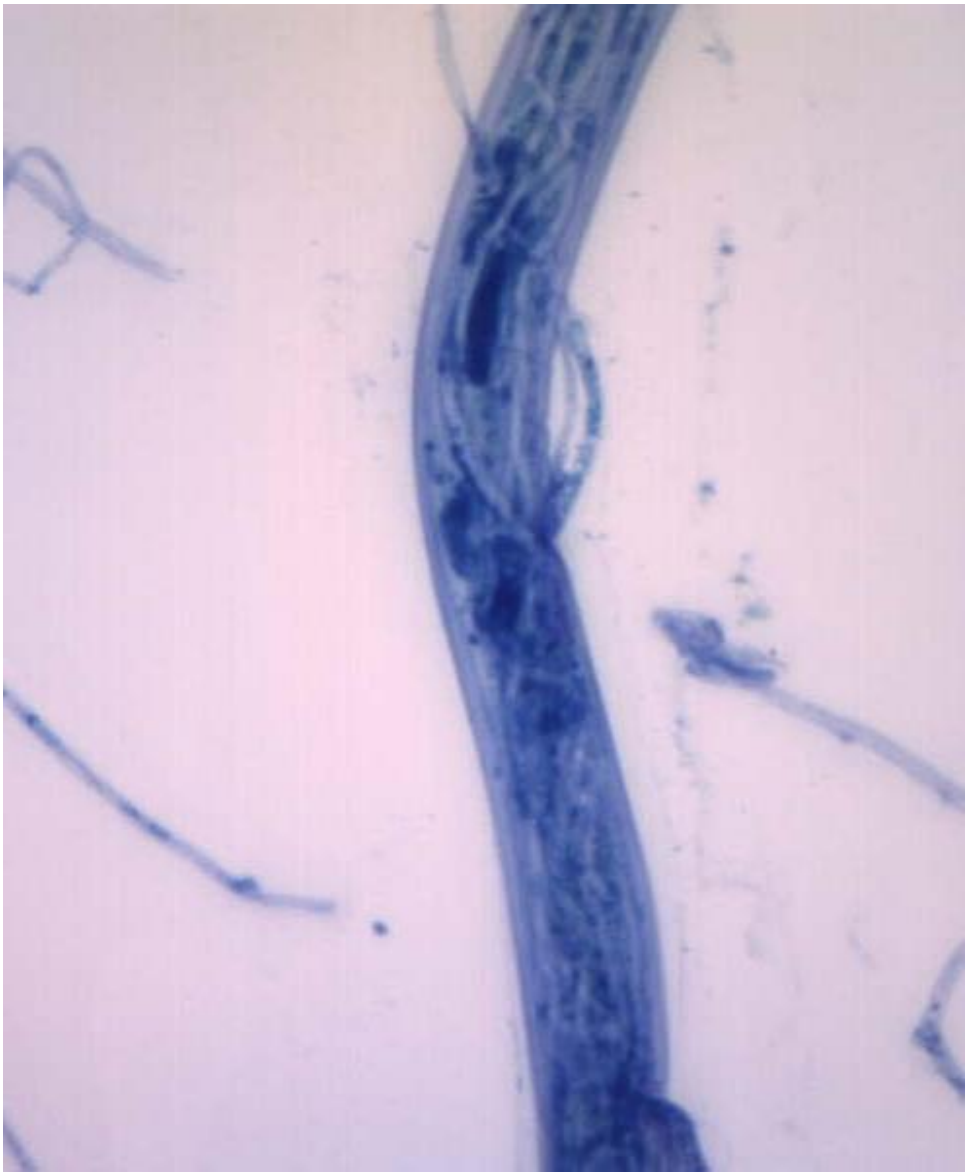

Figure 5.3: Hyphae of *P. ostreatus* colonising the internal structures of *C. elegans*

Before the compound was identified it was given the name ostreatin but has been further classified as 2-decenedioic acid (Kwok et al., 1992). Metabolites were extracted from mycelium grown on wheat straw and purified and characterised by HPLC, MS and NMR (Kwok et al., 1992). This extract immobilised the nematode *Panagrellus redivivus* at a concentration of 300 µg/mL within an hour (Kwok et al., 1992, Degenkolb and Vilcinskas, 2016a). A study into the mechanism of action for nematotoxins extracted from *P. ostreatus* found the substance shrinks the head size

of the nematode and displaces the oesophagus (Satou et al., 2008). However, the compound extracted from this study (Satou et al., 2008) characterised the nematotoxin as linoleic acid and attribute results to its conversion into linoleic acid peroxide (Satou et al., 2008). Interactions with other fatty acids could play a role in this and additionally could also be converted into peroxides (Satou et al., 2008).

5.1.4.2. Hohenbuehelia clelandii

The genus *Hohenbuehelia* are basidiomycetes in the family Pleurotaceae, the same family as *Pleurotus*. The anamorph of this genus was formerly *Nematoctonus*, which are now classified as *Hohenbuehelia* (Thorn, 2013). *Hohenbuehelia* spp. are nematophagous and capable of capturing and digesting nematodes. This genus produces adhesive knobs and conidia as well as nematotoxin as nematode trapping mechanisms (Thorn et al., 2000). Kuikarni and Dighe (2000) describe the need for basidiocarp production in order to sustain a supply of metabolites and developed a method to cultivate fruiting bodies of *H. atrocaerulea*.

Metabolites of the species *Nematoctonus robustus* (*Hohenbuehelia grisea*) were tested for their efficacy against fungi, bacteria, yeasts and nematodes and the bioactives were classified. The bioactives were identified as: dihydropleurotinic acid and pleurotin, two 1,4-naphthoquinone antibiotics and leucopleurotin (Degenkolb and Vilcinskas, 2016a). These displayed weak antifungal activities and medium to weak activities against bacteria and yeasts (Degenkolb and Vilcinskas, 2016a). Pleurotin is a napthoquinone antibiotic which has been isolated from *Hohenbuehelia* and *Pleurotus* species (Shipley et al., 2006). Nematotoxicity of Pleurotin,

dihydropleurotinic acid and leucopleurotin were tested on *C. elegans* but did not display nematicidal activity (Stadler et al., 1994, Degenkolb and Vilcinskas, 2016a).

Hohenbuehelia clelandii is an Australian native species of *Hohenbuhelia*. Found in South Australia, *H. clelandii* is known to trap nematodes (Catcheside et al., 2013). However, to date there are no published records of its nematode trapping efficacy, mechanisms or its capacity to produce nematotoxins. Trapping structures were observed in *H. clelandii* in this study, however toxic droplets were not observed.

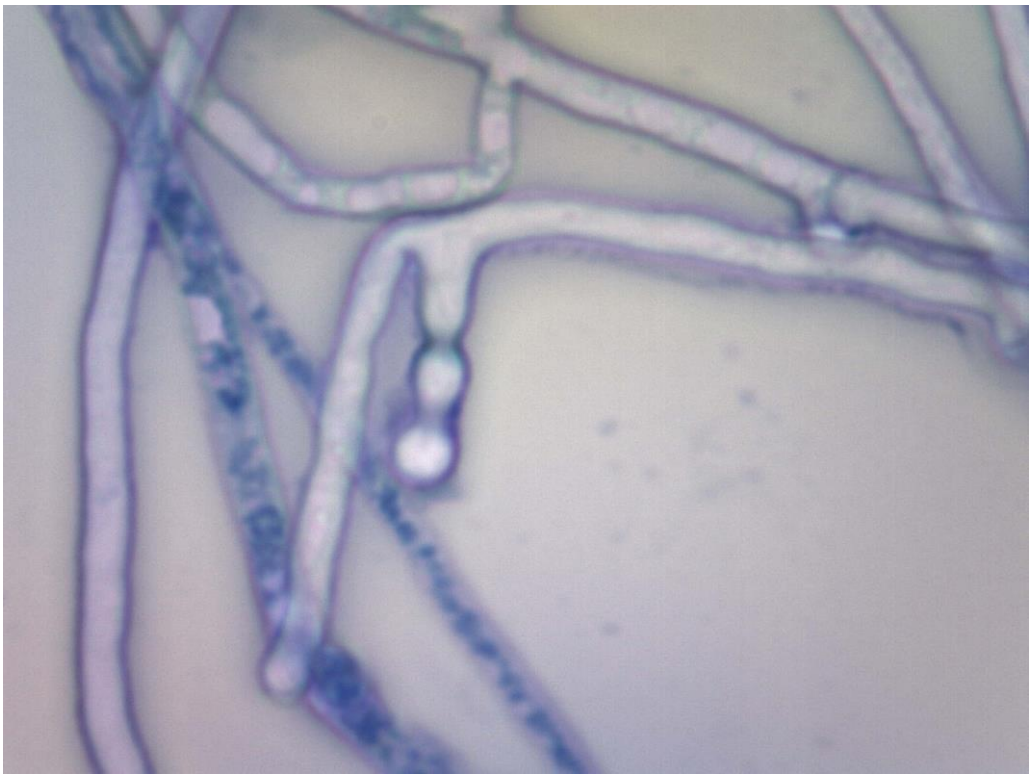

Figure 5.4: Development of adhesive knob from *H. clelandii* hyphae

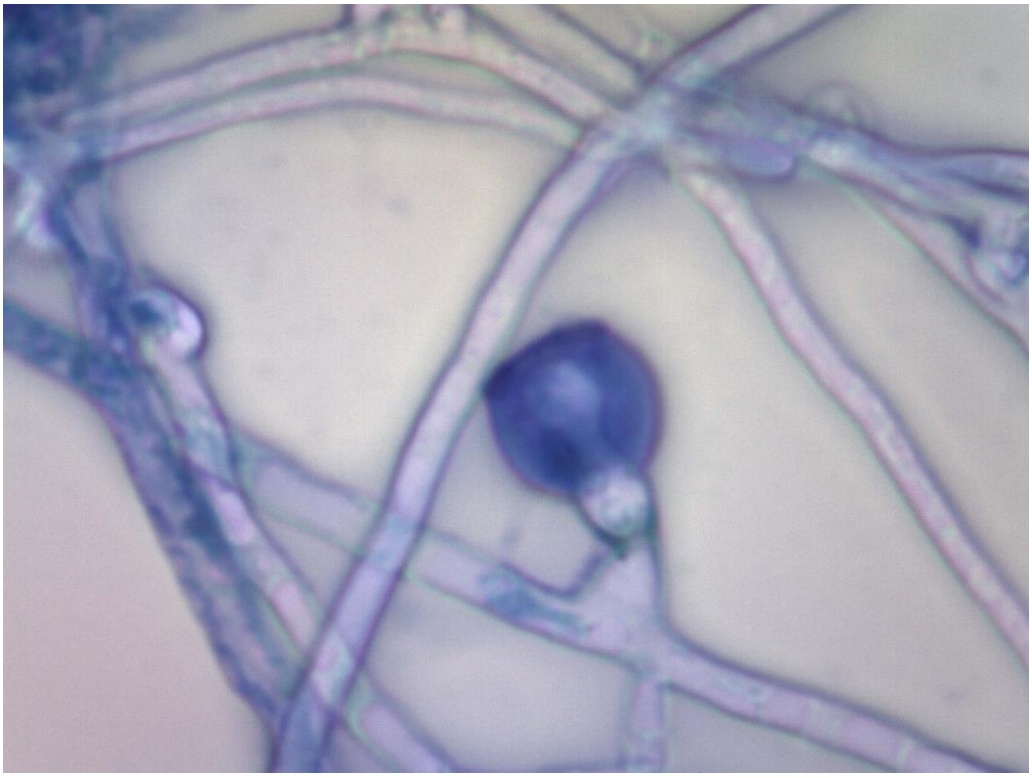

Figure 5.5: Developed adhesive knobs and clamp connections on *H. clelandii* hyphae

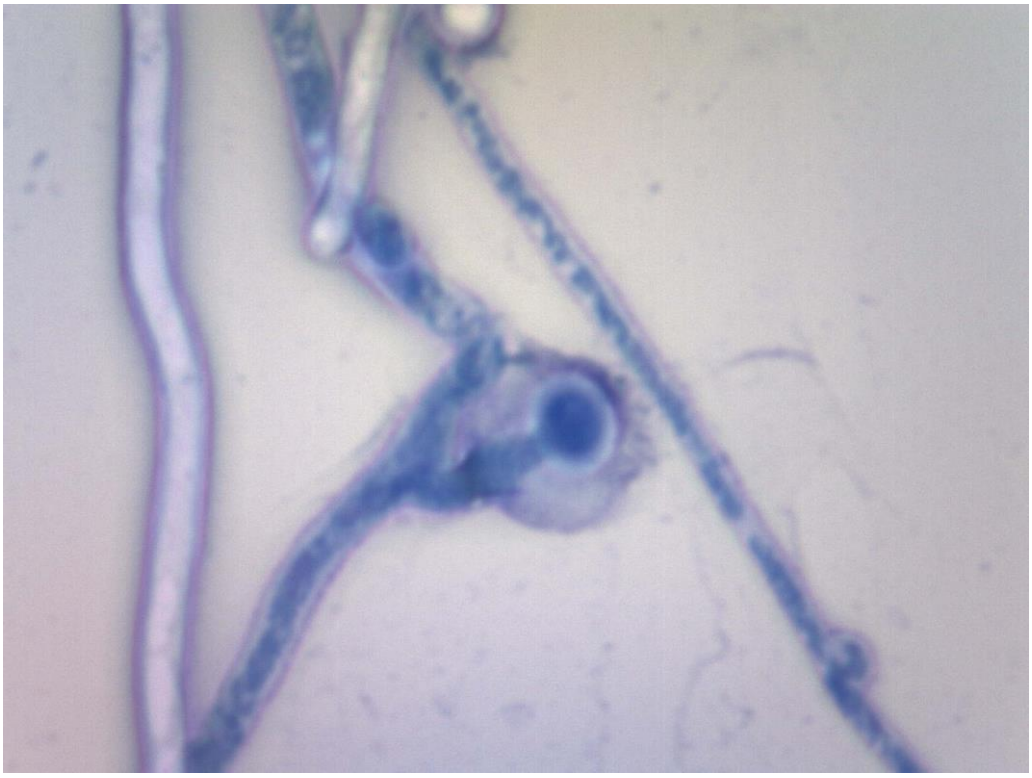

Figure 5.6: Developed adhesive knobs and clamp connections on *H. clelandii* hyphae

5.1.5. Fungal guttation droplets

Guttation droplets are excretions of water and other dissolved materials from plants or fungi, this phenomenon is not known to occur in all fungal species (Gareis and Gareis, 2007). In fungi the droplets are produced on the surface of the mycelium and have been found to contain mycotoxins in some species such as *Penicillium nordicum* and *Penicillium verrucosum* (Gareis and Gareis, 2007). All fungal species examined in this chapter produced guttation droplets in culture after multiple weeks.

5.2. Aims and objectives

This study aimed to isolate nematotoxins from various phases of growth of the nematophagous basidiomycetes *Pleurotus ostreatus, P. ostreatus var. columbinus* and *Hohenbuehelia clelandii* and test their toxicity to *Caenorhabditis elegans* and *Strongyloides* model species *S. ratti.* The nematotoxin extraction methods used were crude extraction methods published for this toxin type without HPLC purification. Crude methods were used as it was a pilot study to investigate toxicity to *S. ratti* and time and required resources for further extraction were not readily available. *P. ostreatus* was used as the model with extractions from mycelium grown on grain and in broth, guttation droplets and from fruiting bodies to assess any variations in metabolites produced and ease of processing.

Additionally a blank extraction was performed to ensure reagents used in the extraction process were not toxic to the nematodes at the point of assay.

5.3. Methods

5.3.1. Fungal cultures

Pleurotus cultures were obtained from cultivated strains kindly provided by Michael Taylor. *Hohenbuehelia clelandii* culture was obtained from a field specimen collected and identified by Pam Catcheside from the South Australian Herbarium. Specimens were tissue cultured onto potato dextrose agar (PDA; Oxoid Ltd).

5.3.2. Fungal growth

Fungal cultures of *Pleurotus ostreatus* and *P. ostreatus. Var. columbinus* were maintained on PDA; at 25 °C. *H. clelandii* was maintained on PDA with 3% ground sawdust and 2 g/L $CaCO_3$. Cultures which produced liquid excretions on the surface of mycelium were put aside. The liquid was collected with 10 µL capillary tubes and stored at -80 °C until required.

5.3.3. Grain

Wheat grain was soaked overnight in tap water and strained, three glass jars with air exchange filtered lids were each filled with 240 g of the soaked wheat grain. Jars were autoclaved at 121 °C for 30 minutes. Jars were allowed to cool overnight and then aseptically inoculated with either *P. ostreatus, P. ostreatus var. columbinus* or *H. clelandii* from PDA culture plates. Jars were incubated at 25 °C until mycelium had completely colonised the grain.

5.3.4. Broth

Half strength malt yeast extract broth was used to facilitate fungal growth in liquid.

<u>Half Strength Malt Yeast Extract Broth:</u>

- 1 L MilliQ water
- 1.5 g Yeast extract (BD Difco™)
- 2.5 g Bacto Peptone (BD Difco™)
- 5 g Dextrose (Fisher Scientific Co.)
- 1.5 g Malt extract (Oxoid LTD)

200 mL of half strength malt yeast extract broths in 450 mL Erlenmeyer flasks were inoculated in duplicate with *Pleurotus ostreatus, P. ostreatus. Var. columbinus* or *Hoehuenbuhelia clelandii* and incubated in shaking incubator at 25˚C for 1 month.

Contents of broths were filtered through 110 mm 1.2µm GF filter paper (FisherBrand). Filtrates were saved and stored at 4 ˚C. Filter paper was retained for toxin extraction as described below.

5.3.5. Fruiting body (Mushroom)

5.3.5.1. *P. ostreatus* and *P. ostreatus* var. columbinus cultivation

Wheat grain spawn was produced as per grain method above. Wheaten straw and expended coffee grounds (9:1) were pasteurised at 70 ˚C for 1 hour and then drained and allowed to cool to room temperature. Straw coffee substrate was layered into plastic bags with grain spawn (9:1) and packed tightly. Bags were sealed and a hook attached to the top, incisions were made to allow for air exchange. Inoculated substrate was incubated at 25 ˚C until mycelium completely colonised bag. Bags were then transferred to a fruiting chamber at 20 ˚C and 99% humidity with fan to keep

CO_2 levels low. Bags were allowed to fruit and mushrooms were collected for toxin extraction once developed.

P. ostreatus and *P. ostreatus* var. columbinus will be referred to by their common names for the remainder of the chapter: pearl oyster mushroom and blue oyster mushroom respectively.

5.3.5.2. *H. clelandii* cultivation attempts

Methods used to cultivate *Pleruotus* spp. were attempted for *H. clelandii* but resulted in slow growth and contamination. Work by Kuikarni and Dighe used an adaptation of this method to successfully cultivate similar species *H. atrocaerulea* (Kuikarni and Dighe, 2000).

The following combinations were trialled in an attempt to cultivate this species, spawn was sterilised by autoclaving and substrate was pasteurised at 70 ˚C:

Spawn: mung beans, 10:1 sawdust: bran, compost, 1:1 coffee: sawdust

Substrate: 1:2 coffee: sawdust as casing layer to sawdust bran spawn and colonised compost, wheaten straw.

5.3.6. Toxin extraction

Methods were adapted from Kwok and Satou's extraction methods, however, purification and characterisation by HPLC, MS and NMR was not completed (Kwok et al., 1992, Satou et al., 2008).

Substrate for extraction (mushroom tissue or filter paper from broth) were added to 200 mL of 1:1 methanol: water and stirred with magnetic stirrer for 15 hours. Grain

extraction was performed in soxhlet extraction glassware, with 45 g of *P. ostreatus* colonised grain with 250 mL of 1:1 methanol (Merck): water to extract metabolites from mycelium for 2 hours.

Solutions were filtered through 110 mm 1.2µm GF filter paper (FisherBrand) and filtrates were transferred to shallow trays and stored in fume hood to evaporate. Residues were dissolved in acetone (Merck) and transferred to 50 mL centrifuge tubes, acetone was then evaporated in fume hood. Extracts were stored at -80 ˚C.

This procedure was repeated without mushroom tissue and used as a blank extract.

5.3.7. Nematode cultures

5.3.7.1. C. elegans

C. elegans were obtained and maintained as described in Chapter 3: isolation and characterisation of nematophagous fungi from Northern Territory soils

5.3.7.2. Strongyloides ratti

S. ratti were kindly provided by Rogan Lee and John Clancy from laboratory maintained and infected rats at Westmead Hospital in Sydney. Freshly collected rat faeces were rehydrated with water and then homogenised. A coproculture was prepared by adding approximately 10% activated charcoal was added to the faeces and faecal slurry was transferred to a 90 mm petri dish with Whatman no.1 filter paper (Whatman plc) in the bottom then incubated at 26 ˚C (Lok, 2007).

After 3-5 days infective larvae were extracted from coprocultures via the Baermann technique (Lok, 2007, Khieu, 2013).

5.3.8. Bioassay

Methods were adapted from Stadler's microwell plate assay for nematicidal activity (Stadler et al., 1993a). Bioassays were conducted in 96 well plates with 500 µL of 2% water agar in each well. Nematodes were washed (Stiernagle, 2006) and diluted to appropriate concentration and approximately 50 nematodes were pipetted into each well. Liquid from nematode transfer was allowed to evaporate from wells. Nematotoxins were diluted 1/10, 1/100, 1/1000 and 1/10000 and 5 µL of toxins were added to wells containing nematodes in duplicate. Sterile MilliQ water was used as a negative control for every nematotoxin type and positive controls were conducted using copper sulphate at 1000, 100, 10, 1 and 0.1 mg/L.

Nematodes were counted under dissecting microscope after 1, 2, 6 and 12 hours and classified as motile or immotile.

5.3.9. Statistics

Statistics were completed using IMB SPSS statistics and GenStat 17th Ed. Normality and homogeneity were assessed using residuals plots. ANOVAs were completed for individual toxin and nematode types. Tukeys HSDs were performed to assess differences between individual concentrations and time points. A probit analysis was used to generate LC50's for each toxin and nematode type.

5.4. Results

5.4.1. Cultivation of *P. ostreatus* mushrooms

P. ostreatus and *P. ostreatus var. columbinus* were successfully cultivated however the extraction process was only formed on *P. ostreatus*.

Figure 5.7: Young fruiting bodies of *P. ostreatus* var. columbinus on wheaten straw and expended coffee grounds

5.4.2. Cultivation of *H. clelandii* mushrooms

Cultivation of *H. clelandii* was unsuccessful, the culture of this species was very slow growing. Despite multiple attempts and supplementations and additions to culture, spawn and substrate media the culture was outcompeted by moulds.

5.4.3. Bioassays

The assays showed extracts of all three fungal species displayed toxicity towards *C. elegans*, however, guttation droplets showed no toxicity. Copper was toxic to *C.*

elegans, however toxicity to *S. ratti* was not displayed in either copper or fungal extracts. Some difficulties were encountered with the use of *S. ratti* in assays which will be further discussed below.

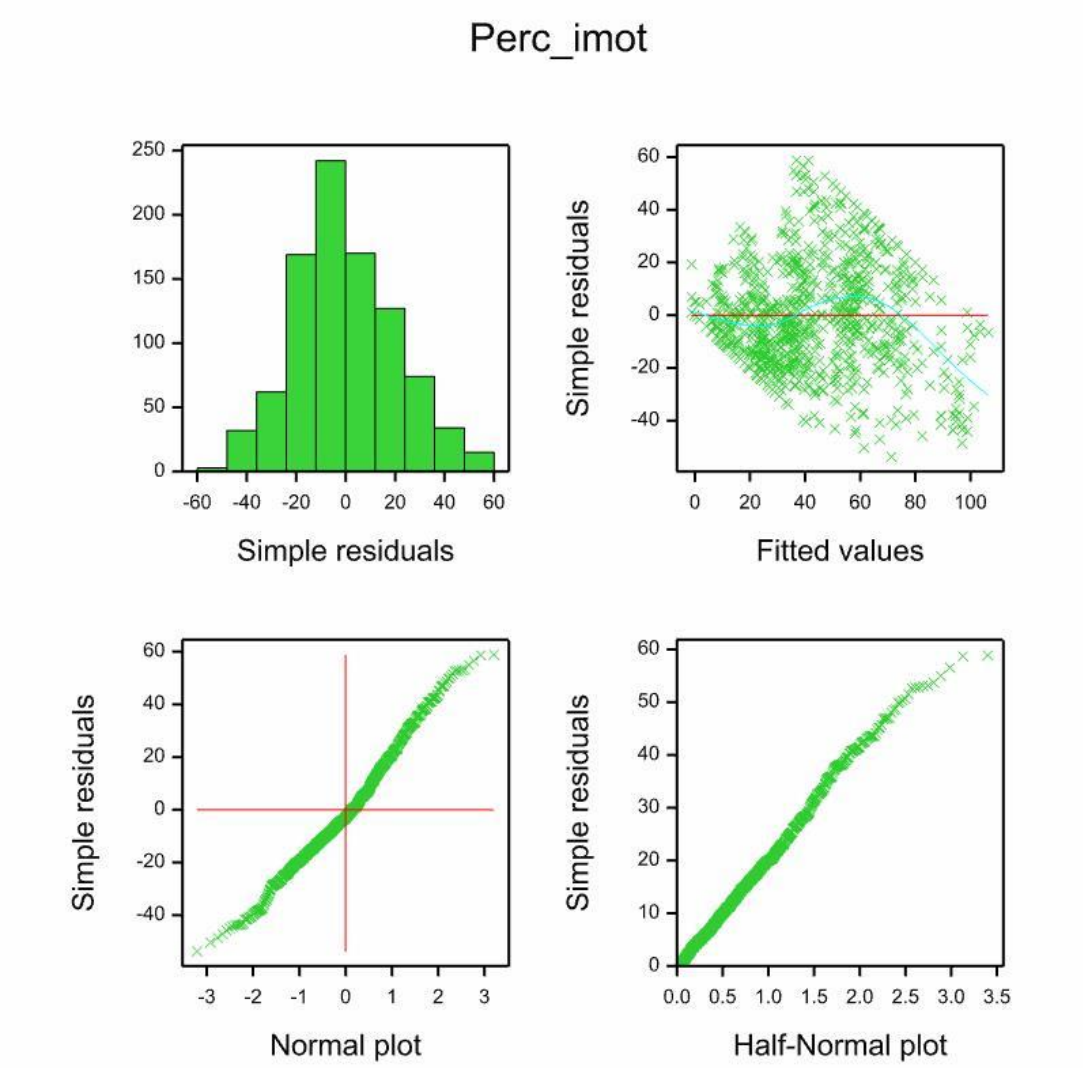

Figure 5.8: Residual plots for normality and homogeneity analysis, simple residuals histogram shows normal distribution

5.4.4. Copper and *C. elegans*

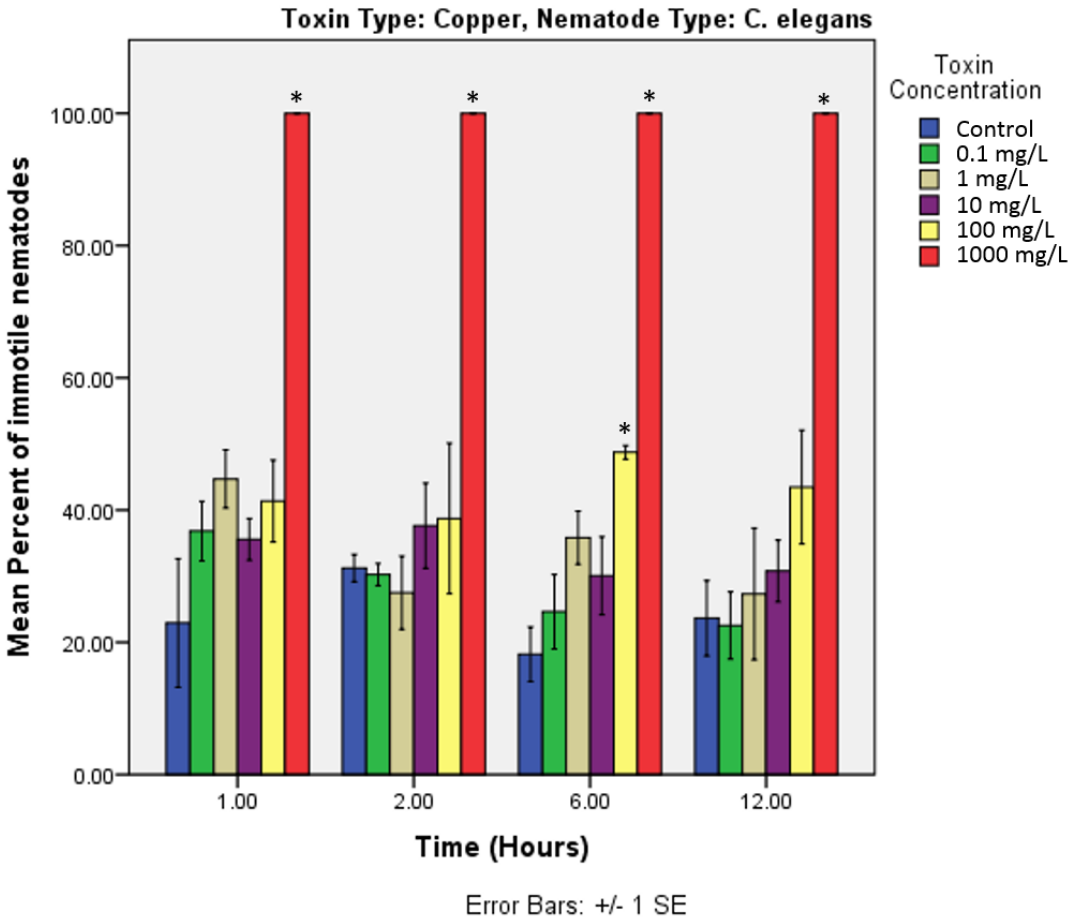

Figure 5.9: Toxicity of copper sulphate up to 1000 mg/L against *C. elegans*, showing 100% immotility at 1000 mg/L. * Indicates significant differences between treatment and control ($p<0.05$)

Table 5.1: SPSS descriptive statistics output for copper sulphate and *C. elegans*

Percent of immotile nematodes

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
Control	16	24.0000	11.92029	2.98007	17.6481	30.3519	5.90	47.80
0.1 mg/L	16	28.5625	9.85291	2.46323	23.3123	33.8127	10.50	50.00
1 mg/L	16	33.8375	13.65884	3.41471	26.5592	41.1158	0.00	56.00
10 mg/L	16	33.5125	9.86610	2.46652	28.2552	38.7698	17.60	56.50
100 mg/L	16	43.0688	14.45096	3.61274	35.3684	50.7691	18.20	69.60
1000 mg/L	16	100.0000	0.00000	0.00000	100.0000	100.0000	100.00	100.00
Total	96	43.8302	28.05840	2.86370	38.1450	49.5154	0.00	100.00

Table 5.2: ANOVA and Tukey HSD outputs displaying significant differences ($p<0.05$) between controls and 1000 mg/L and controls and 100 mg/L for copper sulphate and *C. elegans*

ANOVA

	Sig.
Between Groups	0.000

Tukey HSD – toxin concentration means

Toxin Concentration		Mean Difference	Sig.
Control	0.1 mg/L	-4.56250	0.851
	1 mg/L	-9.83750	0.129
	10 mg/L	-9.51250	0.155
	100 mg/L	-19.06875*	0.000
	1000 mg/L	-76.00000*	0.000

*. The mean difference is significant at the 0.05 level.

Table 5.3: ANOVAs and Tukey HSD outputs for each time period and toxin concentration displaying significant differences in immotility between controls and 1000 mg/L at all time periods and between the control and 100 mg/L at 6 hours for copper and *C. elegans*

ANOVA - time: 1 hour				**ANOVA - time: 6 hours**			
			Sig.				Sig.
Between Groups			.000	Between Groups			.000
Tukey HSD - time 1 hour				**Tukey HSD - time 6 hours**			
Toxin Concentration		Mean Difference	Sig.	Toxin Concentration		Mean Difference	Sig.
Control	0.1 mg/L	-13.90000	.501	Control	0.1 mg/L	-6.42500	.871
	1 mg/L	-21.77500	.105		1 mg/L	-17.62500	.065
	10 mg/L	-12.65000	.596		10 mg/L	-11.87500	.354
	100 mg/L	-18.42500	.221		100 mg/L	-30.52500*	.001
	1000 mg/L	-77.07500*	.000		1000 mg/L	-81.80000*	.000
ANOVA - time: 2 hours				**ANOVA - time: 12 hours**			
			Sig.				Sig.
Between Groups			.000	Between Groups			.000
Tukey HSD - time 2 hours				**Tukey HSD - time 12 hours**			
Toxin Concentration		Mean Difference	Sig.	Toxin Concentration		Mean Difference	Sig.
Control	0.1 mg/L	.97500	1.000	Control	0.1 mg/L	1.10000	1.000
	1 mg/L	3.72500	.997		1 mg/L	-3.67500	.998
	10 mg/L	-6.40000	.970		10 mg/L	-7.12500	.968
	100 mg/L	-7.50000	.942		100 mg/L	-19.82500	.303
	1000 mg/L	-68.77500*	.000		1000 mg/L	-76.35000*	.000

5.4.4.1. Summary

There was a statistically significant difference between treatment groups for the copper positive control as shown through ANOVA (Table 5.3). Copper effectively immobilised *C. elegans* at 1000 mg/L at all time periods in comparison to the control. Copper also displayed effective immobilisation of *C. elegans* at 100 mg/L compared to the control at 6 hours. Concentration had a greater impact on toxicity than time, as exposure to concentrations lower than 100 mg/L did not show significant toxicity (Table 5.3). Concentrations of 100 mg/L or above demonstrated acute affects in *C. elegans,* with significant toxicity when compared to the control at some time points but not others.

5.4.5. Pearl oyster and *C. elegans*

5.4.5.1. Pearl oyster extracted from wheat grain

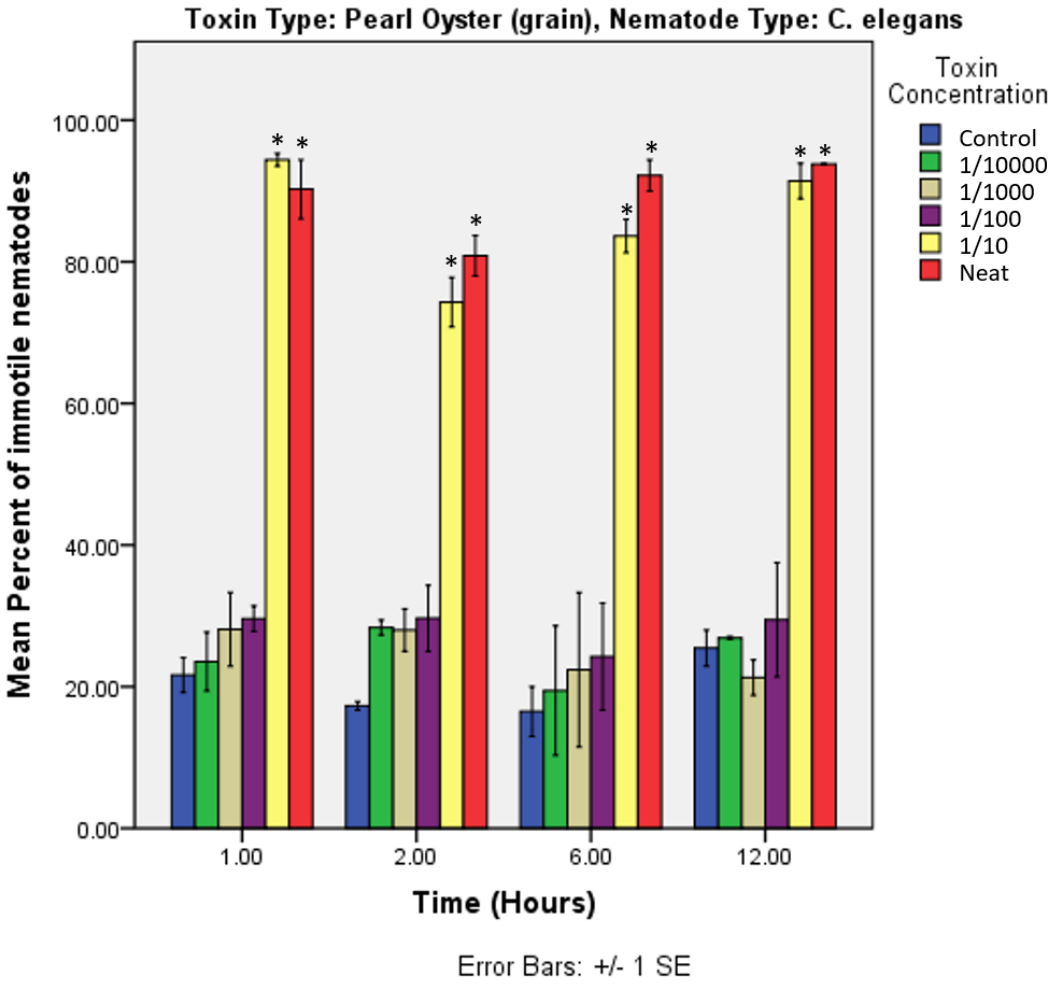

Figure 5.10: Toxicity of pearl oyster grain extract dilutions towards *C. elegans*, showing immotility significantly different to controls at neat and 1/10 concentrations at all time periods. * Indicates significant differences between treatment and control ($p<0.05$)

Table 5.4: SPSS descriptive statistics outputs for toxicity of pearl oyster grain extracts towards *C. elegans*

Percent of immotile nematodes

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
Control	8	20.2250	4.68668	1.65699	16.3068	24.1432	13.00	28.00
1/10000	8	24.5625	6.52598	2.30728	19.1066	30.0184	10.30	29.40
1/1000	8	24.9500	7.56212	2.67361	18.6279	31.2721	11.50	33.30
1/100	8	28.2375	6.92592	2.44868	22.4473	34.0277	16.70	37.50
1/10	8	85.9375	8.73334	3.08770	78.6362	93.2388	70.80	95.30
Neat	8	89.2750	6.12133	2.16422	84.1574	94.3926	78.00	94.40
Total	48	45.5313	30.86541	4.45504	36.5689	54.4936	10.30	95.30

Table 5.5: ANOVA and Tukey HSD outputs displaying significant differences ($p<0.05$) in immotility of *C. elegans* between controls and both the neat and 1/10 dilution of the extract from pearl oyster grain cultures

ANOVA – toxin concentration means

	Sig.
Between Groups	0.000

Tukey HSD – toxin concentration means

Toxin Concentration		Mean Difference	Sig.
Control	1/10000	-4.33750	0.803
	1/1000	-4.72500	0.741
	1/100	-8.01250	0.205
	1/10	-65.71250*	0.000
	Neat	-69.05000*	0.000

*. The mean difference is significant at the 0.05 level.

Table 5.6: ANOVAs and Tukey HSD outputs for each time period and toxin concentration displaying significant ($p<0.05$) differences in mobility of *C. elegans* between controls, neat and 1/10 dilution of the extract from pearl oyster grain cultures at all time periods.

ANOVA - time: 1 hour				**ANOVA - time: 6 hours**			
			Sig.				Sig.
Between Groups			.000	Between Groups			.001
Tukey HSD - time 1 hour				**Tukey HSD - time 6 hours**			
Toxin Concentration		Mean Difference	Sig.	Toxin Concentration		Mean Difference	Sig.
Control	1/10000	-1.90000	.998	Control	1/10000	-2.95000	.999
	1/1000	-6.45000	.767		1/1000	-5.90000	.986
	1/100	-7.95000	.612		1/100	-7.75000	.958
	1/10	-72.75000*	.000		1/10	-67.15000*	.003
	Neat	-68.60000*	.000		Neat	-75.70000*	.002
ANOVA - time: 2 hours				**ANOVA - time: 12 hours**			
			Sig.				Sig.
Between Groups			.000	Between Groups			.000
Tukey HSD - time 2 hours				**Tukey HSD - time 12 hours**			
Toxin Concentration		Mean Difference	Sig.	Toxin Concentration		Mean Difference	Sig.
Control	1/10000	-11.05000	.219	Control	1/10000	-1.45000	1.000
	1/1000	-10.70000	.240		1/1000	4.15000	.960
	1/100	-12.35000	.154		1/100	-4.00000	.966
	1/10	-57.00000*	.000		1/10	-65.95000*	.000
	Neat	-63.55000*	.000		Neat	-68.35000*	.000

5.4.5.2. Pearl oyster extracted from broth

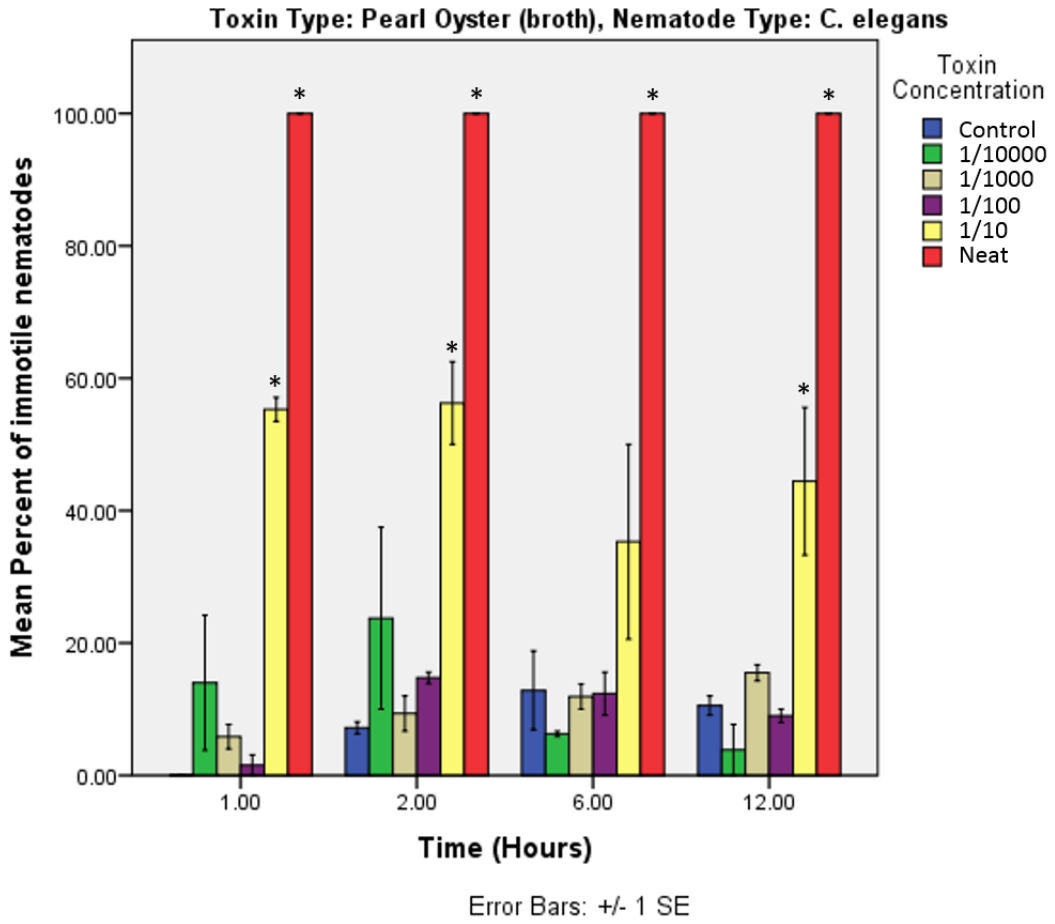

Figure 5.11: Toxicity of pearl oyster broth extract dilutions against *C. elegans*, showing immotility significantly different to controls at neat and 1/10 dilution of broth extracts at all time periods. * Indicates significant differences between treatment and control ($p<0.05$)

Table 5.7: SPSS descriptive statistics outputs for the toxicity of pearl oyster broth extracts towards *C. elegans*

Percent of immotile nematodes

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
Control	8	7.6500	6.15258	2.17527	2.5063	12.7937	0.00	18.80
1/10000	8	11.9750	12.52492	4.42823	1.5039	22.4461	0.00	37.50
1/1000	8	10.6500	4.31972	1.52725	7.0386	14.2614	4.00	16.70
1/100	8	9.4125	5.70199	2.01596	4.6455	14.1795	0.00	15.60
1/10	8	47.8250	13.91646	4.92021	36.1905	59.4595	20.60	62.50
Neat	8	100.0000	0.00000	0.00000	100.0000	100.0000	100.00	100.00
Total	48	31.2521	35.04559	5.05840	21.0759	41.4283	0.00	100.00

Table 5.8: ANOVA and Tukey HSD outputs displaying significant differences between controls and neat concentration as well as between controls and 1/10 dilution for pearl oyster broth and *C. elegans*

ANOVA			
			Sig.
Between Groups			0.000
Tukey HSD – toxin concentration means			
Toxin Concentration		Mean Difference	Sig.
Control	1/10000	-4.32500	0.912
	1/1000	-3.00000	0.981
	1/100	-1.76250	0.998
	1/10	-40.17500*	0.000
	Neat	-92.35000*	0.000

*. The mean difference is significant at the 0.05 level.

Table 5.9: ANOVAs and Tukey HSD outputs for each time period and toxin concentration displaying significant ($p<0.05$) differences in mobility of *C. elegans* between controls, neat and 1/10 dilution of the extract from pearl oyster broth cultures at all time periods except at 6 hours incubation.

ANOVA - time: 1 hour				**ANOVA - time: 6 hours**			
			Sig.				Sig.
Between Groups			.000	Between Groups			.000
Tukey HSD - time 1 hour				**Tukey HSD - time 6 hours**			
Toxin Concentration		Mean Difference	Sig.	Toxin Concentration		Mean Difference	Sig.
Control	1/10000	-14.00000	.325	Control	1/10000	6.55000	.976
	1/1000	-5.85000	.918		1/1000	.95000	1.000
	1/100	-1.55000	1.000		1/100	.50000	1.000
	1/10	-55.30000*	.001		1/10	-22.45000	.290
	Neat	-100.00000*	.000		Neat	-87.15000*	.001
ANOVA - time: 2 hours				**ANOVA - time: 12 hours**			
			Sig.				Sig.
Between Groups			.000	Between Groups			.000
Tukey HSD - time 2 hours				**Tukey HSD - time 12 hours**			
Toxin Concentration		Mean Difference	Sig.	Toxin Concentration		Mean Difference	Sig.
Control	1/10000	-16.55000	.496	Control	1/10000	6.70000	.913
	1/1000	-2.15000	1.000		1/1000	-4.95000	.973
	1/100	-7.55000	.946		1/100	1.55000	1.000
	1/10	-49.05000*	.011		1/10	-33.90000*	.020
	Neat	-92.80000*	.000		Neat	-89.45000*	.000

5.4.5.3. Pearl oyster extracted from fruiting body

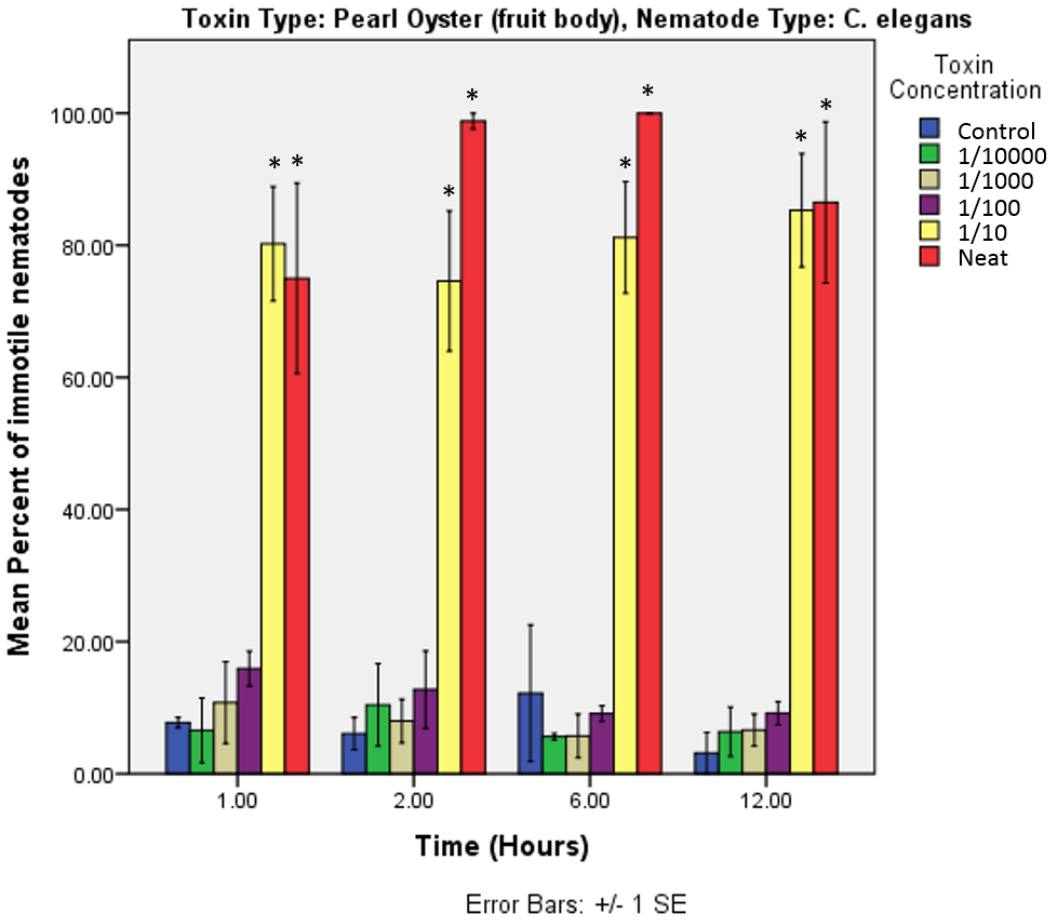

Figure 5.12: Toxicity of pearl oyster fruiting body extract dilutions against *C. elegans*, showing immotility significantly different to controls at neat and 1/10 concentrations at all time periods. * Indicates significant differences between treatment and control ($p<0.05$)

Table 5.10: SPSS descriptive statistics outputs for the toxicity of pearl oyster fruiting body extracts towards *C. elegans*

Percent of immotile nematodes

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
Control	16	7.2875	10.48274	2.62068	1.7016	12.8734	0.00	42.90
1/10000	16	7.2438	8.08752	2.02188	2.9342	11.5533	0.00	25.00
1/1000	16	7.7688	7.52841	1.88210	3.7571	11.7804	0.00	27.30
1/100	16	11.7438	6.71664	1.67916	8.1647	15.3228	0.00	27.30
1/10	16	80.3250	16.77063	4.19266	71.3886	89.2614	55.60	100.00
Neat	16	90.0750	19.93815	4.98454	79.4507	100.6993	50.00	100.00
Total	96	34.0740	38.49676	3.92906	26.2738	41.8741	0.00	100.00

Table 5.11: ANOVA and Tukey HSD outputs displaying significant differences between controls and neat concentration as well as between controls and 1/10 dilution for pearl oyster fruiting body and *C. elegans*

ANOVA

	Sig.
Between Groups	0.000

Tukey HSD – toxin concentration means

Toxin Concentration		Mean Difference	Sig.
Control	1/10000	0.04375	1.000
	1/1000	-0.48125	1.000
	1/100	-4.45625	0.917
	1/10	-73.03750*	0.000
	Neat	-82.78750*	0.000

*. The mean difference is significant at the 0.05 level.

Table 5.12: ANOVAs and Tukey HSD outputs for each time period and toxin concentration displaying significant ($p<0.05$) differences in mobility of *C. elegans* between controls, neat and 1/10 dilution of the extract from pearl oyster fruiting bodies.

ANOVA - time: 1 hour				**ANOVA - time: 6 hours**			
			Sig.				Sig.
Between Groups			.000	Between Groups			.000
Tukey HSD - time 1 hour				**Tukey HSD - time 6 hours**			
Toxin Concentration		Mean Difference	Sig.	Toxin Concentration		Mean Difference	Sig.
Control	1/10000	1.17500	1.000	Control	1/10000	6.57500	.959
	1/1000	-3.02500	1.000		1/1000	6.47500	.961
	1/100	-8.17500	.972		1/100	3.07500	.999
	1/10	-72.47500*	.000		1/10	-68.97500*	.000
	Neat	-67.25000*	.000		Neat	-87.80000*	.000
ANOVA - time: 2 hours				**ANOVA - time: 12 hours**			
			Sig.				Sig.
Between Groups			.000	Between Groups			.000
Tukey HSD - time 2 hours				**Tukey HSD - time 12 hours**			
Toxin Concentration		Mean Difference	Sig.	Toxin Concentration		Mean Difference	Sig.
Control	1/10000	-4.35000	.994	Control	1/10000	-3.22500	.999
	1/1000	-1.90000	1.000		1/1000	-3.47500	.999
	1/100	-6.67500	.962		1/100	-6.05000	.985
	1/10	-68.52500*	.000		1/10	-82.17500*	.000
	Neat	-92.72500*	.000		Neat	-83.37500*	.000

5.4.5.4. Pearl oyster guttation droplet

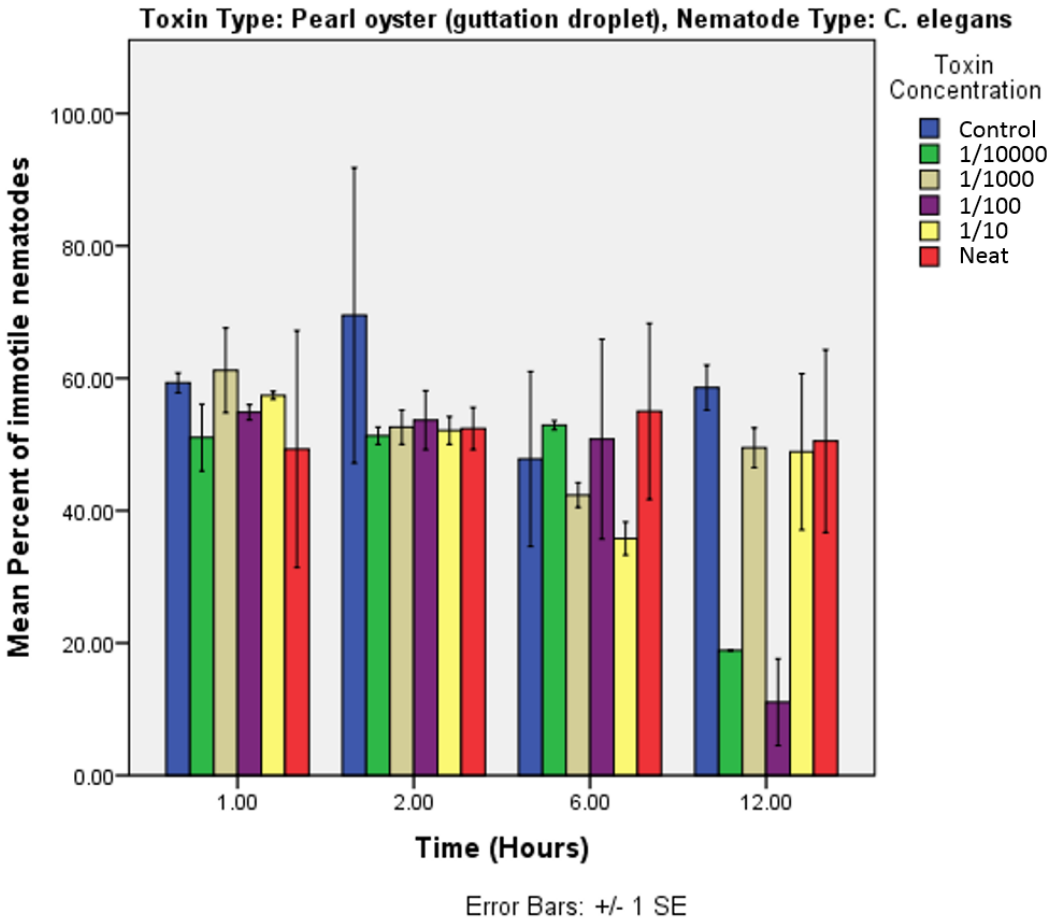

Figure 5.13: Toxicity of pearl oyster guttation droplet dilutions against *C. elegans*, showing no significant differences between controls and toxin

Table 5.13: SPSS descriptive statistics outputs for the toxicity of pearl oyster guttation extracts towards *C. elegans*

Percent of immotile nematodes

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
Control	8	58.8000	16.22274	5.73560	45.2375	72.3625	34.60	91.80
1/10000	8	43.5375	15.48316	5.47413	30.5933	56.4817	18.80	56.10
1/1000	8	51.4125	8.33468	2.94676	44.4445	58.3805	40.50	67.60
1/100	8	42.5875	21.55951	7.62244	24.5633	60.6117	4.50	65.90
1/10	8	48.5500	10.74204	3.79788	39.5694	57.5306	33.30	60.70
Neat	8	51.8000	14.30804	5.05866	39.8382	63.7618	31.40	68.30
Total	48	49.4479	15.25551	2.20194	45.0182	53.8777	4.50	91.80

Table 5.14: ANOVA output displaying no significant difference between means therefore Tukey HSDs were not completed for toxin concentrations for pearl oyster guttation droplet and *C. elegans*

ANOVA	
	Sig.
Between Groups	0.294

5.4.5.5. Summary

All pearl oyster extracts with the exception of the guttation droplets were successful at immobilising *C. elegans* in the neat forms. Extracts from grain and fruiting bodies displayed similar activity at 1/10 dilutions. The broth extract additionally displayed some nematicidal activity towards *C. elegans* at the 1/10 dilution but not as effective as other extracts.

These results were reflected in analysis and there was a statistically significant difference ($p<0.05$) between treatment groups in the extracts from pearl oyster grain, broth and fruiting body, there was no statistically significant difference ($p>0.05$) between treatment groups for pearl oyster guttation droplets as shown

through ANOVAs. Tukeys HSDs indicated pearl oyster grain, broth and fruiting body extracts were significantly different ($p<0.05$) to controls in neat concentrations at all times. These extracts were also significantly different to controls in the 1/10 dilution of neat toxin. Other concentrations of the extracts were not significantly different to the control.

Pearl oyster guttation droplets did not display any significant difference in *C. elegans* immobilisation from the control at any concentration or time period.

5.4.6. Blue oyster and *C. elegans*

5.4.6.1. Blue oyster extracted from broth

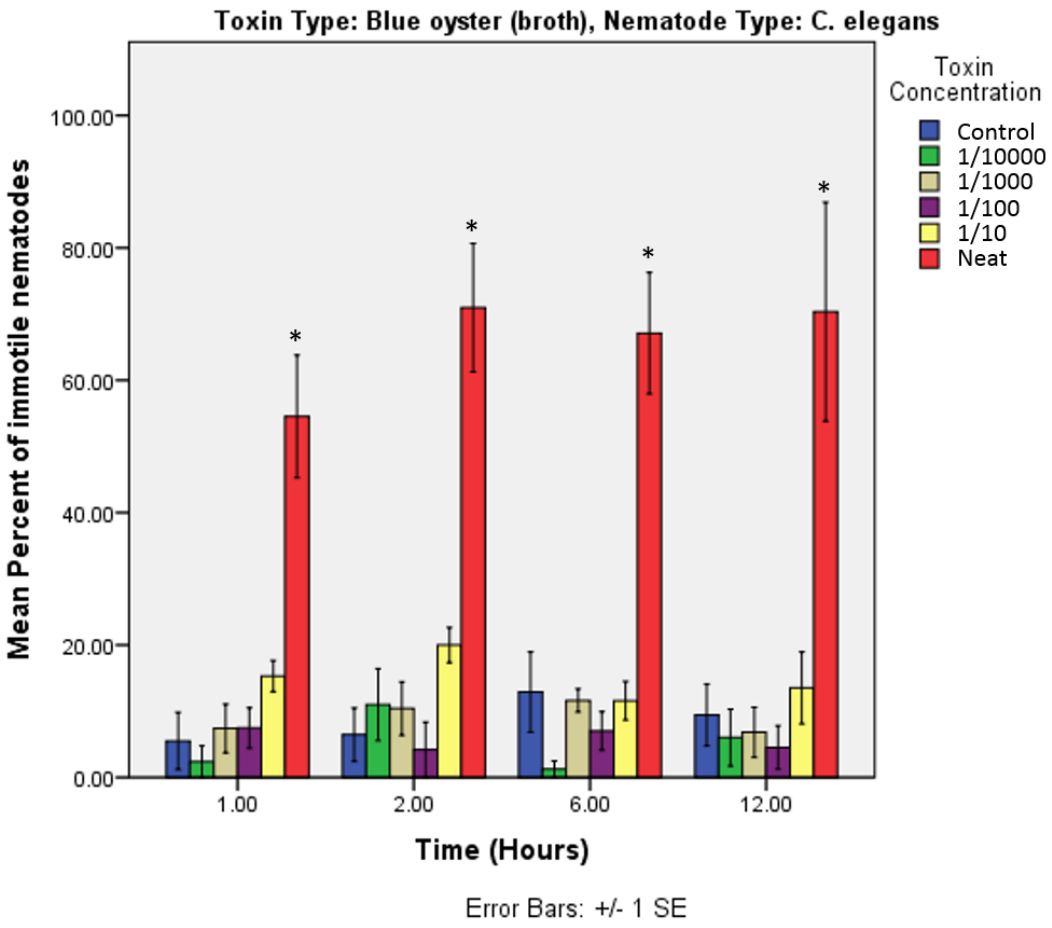

Figure 5.14: Toxicity of blue oyster broth extract dilutions against *C. elegans*, showing immotility significantly different to controls at neat concentrations at all time periods. * Indicates significant differences between treatment and control ($p<0.05$)

Table 5.15: SPSS descriptive statistics outputs for the toxicity of blue oyster broth extracts towards *C. elegans*

Percent of immotile nematodes

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
Control	16	8.5688	9.15429	2.28857	3.6908	13.4467	0.00	28.60
1/10000	16	5.1625	7.71595	1.92899	1.0510	9.2740	0.00	21.40
1/1000	16	9.0625	6.48525	1.62131	5.6068	12.5182	0.00	18.80
1/100	16	5.8000	6.25716	1.56429	2.4658	9.1342	0.00	16.70
1/10	16	15.1000	7.11908	1.77977	11.3065	18.8935	3.00	27.30
Neat	16	65.7375	21.83486	5.45872	54.1025	77.3725	29.00	100.00
Total	96	18.2385	24.19011	2.46889	13.3372	23.1399	0.00	100.00

Table 5.16: ANOVA and Tukey HSD outputs displaying significant differences between controls and neat toxin concentration for blue oyster broth and *C. elegans*

ANOVA

	Sig.
Between Groups	0.000

Tukey HSD – toxin concentration means

Toxin Concentration		Mean Difference	Sig.
Control	1/10000	3.40625	0.955
	1/1000	-0.49375	1.000
	1/100	2.76875	0.982
	1/10	-6.53125	0.568
	Neat	-57.16875*	0.000

*. The mean difference is significant at the 0.05 level.

Table 5.17: ANOVAs and Tukey HSD outputs for each time period and toxin concentration displaying significant ($p<0.05$) differences in immobility of *C. elegans* between controls and the neat extract from blue oyster broth culture at all incubation times.

ANOVA - time: 1 hour				**ANOVA - time: 6 hours**			
			Sig.				Sig.
Between Groups			.000	Between Groups			.000
Tukey HSD - time 1 hour				**Tukey HSD - time 6 hours**			
Toxin Concentration		Mean Difference	Sig.	Toxin Concentration		Mean Difference	Sig.
Control	1/10000	3.12500	.997	Control	1/10000	11.65000	.555
	1/1000	-1.90000	1.000		1/1000	1.27500	1.000
	1/100	-1.97500	1.000		1/100	5.87500	.953
	1/10	-9.80000	.703		1/10	1.32500	1.000
	Neat	-49.02500*	.000		Neat	-54.20000*	.000
ANOVA - time: 2 hours				**ANOVA - time: 12 hours**			
			Sig.				Sig.
Between Groups			.000	Between Groups			.000
Tukey HSD - time 2 hours				**Tukey HSD - time 12 hours**			
Toxin Concentration		Mean Difference	Sig.	Toxin Concentration		Mean Difference	Sig.
Control	1/10000	-4.55000	.991	Control	1/10000	3.40000	1.000
	1/1000	-3.95000	.995		1/1000	2.60000	1.000
	1/100	2.27500	1.000		1/100	4.90000	.997
	1/10	-13.55000	.521		1/10	-4.10000	.999
	Neat	-64.52500*	.000		Neat	-60.92500*	.000

5.4.6.2. Blue oyster guttation droplet

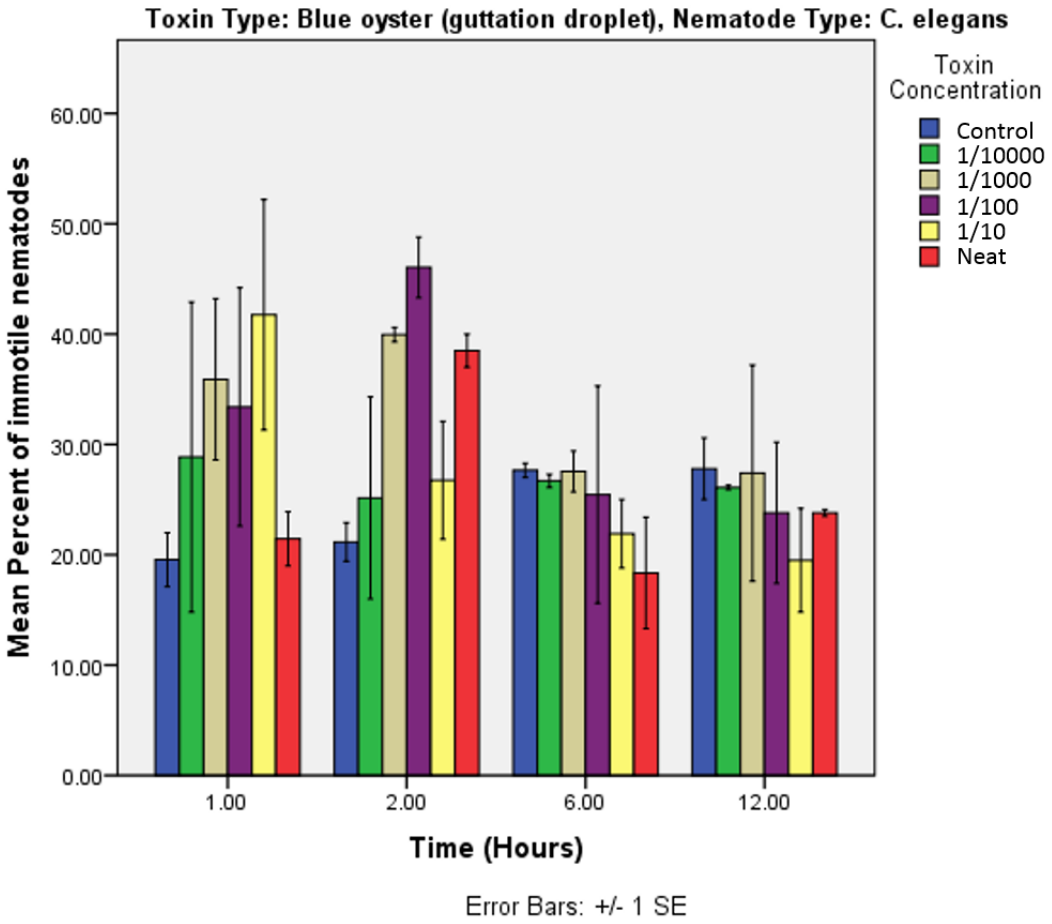

Figure 5.15: Toxicity assay of blue oyster guttation droplet dilutions against *C. elegans*, showing no significant difference in immotility between controls and any droplet dilution at any time period.

Table 5.18: SPSS descriptive statistics outputs for the toxicity of dilutions of blue oyster guttation droplets towards *C. elegans*

Percent of immotile nematodes

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
Control	8	24.0375	4.56726	1.61477	20.2192	27.8558	17.10	30.60
1/10000	8	26.7000	9.08547	3.21220	19.1044	34.2956	14.80	42.90
1/1000	8	32.7000	8.79269	3.10869	25.3491	40.0509	17.60	43.20
1/100	8	32.1750	12.77908	4.51809	21.4914	42.8586	15.60	48.80
1/10	8	27.4750	11.56976	4.09053	17.8024	37.1476	14.80	52.20
Neat	8	25.5250	8.83593	3.12397	18.1380	32.9120	13.30	40.00
Total	48	28.1021	9.67305	1.39618	25.2933	30.9108	13.30	52.20

Table 5.19: ANOVA output displaying no significant difference between means therefore Tukey HSDs were not completed for toxin concentrations for blue oyster guttation droplet and *C. elegans*

ANOVA	
	Sig.
Between Groups	0.379

5.4.6.3. Summary

Blue oyster broth immobilised up to 100% of *C. elegans* in neat concentrations of the extract the mean however was 65% (Table 5.15). There was a statistically significant difference between treatment groups in the extract from blue oyster broth, there was no statistically significant difference between treatment groups for blue oyster guttation droplets as shown through ANOVAs. Blue oyster broth extract effectively immobilised *C. elegans* in neat concentrations at all time periods in comparison to the control. Other concentrations of the extracts were not significantly different to the control. Blue oyster guttation droplets did not display any significant difference in *C. elegans* immobilisation from the control at any concentration or time period.

5.4.7. *H. clelandii* and *C. elegans*

5.4.7.1. *H. clelandii* extraction from broth

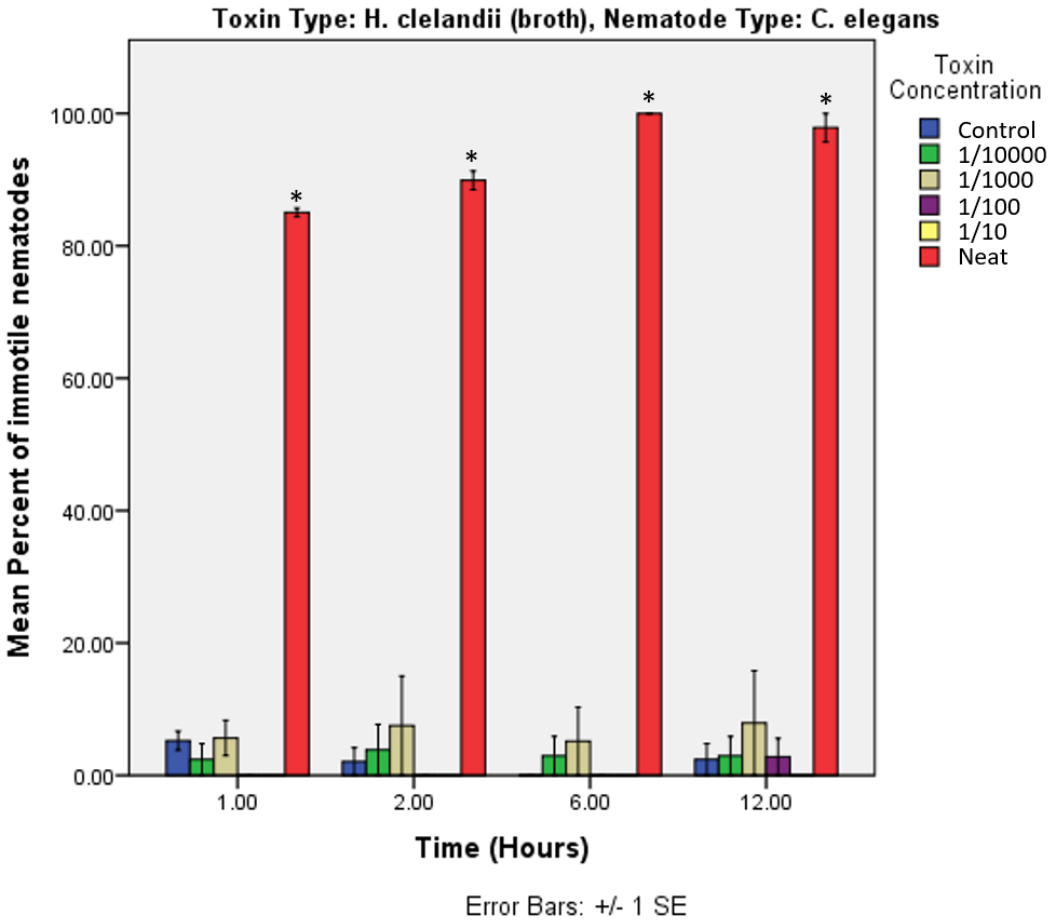

Figure 5.16: Toxicity of *H. clelandii* broth extract dilutions against *C. elegans*, showing immotility significantly different to controls at neat concentrations at all time periods. * Indicates significant differences ($p<0.05$)

Table 5.20: SPSS descriptives output for *H. clelandii* broth and *C. elegans*

Percent of immotile nematodes

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
Control	8	2.4375	2.73806	0.96805	0.1484	4.7266	0.00	6.70
1/10000	8	3.0375	3.34106	1.18124	0.2443	5.8307	0.00	7.70
1/1000	8	6.5500	6.71246	2.37321	0.9382	12.1618	0.00	15.80
1/100	8	0.7000	1.97990	0.70000	-0.9552	2.3552	0.00	5.60
1/10	4	0.0000	0.00000	0.00000	0.0000	0.0000	0.00	0.00
Neat	8	93.2000	6.59394	2.33131	87.6873	98.7127	84.40	100.00
Total	44	19.2591	35.57373	5.36294	8.4437	30.0745	0.00	100.00

Table 5.21: ANOVA and Tukey HSD outputs displaying significant differences between controls and neat concentration *H. clelandii* and *C. elegans*

ANOVA

	Sig.
Between Groups	0.000

Tukey HSD – toxin concentration means

Toxin Concentration		Mean Difference	Sig.
Control	1/10000	-0.60000	1.000
	1/1000	-4.11250	0.467
	1/100	1.73750	0.971
	1/10	2.43750	0.949
	Neat	-90.76250*	0.000

*. The mean difference is significant at the 0.05 level.

Table 5.22: ANOVAs and Tukey HSD outputs for each time period and toxin concentration displaying significant differences between controls and neat dilutions at all time periods for *H. clelandii* broth and *C. elegans*

ANOVA - time: 1 hour				**ANOVA - time: 6 hours**			
			Sig.				Sig.
Between Groups			.000	Between Groups			.000
Tukey HSD - time 1 hour				**Tukey HSD - time 6 hours**			
Toxin Concentration		Mean Difference	Sig.	Toxin Concentration		Mean Difference	Sig.
Control	1/10000	2.85000	.795	Control	1/10000	-2.95000	.943
	1/1000	-.40000	1.000		1/1000	-5.15000	.676
	1/100	5.25000	.310		1/100	.00000	1.000
	1/10	5.25000	.310		1/10	.00000	1.000
	Neat	-79.80000*	.000		Neat	-100.00000*	.000
ANOVA - time: 2 hours				**ANOVA - time: 12 hours**			
			Sig.				Sig.
Between Groups			.000	Between Groups			.000
Tukey HSD - time 2 hours				**Tukey HSD - time 12 hours**			
Toxin Concentration		Mean Difference	Sig.	Toxin Concentration		Mean Difference	Sig.
Control	1/10000	-1.75000	.999	Control	1/10000	-.55000	1.000
	1/1000	-5.40000	.880		1/1000	-5.50000	.900
	1/100	2.10000	.998		1/100	-.40000	1.000
	1/10	2.10000	.998		1/10	2.40000	.997
	Neat	-87.80000*	.000		Neat	-95.45000*	.000

5.4.7.2. *H. clelandii* guttation droplets

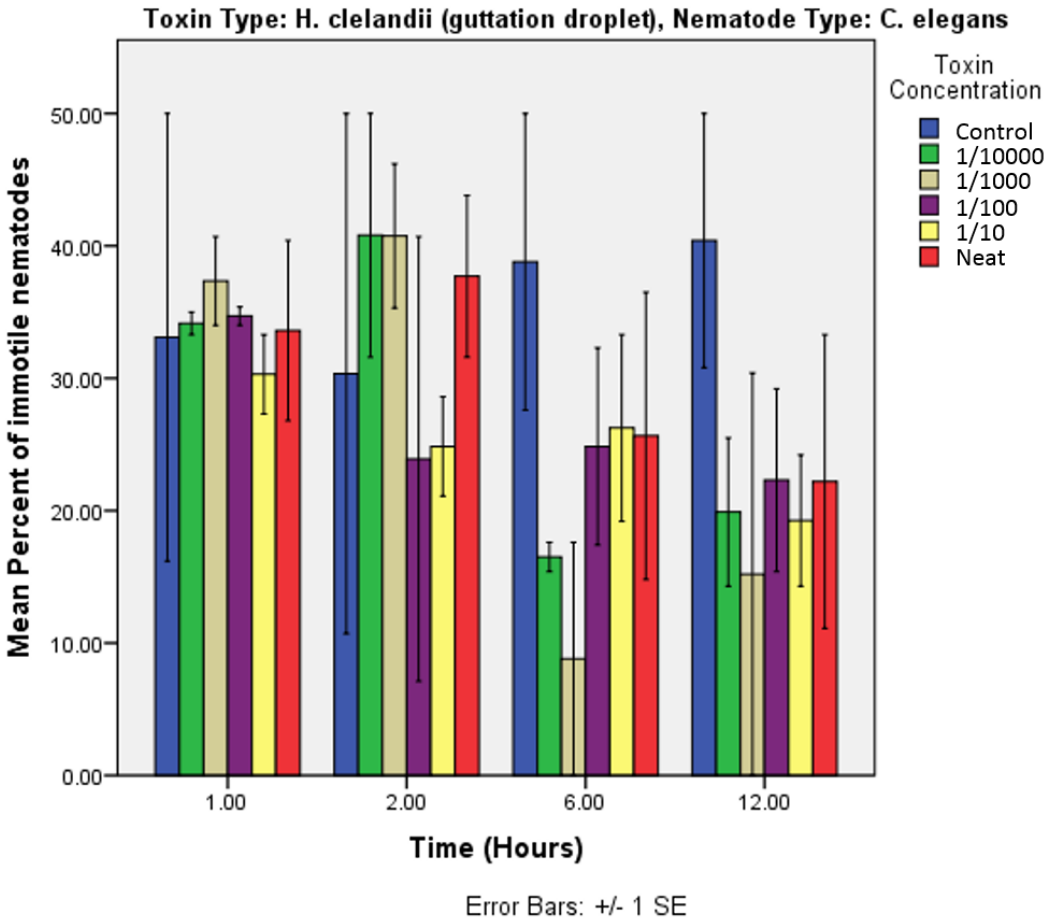

Figure 5.17: Toxicity of *H. clelandii* guttation droplet dilutions against *C. elegans*, showing no significant difference between controls at neat concentrations at any time period.

Table 5.23: SPSS descriptive statistics outputs for the toxicity of *H. clelandii* guttation droplets towards *C. elegans*

Percent of immotile nematodes

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
Control	8	35.6625	16.53057	5.84444	21.8426	49.4824	10.70	50.00
1/10000	8	27.8375	12.15788	4.29846	17.6733	38.0017	14.30	50.00
1/1000	8	25.5250	17.78738	6.28879	10.6544	40.3956	0.00	46.20
1/100	8	26.4375	11.71311	4.14121	16.6451	36.2299	7.10	40.70
1/10	8	25.1625	6.75911	2.38971	19.5117	30.8133	14.30	33.30
Neat	8	29.7875	11.66453	4.12403	20.0357	39.5393	11.10	43.80
Total	48	28.4021	13.05922	1.88494	24.6101	32.1941	0.00	50.00

Table 5.24: ANOVA output displaying no significant difference between means therefore Tukey HSDs were not completed for toxin concentrations for *H. clelandii* guttation droplet and *C. elegans*

ANOVA	
	Sig.
Between Groups	0.623

5.4.7.3. Summary

There was a statistically significant difference between treatment groups in the extract from *H. clelandii* broth, there was no statistically significant difference between treatment groups for *H. clelandii* guttation droplets as shown through ANOVAs and independent samples Kruskal-Wallis tests. *H. clelandii* broth extract effectively immobilised *C. elegans* in neat concentrations at all time periods in comparison to the control. Other concentrations of the extracts were not significantly different to the control. *H. clelandii* guttation droplets did not display any significant difference in *C. elegans* immobilisation from the control at any concentration or time period.

5.4.8. Blank extract and *C. elegans*

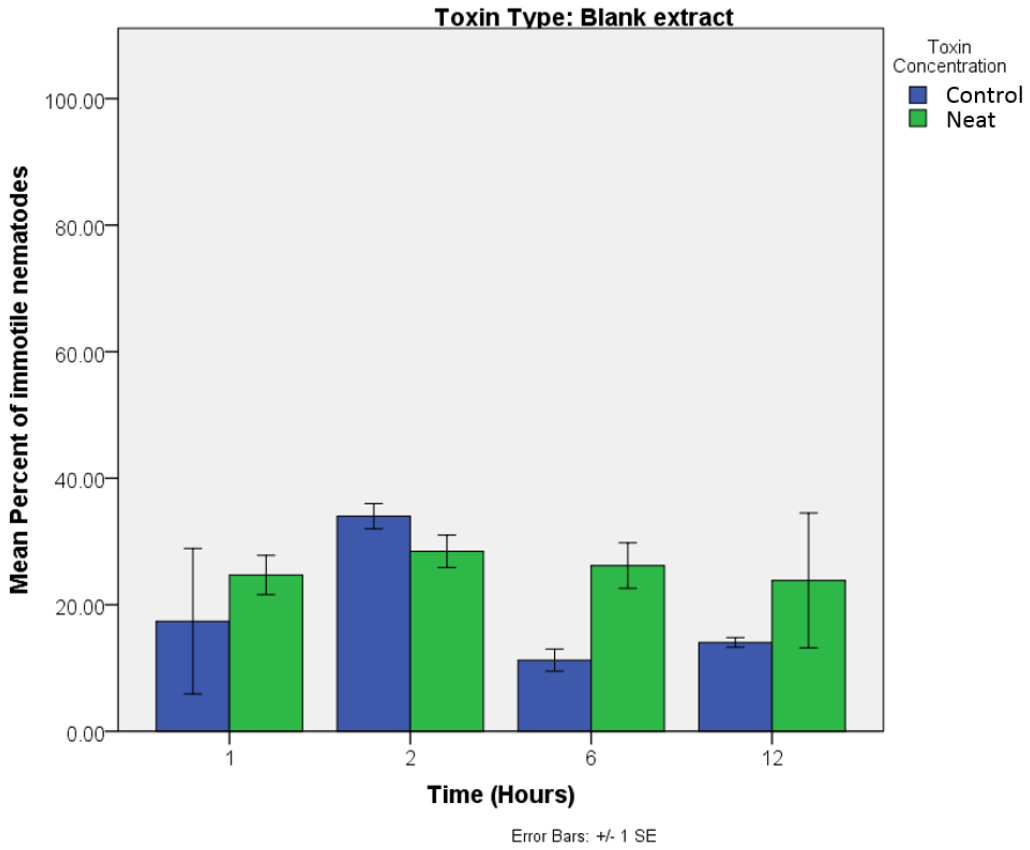

Figure 5.18: Toxicity of blank extract against *C. elegans*, showing no significant difference between controls at extract at any time period.

Table 5.25: SPSS descriptive statistics outputs for the toxicity of the blank extract towards *C. elegans*

Percent of immotile nematodes

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
Control	8	19.1750	11.36256	4.01727	9.6757	28.6743	5.90	36.00
Neat	8	25.8000	6.64809	2.35046	20.2421	31.3579	13.20	34.50
Total	16	22.4875	9.62184	2.40546	17.3604	27.6146	5.90	36.00

Table 5.26: ANOVA output displaying no significant difference between means therefore Tukey HSDs were not completed for the blank extract and *C. elegans*

ANOVA	
	Sig.
Between Groups	0.177

5.4.8.1. Summary

No significant differences were shown between the blank extract and the controls indicating the extraction method of the toxins did not increase their toxicity.

5.4.9. S. ratti and copper toxicity

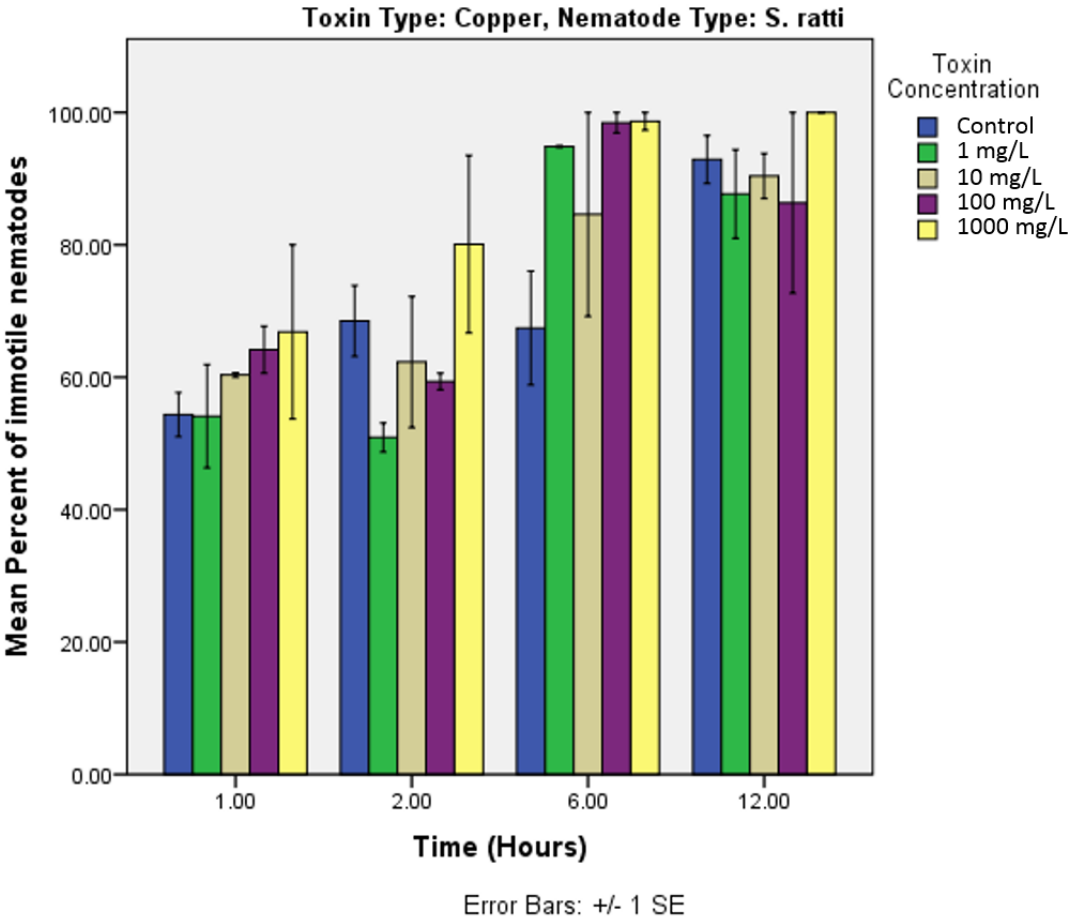

Figure 5.19: Toxicity of copper sulphate up to 1000 mg/L against *S. ratti*, showing no significant difference between controls and copper sulphate solution at any concentration or time period

Table 5.27: SPSS descriptive statistics outputs for the toxicity of copper sulphate dilutions towards *S. ratti*

Percent of immotile nematodes

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
Control	12	70.8000	16.74830	4.83482	60.1586	81.4414	48.90	100.00
1/1000	8	71.8875	21.67800	7.66433	53.7642	90.0108	46.30	95.00
1/100	8	74.4125	17.32632	6.12578	59.9273	88.8977	52.40	100.00
1/10	8	77.0750	18.72384	6.61988	61.4215	92.7285	58.10	100.00
Neat	8	86.4000	17.81829	6.29972	71.5035	101.2965	53.70	100.00
Total	44	75.6318	18.36423	2.76851	70.0486	81.2151	46.30	100.00

Table 5.28: ANOVA output displaying no significant difference between means therefore Tukey HSDs were not completed for toxin concentrations for copper sulphate dilutions and *S. ratti*

ANOVA	
	Sig.
Between Groups	0.419

5.4.9.1. Summary

There was no statistically significant difference between treatment groups for the copper positive control as shown through ANOVAs. Copper did not effectively immobilise *S. ratti* at any concentration or time period in comparison to the control. There was a general trend showing immobilisation of *S. ratti* over time in controls and copper dilutions.

5.4.10. Pearl oyster and *S. ratti*

5.4.10.1. Pearl oyster extracted from wheat grain

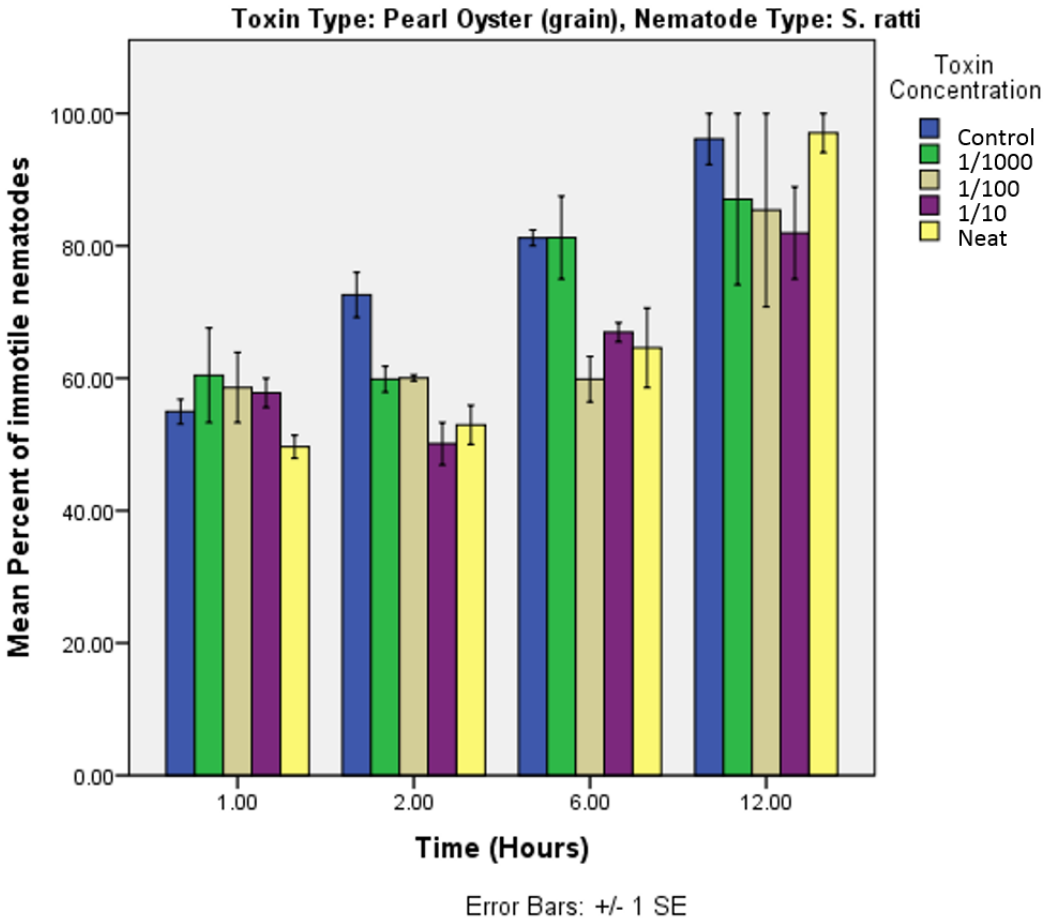

Figure 5.20: Toxicity of pearl oyster grain extract dilutions against *S. ratti*, showing no significant differences between controls and extracts.

Table 5.29: SPSS descriptive statistics outputs for the toxicity of pearl oyster grain extract dilutions towards *S. ratti*

Percent of immotile nematodes

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
Control	8	76.2250	16.20201	5.72827	62.6798	89.7702	53.10	100.00
1/1000	8	72.1500	15.62681	5.52491	59.0857	85.2143	53.30	100.00
1/100	8	65.9750	14.71343	5.20198	53.6743	78.2757	53.30	100.00
1/10	8	64.2000	13.39382	4.73543	53.0025	75.3975	46.90	88.90
Neat	8	66.0625	20.42505	7.22135	48.9867	83.1383	47.90	100.00
Total	40	68.9225	16.06210	2.53964	63.7856	74.0594	46.90	100.00

Table 5.30: ANOVA output displaying no significant difference between means therefore Tukey HSDs were not completed for toxin concentrations for pearl oyster grain extract and *S. ratti*

ANOVA	
	Sig.
Between Groups	0.546

5.4.10.2. Pearl oyster extracted from broth

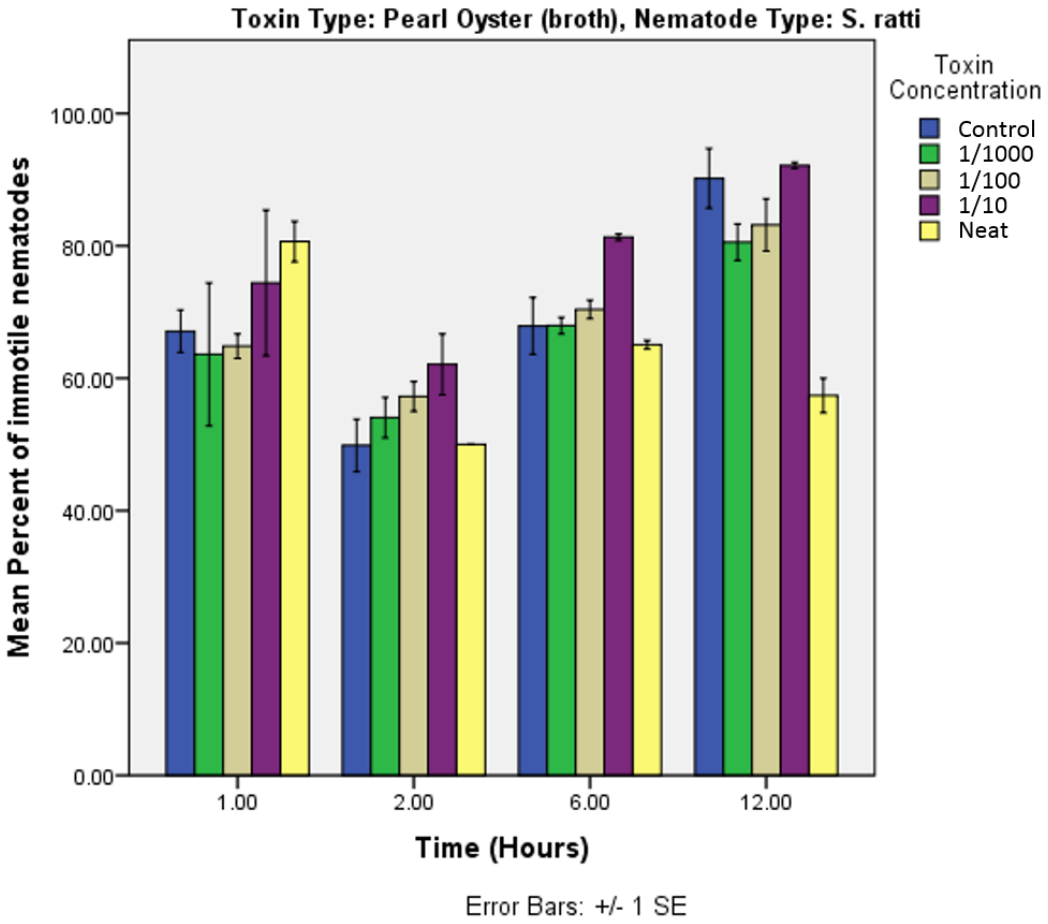

Figure 5.21: Toxicity of pearl oyster broth extract dilutions against *S. ratti*, showing no significant differences between controls and extracts.

Table 5.31: SPSS descriptive statistics outputs for the toxicity of pearl oyster broth extract dilutions towards *S. ratti*

Percent of immotile nematodes

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
Control	8	68.7625	15.90453	5.62310	55.4660	82.0590	45.90	94.70
1/1000	8	66.5375	11.92811	4.21722	56.5654	76.5096	51.00	83.30
1/100	8	68.9125	10.46742	3.70079	60.1615	77.6635	55.00	87.10
1/10	8	77.4875	13.29279	4.69971	66.3744	88.6006	57.50	92.60
Neat	8	63.2750	12.33193	4.35999	52.9653	73.5847	50.00	83.70
Total	40	68.9950	13.12910	2.07589	64.7961	73.1939	45.90	94.70

Table 5.32: ANOVA output displaying no significant difference between means therefore Tukey HSDs were not completed for toxin concentrations for pearl oyster broth extract and *S. ratti*

ANOVA	
	Sig.
Between Groups	0.278

5.4.10.3. Pearl oyster fruiting body extract

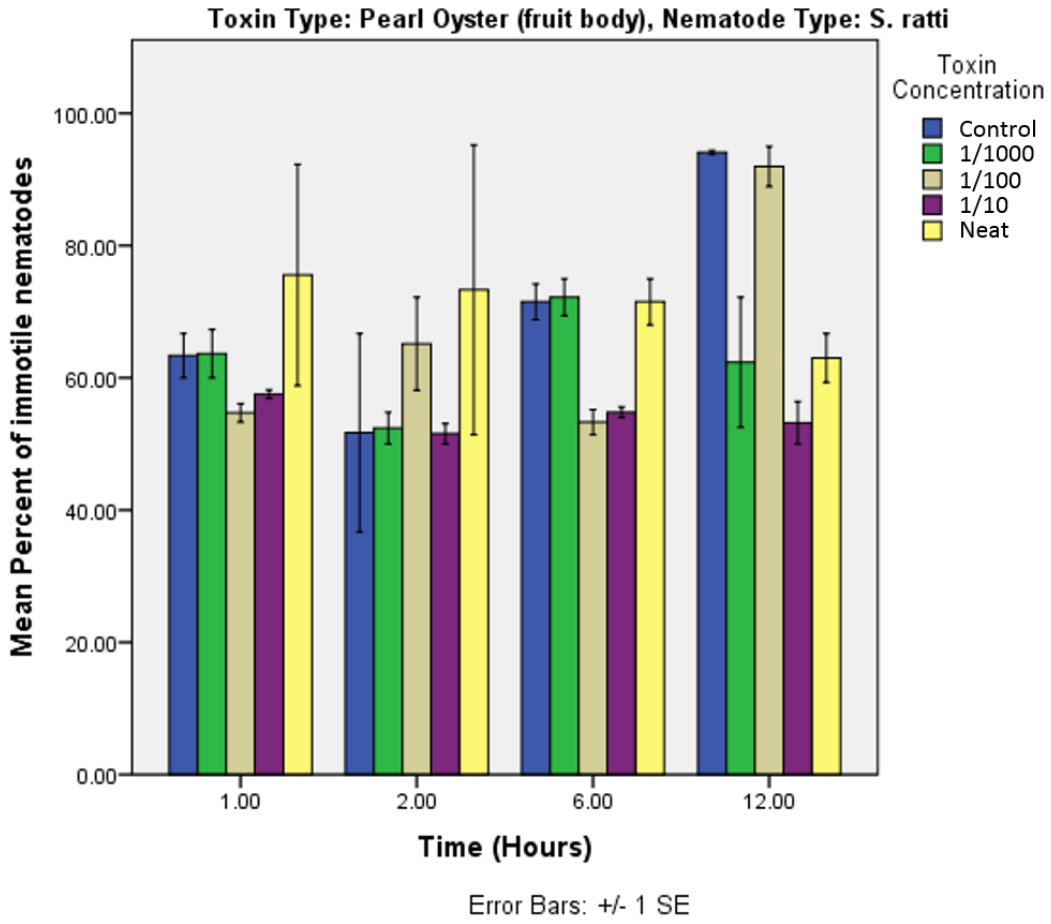

Figure 5.22: Toxicity of pearl oyster fruiting body extract dilutions against *S. ratti*, showing no significant differences between controls and extracts.

Table 5.33: SPSS descriptive statistics outputs for the toxicity of pearl oyster fruiting body extract dilutions towards *S. ratti*

Percent of immotile nematodes

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
Control	8	70.1625	18.56009	6.56198	54.6459	85.6791	36.70	94.40
1/1000	8	62.6500	9.58123	3.38748	54.6399	70.6601	50.00	75.00
1/100	8	66.2750	17.13290	6.05740	51.9515	80.5985	51.40	95.00
1/10	8	54.2750	3.08394	1.09034	51.6968	56.8532	50.00	58.20
Neat	8	70.8375	15.82267	5.59416	57.6094	84.0656	51.40	95.20
Total	40	64.8400	14.66721	2.31909	60.1492	69.5308	36.70	95.20

Table 5.34: ANOVA output displaying no significant difference between means therefore Tukey HSDs were not completed for toxin concentrations for pearl oyster fruiting body extract and *S. ratti*

ANOVA	
	Sig.
Between Groups	0.142

5.4.10.4. Summary

There was no statistically significant difference($p>0.05$) between treatment groups for the pearl oyster grain, broth or fruiting body extract toxicity towards *S.ratti* as shown by ANOVAs. These extracts did not effectively immobilise *S. ratti* at any concentration or time period in comparison to the control. Extracts from broth and grain showed a trend of immobilisation increasing over time in controls and extracts, this was not significant ($p>0.05$).

5.4.11. Blue oyster and *S. ratti*

5.4.11.1. Blue oyster broth extract

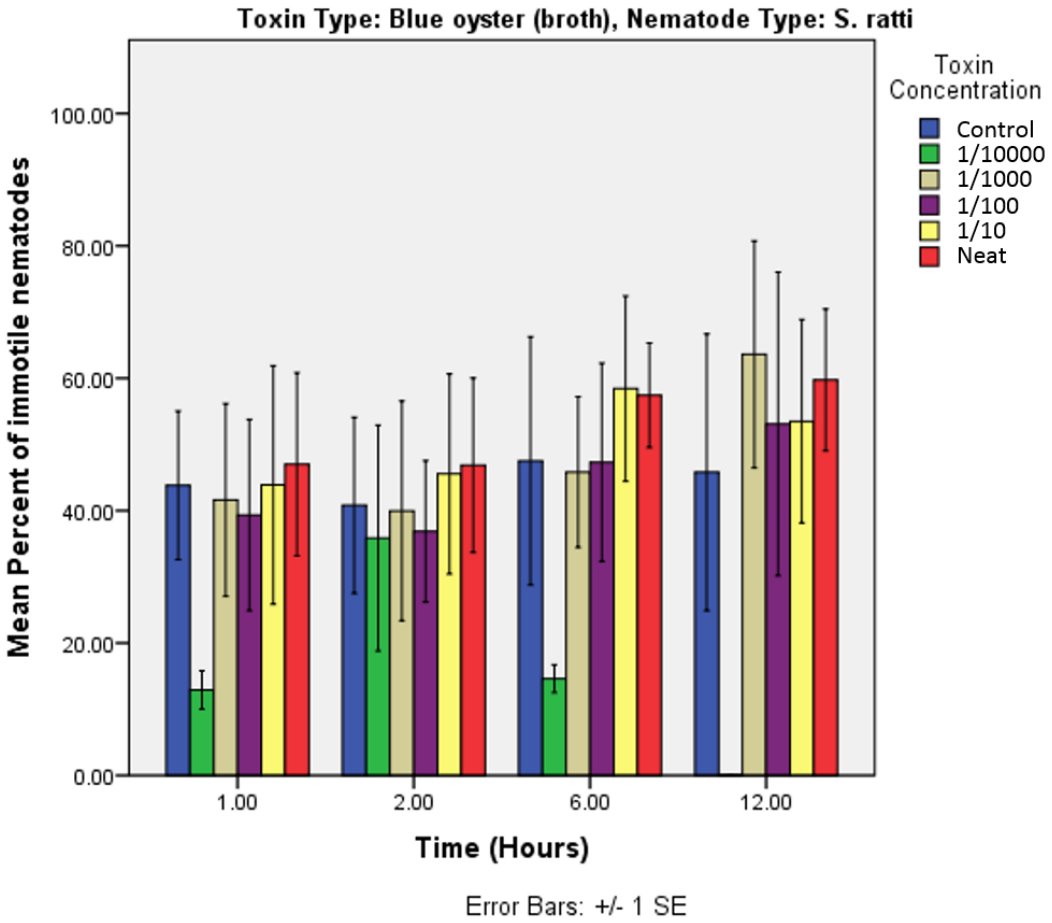

Figure 5.23: Toxicity of blue oyster broth extract dilutions against *S. ratti*, showing no significant differences between controls and extracts.

Table 5.35: SPSS descriptive statistics outputs for the toxicity of blue oyster broth extract dilutions towards *S. ratti*

Percent of immotile nematodes

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
Control	16	44.4813	29.65291	7.41323	28.6803	60.2822	5.30	90.90
1/10000	8	15.8375	16.60473	5.87066	1.9556	29.7194	0.00	52.90
1/1000	16	47.7438	28.68609	7.17152	32.4580	63.0295	5.90	93.30
1/100	16	44.1500	30.02701	7.50675	28.1497	60.1503	0.00	96.00
1/10	16	50.3375	28.72474	7.18118	35.0312	65.6438	7.70	85.70
Neat	16	52.7625	21.69285	5.42321	41.2032	64.3218	17.60	84.00
Total	88	44.9807	28.11763	2.99735	39.0231	50.9382	0.00	96.00

Table 5.36: ANOVA output displaying no significant difference between means therefore Tukey HSDs were not completed for toxin concentrations for blue oyster broth extract and *S. ratti*

ANOVA	
	Sig.
Between Groups	0.055

5.4.11.2. Summary

There was no statistically significant difference in toxicity ($p>0.05$) between treatment groups for the blue oyster broth extract as shown through ANOVAs. This extract did not effectively immobilise *S. ratti* at any concentration or time period in comparison to the control.

5.4.12. *H. clelandii* and *S. ratti*

5.4.12.1. *H. clelandii* broth extract

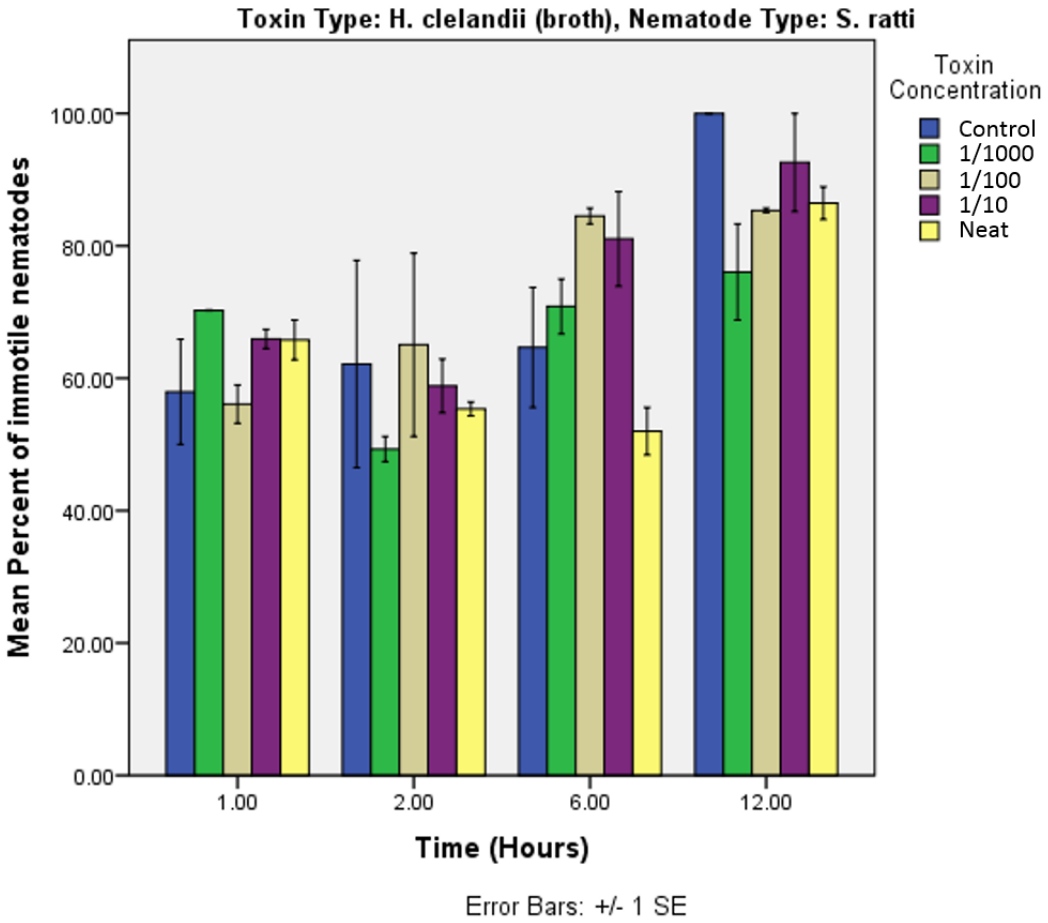

Figure 5.24: Toxicity of *H. clelandii* broth extract dilutions against *S. ratti*, showing no significant differences between controls and extracts.

Table 5.37: SPSS descriptive statistics outputs for the toxicity of *H. clelandii* broth extract dilutions towards *S. ratti*

Percent of immotile nematodes

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
Control	8	71.1875	20.83839	7.36748	53.7662	88.6088	46.50	100.00
1/1000	8	66.6125	11.87283	4.19768	56.6866	76.5384	47.40	83.30
1/100	8	72.7500	15.44696	5.46133	59.8360	85.6640	51.20	85.70
1/10	8	74.6125	15.23974	5.38806	61.8718	87.3532	54.80	100.00
Neat	8	64.9000	14.65693	5.18201	52.6465	77.1535	48.40	88.90
Total	40	70.0125	15.49861	2.45054	65.0558	74.9692	46.50	100.00

Table 5.38: ANOVA output displaying no significant difference between means therefore Tukey HSDs were not completed for toxin concentrations for *H. clelandii* broth extract and *S. ratti*

ANOVA	
	Sig.
Between Groups	0.709

5.4.12.2. Summary

There was no statistically significant difference in toxicity between treatment groups for the *H. clelandii* broth extract as shown through ANOVAs. This extract did not effectively immobilise *S. ratti* at any concentration or time period in comparison to the control. There was a general trend showing immobilisation of *S. ratti* over time in controls and extracts.

5.4.13. LC50

Table 5.39: Probit analysis LC_{50} values for all toxin and nematode types

Fungus	Extraction	*C. elegans*	*S. ratti*
Pearl oyster	Broth	0.07607	NS
	Grain	0.03586	NS
	Fruiting body	0.04726	NS
	Droplet	NS	
Blue oyster	Broth	1.222	NS
	Droplet	NS	
H. clelandii	Broth	0.1913	NS
	Droplet	NS	
Copper		0.08185	NS

The probit analysis estimated LC_{50} values for all toxin and nematode types which displayed significance in the ANOVAs. Therefore LC_{50} values were not generated for *S. ratti* assays or guttation droplet assays. As the toxins used were not purified the LC_{50} concentrations equate to dilution factors as used in the assays with 1 equating to the neat toxin. LC_{50}s for pearl oyster extracts (excluding guttation droplets) were all lower than the LC_{50} value for copper. LC_{50} for *H. clelandii* was still less than one indicating that 50% of *C. elegans* were killed at lower than the neat concentration. The LC_{50} for blue oyster was however over the neat concentration at 1.222 indicating that a concentration higher than the current neat extract would be required to kill 50% of the *C.elegans.*

5.5. Discussion

Nematotoxic capabilities of fungal extracts were more effective against *C. elegans* than *S. ratti*. This was also evident in the copper control. Neat extracts were the most effective against *C. elegans* in all fungal extracts. Pearl oyster extracts were the most toxic to *C. elegans* and the 1/10 dilutions were significantly different from the controls in all of the extracts. This was reflected in the LC_{50} values and the dose ranged from 0.035 to 0.076 indicating the toxins in the extracts were effective at doses lower than the 1/10 dilutions. Additionally they were all lower than the LC_{50} value for copper. *H. clelandii* extract from broth was effective at immobilising *C. elegans* in the neat concentration. This is the first time *H. clelandii* extracts have been tested against *C. elegans* and the responses suggests confounding results to its relatives *Nematoctonus concurrens* and *N. robustus* which were found to have antimicrobial activity but not nematotoxic activity (Anke et al., 1995).

However, there was no nematode toxicity displayed in either nematode type for the fungal guttation droplets. This indicates they either did not contain nematotoxins or the concentration was low enough to have no visual effects on the nematodes.

Despite the effectiveness of the fungal extracts on *C. elegans, S. ratti* did not respond in the same manner. Additionally *S. ratti* did not respond to the copper control, this was unexpected considering the responses of *C. elegans* to both fungal extracts and copper controls. The difference in toxicity between nematode types could be due to a multitude of factors including nematode behaviour rather than toxicity.

Copper has been used for toxicity testing against *C. elegans* in other studies, (Boyd and Williams, 2003) found *C. elegans* had a similar susceptibility to copper chloride

as *C. elegans* did to copper sulphate in this thesis with LC_{50}s 85.4 mg/L and 81.8 mg/L respectively. This indicates the methods for the assay used in this thesis were highly comparable and reproducible to other studies.

The effects of copper toxicity can be influenced by organic matter including humic acids, amino acids, polypeptides and polysaccharides in the extracellular environment. However, the exact mechanisms and mode of action for copper toxicity in microorganisms is not clearly understood. The effects are through to be able to cause protein damage and lipid peroxidation which has flow on effects on multiple cellular functions (Dupont et al., 2011).

Working with nematodes adds additional layers of complexity when compared to single cellular microorganisms such as bacteria. Nematodes have more complex lifecycles often involving males and females, eggs and larval stages. They can also differ between species in behaviour, morphology, and feeding type (Boyd and Williams, 2003).

C. elegans have frequently been used as a model nematode for parasitic nematodes and anthelmintic screening (Simpkin and Coles, 1981, James and Davey, 2009). There are similarities between *C. elegans* dauer larvae and the infective larvae of *Strongyloides* spp. contributing to *C. elegans* use as a model nematode (Viney and Lok, 2007). *C. elegans* and *S. ratti* have some fundamental differences linked to behaviour, morphology and feeding types that need to be considered as they could have impacted on the results of this study.

5.5.1. Behavioural differences

C. elegans and *S. ratti* behaved differently during toxicity assays. After the nematodes were harvested either from plate or faeces they were processed and handled following the same procedures. *C. elegans* maintained their moderate level of activity once in the wells for the assay unless they were affected by the treatment in which case they became immotile. *S. ratti* were relatively active in liquid culture before transportation into wells for assays, once in the wells their activity diminished to almost immotile. Assessments for the assay were conducted under a dissecting microscope. The light and warmth produced by the microscope increased the motility of the *S. ratti*, enabling counting and differentiation between motile and immotile nematodes to occur. The nematodes also nictated in the wells indicating they had not ceased searching for a host. Nictation is a behaviour involving the nematode standing on their tails and swaying back and forth (Karp, 2016). Infective larvae of parasitic nematodes are known to perform this behaviour as a host searching mechanism, dauer larvae are the only other known nematode to display this behaviour (Lee et al., 2011). The dauer larvae are thought to perform this behaviour as a mechanism to escape when conditions are not favourable such as when agar plates become contaminated (Lee et al., 2011). *C. elegans* did not nictate during this assay.

The other behavioural trait displayed by *S. ratti* was their inability to stay within the well of the plates. The nematodes were moving up the sides and out of the wells during the course of the experiment. This meant they were reducing their movement whilst in the toxin and removing themselves from it. *C. elegans* however, rarely

moved out of the toxin or wells. *S. ratti's* movement out of the wells would have contributed to the trends in the data implying the immotility slightly increased over time. These behavioural issues could be overcome by performing the assay in a liquid substrate in sealed wells.

5.5.2. Nematode feeding types

Nematode feeding type varies greatly which also impacts on nematode behaviour. *C. elegans* are bacterial feeders which means they are constantly grazing for bacteria (Stirling, 2014). This means they are consuming particles from their external surroundings. *S. ratti* are animal parasites and whilst are similar to *C. elegans* when they are in their infective larval stage they are not feeding on bacteria. *S. ratti* infective larvae are non-feeding and contain enough nutrition internally to survive with only moisture for approximately three weeks when they either find a host or perish (Viney, 1996). This enables them to become a somewhat enclosed unit during this phase of their lifecycle and are therefore not likely to be consuming particles from their external environments.

5.5.3. Morphology

5.5.3.1. Nematode cuticles

The outer surface of a nematode is called the cuticle which a non-living layer which is secreted by the hypodermis (Chen et al., 2004). The cuticle covers the entire external surface including the ducts and openings, this provides protection from the

environment (Chen et al., 2004). The composition varies between both nematode taxa and growth stages.

S. ratti has a strongly negatively charged outer cortical layer of cuticle (Chen et al., 2004, Murrell et al., 1983). It has been suggested that this could aid desiccation tolerance by keeping a water coat over the cuticle (Perry and Wharton, 2011). Nematode sheaths are cuticles that are retained post moulting and potentially play a role in protection against pathogenic fungi (Perry and Wharton, 2011). Other species of parasitic nematode such as *Ancylostoma caninum* (hookworm) possess a sheath which is shed upon skin penetration of the new host, *S. ratti* infective larvae were previously not thought to possess a sheath (Grove et al., 1987). However, it is now understood that a surface coat, potentially the epicuticle or similar rather than a sheath is lost during skin penetration in the infection process (Grove et al., 1987).

C. elegans shed their old cuticles immediately post moulting and therefore do not retain sheaths (Cassada and Russell, 1975). Dauer larvae of *C. elegans* have a cuticle which entirely encloses them and prevents them from feeding and has improved chemical resistance, this acts as a protective mechanism from the environment (Corsi et al., 2015). It is also thought that a sheath is maintained post moulting but is lost with aging and washing (Cassada and Russell, 1975).

Further information is required to determine the modes of action and mechanisms of entry for the nematotoxins tested. Despite the robustness of the cuticle and the protective measures from the environment it provides, permeation of the nematode cuticle is one of the potential mechanisms of entry for these nematotoxins. Cyclotides are plant pesticides that are active on nematodes directly via cuticle

penetration, ingestion by the nematode is not necessary. The cyclotides increase the chemical permeability of the nematode cuticle potentially by disruption of the lipid rich epicuticle (Colgrave et al., 2010). Acetic acid is also known for permeation of the cuticle and is used in fixation and preparation of mounting nematode slides. It is often used in the form of glacial acetic acid which helps to penetrate and soften the tissue (Berland, 1984). Additionally acetic acid has been found in antagonistic fungi as a nematode killing mechanism. Nematotoxins extracted from *Paecilomyces lilacinus* and *Trichoderma longibrachiatum* were characterized as acetic acid (Djian et al., 1991). Some of the compounds isolated from *Pleurotus* spp. and *Hohenbuehelia* spp. have been characterized as acids such as 2-decenedioic acid, linoleic acid and dihydropleurotinic acid (Degenkolb and Vilcinskas, 2016a). This raises questions as to whether these acids have similar modes of action to acetic acid.

5.5.4. Impacts of differences between *C. elegans* and *S. ratti*

The differences between *C. elegans* and *S. ratti* in behaviour, feeding type and morphology are relevant to the mechanism of entry for the toxins tested. The mechanism in which the toxins enter the nematode could vary between the fungal species tested. The nematodes cuticle and behaviour could influence the effect the toxins have if the mechanism of entry is contact through epidermis. If the mechanism of entry is via ingestion then this could also impact the effect of the toxin. Both of these mechanisms of entry would favour survival of *S. ratti* over *C. elegans*. *S. ratti*'s behaviour often involved removing themselves from the well which removed them from the toxin, reducing the capability of the toxin to permeate the nematode cuticle. As *S. ratti* are also parasitic, they are not grazers and therefore not ingesting

things from their environments, including the toxins in the assay. These factors may have influenced the results of the assay and many help to explain why *C. elegans* was more sensitive to the toxins than *S. ratti*.

With a broad array of nematotoxin types and modes of action screening metabolites against an array of nematodes types is essential (Degenkolb and Vilcinskas, 2016a). *C. elegans* is often more sensitive to nematotoxins than other species (Degenkolb and Vilcinskas, 2016a, Anke et al., 1995). This was observed in these experiments and was also the case with the copper control. There could be potential for greater uptake and effects from these toxins to *S. ratti* with the addition of a surfactant. Surfactants are an often essential addition to agricultural chemicals, particularly those applied to plants and enable uptake of the chemical into the plant (Stock and Holloway, 1993). If these extracts were to be used in the environment and applied to soil formulation with surfactant may be an important consideration in the effectiveness and uptake.

Correlation between *C. elegans* and *S. ratti* data may have been better if *C. elegans* dauer larvae were used instead of the range of life cycle stages (predominantly L3) available from culture. Working with all lifecycle stages of *C. elegans* is more comparable to the natural ecology of the nematode and how it would normally be encountered by the toxin secreting fungi. As mentioned dauer larvae are much more similar to the infective larvae of *S. ratti* both morphologically and behaviourally. However, working with the nematodes proved difficult for both species each coming with their own challenges. *C. elegans* whilst easy to maintain can become very sensitive through handling processes. On multiple occasions assays were planned but

postponed due to *C. elegans* numbers or viability. Eventually an optimal harvest time was recognised at 7-10 days post transfer to fresh NGM plates. This time period yielded high numbers of nematodes at L2-3 stage that retained high viability through washing procedures in preparation for addition to assays. Worm stars were also an issue and had to be carefully avoided by adhering to strict time periods when working with nematodes in liquid culture. Worm stars are a phenomenon caused by *Leucobacter* spp., bacteria that adhere to the tails of nematodes, when infected nematodes move past one another their tails adhere together (Clark and Hodgkin, 2015, Hodgkin et al., 2013). This causes the nematodes to move rapidly and collide with more nematodes which subsequently adhere, the tails eventually forming a worm star, this exhausts and kills the nematodes (Hodgkin et al., 2013). Nematodes caught in worm stars are unsuitable for use in assays.

It was therefore determined that induction of the dauer larvae phase of the lifecycle added another layer of complexity to the assay and was not beneficial. *C. elegans* that were in other phases of the lifecycle provided a sound starting point and an indicator of responses of free-living nematodes to the toxins. *S. ratti* would be tested separately and act as a more direct indicator of *S. stercoralis* responses.

5.5.5. Future of fungal extracts in biological control and nematode control

Whilst a multitude of reasons could be responsible for toxicity of fungal extracts and copper being greater in *C. elegans* than *S. ratti* the lack of response in *S. ratti* reduces incentive to continue this work. This research was carried out with crude fungal extracts and the toxins were not characterised or clarified. These processes are

expensive, time consuming and require particular expertise. This was outside the scope and budget of the project and without strong responses with *S. ratti* it was not worth investigating for *S. stercoralis* control. Both economically and biologically, the application of fungal toxins to areas with environmental contamination with *S. stercoralis* holds challenges.

Biologically *S. stercoralis* infection can occur from as little as one viable larvae, as the toxins were not capable of effectively immobilizing significantly more *S. ratti* than the control they would be ineffective. The toxins would need to be capable of immobilising 100% of larvae to be considered as a viable option. Otherwise the toxin could be used to reduce numbers of the larvae and reduce infection risk but not to remove it completely. Additionally, the toxin would ideally be applied to areas with environmental contamination of *S. stercoralis*, this contamination comes from faeces and is usually deposited into soil, and therefore the microbial load would be quite high. The concentration and formulation of the toxin would need to be capable of acting effectively against the *S. stercoralis* in a soil and faecal matrix. This was not tested in these experiments but there is evidence of toxins absorbing or quenching in similar environments. For example Miller et al. studied the capacity of cyanobacterial toxin to be absorbed by soil molecules through bank filtration (Miller et al., 2001). There is a possibility this toxin would act in a similar manner with soil particles and the interaction or absorption with other microorganisms is unknown. This could reduce the viability of these fungal extracts if they acted in this way, particularly considering the energy required to produce such a small volume of toxin with less than 100% immobilization in the neat extract.

The volume and concentration required leads into the economics of viably using this method. Production of mushroom fruiting bodies or even fungal mycelium is a technical process which is prone to contamination at multiple stages. Due to the technical expertise, equipment and infrastructure required, economic viability many only work if toxin were extracted from by-products or waste products of existing mushroom cultivation industries. Spent mushroom substrate from oyster mushroom cultivation can be utilised by being composted. However, it would also be possible to utilise this for toxin extraction before the composting process. This could help to make large scale oyster mushroom nematotoxin production financially viable without the capital costs of infrastructure and equipment. Alternatively Thorn and Barron have suggested that direct application of the fungus to soil maybe be a viable option(Thorn and Barron, 1984, Barron and Thorn, 1987). Stamets book *Mycelium Running* contains a multitude of ideas in regards to economic viability of such ideas including the use of waste materials like cow manure and creation of mycofiltration techniques for human pathogens (Stamets, 2005).

Further identification of the active chemicals followed by chemical or biotechnological synthesis could also be investigated. Kotze et al. found the LC_{50} of ivermectin to be 49.5 µM or 43.3 mg/L for *S. ratti*, considering the LD_{50} for copper sulphate was 81.8 mg/L in this thesis and the unpurified extracts from *P. ostreatus* were all more potent than copper, if biosynthesis was available these actives could be in the same realm as ivermectin in terms of potency (Kotze et al., 2004). This could make purification and synthesis of these compounds a viable option as an alternative to ivermectin.

This research did not conclude that the fungal toxin extracts would be effective against the model *Strongyloides sp.*, *S. ratti* and therefore *S. stercoralis* in environmental conditions. However, the toxin was effective against *C. elegans in vitro* and therefore could have potential effects against other nematode species including plant parasitic nematodes. The effects of these extracts have not yet been tested against other nematode species or other microorganisms. The assay with *C. elegans* suggest that the target organism would not be the only organism effected if the extracts were to be used with plant parasitic nematodes.

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6. Chapter Six: General Conclusions

This thesis presents an investigation into the environmental reservoirs of *Strongyloides stercoralis* in Australia and aimed to develop methods to reduce numbers of infective larvae in these areas as part of an integrated multifaceted approach to control the parasite. The use of nematophagous fungi and their extracts were the main focus of infective larval reduction methods.

This work aimed to investigate environmental reservoirs of *S. stercoralis* including their locations, substrate types (canine faeces, soil-faecal matrices) and prevalence in the Northern Territory of Australia. The subsequent aims were to investigate the potential of nematophagous fungi and their extracts as environmental control measures of the infective larvae of this human parasitic nematode.

This is a novel approach to *S. stercoralis* control and the first time that isolation of nematophagous fungi native to Australian soils has been conducted for this purpose. Soil samples were collected from the Northern Territory in Australia and the sprinkle plate used as the technique to isolate potential nematophagous fungi. Characterization of the isolates proved difficult due to the lack of clarity between predatory activity, saprotrophic activity and weak predatory activity. It was concluded that none of the isolates were strictly nematophagous fungi and were weakly or opportunistically predating nematodes, which were among an array of defensive properties displayed by them. The array of isolates displayed the spectrum of fungal diversity ranging from specialised nematode trapping structures through to saprotrophic activity on dead nematodes and the ambiguity in distinguishing where

a particular species falls within this range. Therefore no fungal isolates native to the Northern Territory were suitable candidates to be used in the remainder of the experiments. This impacted the direction of the research slightly and more emphasis was placed on nematophagous fungi that use nematotoxin excretions to immobilize and kill nematodes.

This research is one of very few studies investigating the effect of fungi on *S. stercoralis* and provides a framework for more directed research in this area. Currently the only published work on the use of predatory nematophagous fungi on *S. stercoralis* has been conducted by Braga et al (2010b) using nematophagous species: *Duddingtonia flagrans, Monacrosporium thaumasium* and *Artrobotrys robusta*. Trapping efficacies of these nematophagous species ranged from 73.2-83.7% removal of L3 larvae (Braga et al., 2010b). As discussed in Chapter 5 there are benefits to reducing the number of *S. stercoralis* larvae but only a singular infective larvae is required to cause infection. Testing the nematophagous efficacy of the species used in this research (*A. oligospora*, *Pleurotus ostreatus, P. ostreatus* var. columbinis and *Hohenbuehelia clelandii*) in substrate and nutrient conditions similar to those described in Chapter 4 within a soil-faecal matrix, could have provided interesting insights regarding predation. However, the availability of *S. stercoralis* infective larvae was the significant factor which limited the conduct this work. The environmental samples received in Chapter 1 were initially sought to provide the live infective larvae to be used in the assays, but the age of the samples and inability to detect *S. stercoralis* rendered this not possible. The model organism for *S. stercoralis*, *S. ratti* had potential to be used as an indicator organism to conduct this work, but

access to live larvae of *S. ratti* was also limited. The viability of the *S. ratti* larvae received from Westmead Hospital varied greatly and the nematotoxin assays presented in Chapter 5 took over two years to conduct. This included the preparation and development of the assays using *C. elegans* and receipt of rat faecal samples which did not yield high enough numbers of *S. ratti* infective larvae to conduct the assays. The Westmead laboratory is located in a different state to the Flinders University laboratory and travel time was regarded as the major constraint to *S. ratti* larval yields. Therefore the work in Chapter 4 assessing the efficacy of *A. oligospora* to trap *C. elegans* in a soil-faecal matrix acts as an indicator of how *S. stercoralis* would survive in a similar system. The ability of *A. oligospora* to effectively trap and remove 99.9% of *C. elegans* in a canine faecal matrix was demonstrated, however, in a canine faecal matrix incorporating organic soil trapping efficacy was only 61.2%. This provides valuable insight into the behaviour of nematophagous fungi in high nutrient substrates. This work displayed the ability of nematophagous fungi to colonise and trap more effectively in high nutrient substrates providing enhanced trapping abilities in canine faeces above soil-faecal matrices. *S. stercoralis* infective larvae are likely to occur in either of these substrates, however, the organic soil faecal matrix was the target substrate in this research, simulating the substrate surrounding a leaking septic tank. Therefore the trapping efficacy of 61.2% is relatively low considering only one nematode is required for infection.

The nematotoxic substances produced by *P. ostreatus, P. ostreatus* var. columbinus and *H. clelandii*, which aid in their nematophagous processes were investigated for their toxicity to *C. elegans* and *S. ratti.* The purpose of this work was to identify if

extracts had potential for use as an environmental control measure for free-living *S. stercoralis* larvae. This is the first study that has assessed the toxicity of any of these extracts with *S. ratti* and the first time *H. clelandii* extracts have been extracted or assessed for their toxic effects on any other organism.

The extracts from *H. clelandii* mycelium grown in broth were effective at immobilizing *C. elegans* in neat unpurified extracts. This provides novel insight into this species as nematode toxicity has not been described before. This adds to our understanding of the nematophagous capabilities of *H. clelandii* and provides scope for future work with the species.

None of the extracts were effective at immobilizing *S. ratti*. The *S. ratti* did not respond to the copper sulphate positive controls, this indicates that the methodology of the assay did not suit *S. ratti* infective larvae due to the differences in their behaviour, cuticles and feeding mechanisms. The assay did fully submerge the nematodes in the fungal extracts or copper sulphate which would simulate the contact they would be faced with in a soil faecal matrix if used as an environmental control. The lack of response in these assays limits the potential for these extracts to be a beneficial mechanism for environmental control of *S. stercoralis*. However, the potency of the fungal extracts and low toxicity profile in mammals suggests great potential for their use with other nematode species and further development with biosynthesis. This result provides new information regarding the interactions between nematophagous fungi and their extracts and *S. ratti*. This research attempts to link idealized laboratory assays with non-ideal, environmentally relevant

conditions and applications. This knowledge can now be used to guide the potential future directions of this work.

6.1. Future directions

The scope of future work in the realm of strongyloidiasis alone is broad and a disease that is multifaceted needs a comprehensive and dynamic approach to help tackle the global challenge. Investigating the role of the free-living phase of the lifecycle and understanding the environmental reservoirs in broader detail plays a key role to interjecting transmission and infection. As mentioned previously in this thesis, failed infrastructure plays a big role in Australia and governments, communities and aid organisations should be pooling more resources into improving and maintaining facilities. From a research perspective there is still much that is untapped and further resources, grant funding and whole community approaches should be used to investigate further. This requires researchers to work together with clinicians, environmental health officers, communities and government organisations to build relationships and develop united goals. A combined approach would enable the necessary community engagement and ethical considerations and applications to be achieved respectfully so that further work in this space can commence with more tools in the arsenal.

The use of DNA technology is a powerful tool that should continue to be utilized with *S. stercoralis* to develop our understanding of environmental reservoirs. Greater access to indigenous communities and samples in Australia could confirm the transmission of *S. stercoralis* in canines and detection in areas such as leaking septic tanks could be used to advocate for better facility maintenance. Simple solutions to

S. stercoralis in environmental reservoirs could be investigated further to determine if soil amendments such as salinity, pH or other easily adjusted factors are effective at controlling infective larvae.

Many of the limitations in this thesis were related to the viability of infective larvae of *Strongyloides* spp. This included no or limited viable larvae from faeces and an inability to detect their DNA. It is therefore important to consider whether this tells us more about the biology and behaviour of the larvae in the environment than originally considered. It could provide insight into the longevity of the larvae or that the conditions they require are very specific. This area should be researched further to investigate larval survivability as the results could provide vital information about transmission.

Fungal nematode interactions are only just beginning to be explored in this realm and the outcomes of this thesis highlight the potential they hold. This work provides a framework for further investigations into a neglected human parasite in an Australian context using native fungi to reduce larval loads in environmental reservoirs. The potential of this work spans further than human parasitic nematodes and the author hopes that this research can add to the extensive ongoing work into different species of nematophagous fungi and species of parasitic nematodes of veterinary significance. This includes a broad range of animal parasitic species such as: *Haemonchus contortus, Strongyloides westeri, Strongyloides venezuelenisis, Taenia saginata, Ascaris suum, Amblyomma cajannense, Oxyuris equi, Strongyloides papillosus* along with many others (Braga et al., 2014, Braga et al., 2013, Braga et al., 2010a, Araujo et al., 2010, Braga et al., 2011, Araújo et al., 2008, Araújo et al., 2009,

Chandrawathani et al., 1998). These studies are predominantly focused around the animal bedding where infective free-living larvae survive, they have additionally progressed into a follow on area which looks at the ability of the nematophagous fungi to survive the gastrointestinal tracts of the host animals (Braga et al., 2010a, Araujo et al., 2010, Araujo et al., 2012). This is a vital next step in biological control of parasitic nematodes using nematophagous fungi that enables the fungus to begin colonizing the faecal matter and parasitizing the infective larvae as soon as possible.

Additionally nematotoxic extracts from nematophagous fungi should be further explored for their activity within the host as a replacement or addition to current anthelmenthics. *P. ostreatus* is a commonly cultivated mushroom for its choice edibility and therefore extracts are not harmful to consumers when consumed within the mushroom. This makes this species a viable candidate for further research into parasitic nematode control.

6.2. References

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