NATURAL PRODUCT ANTHELMINTIC COMPOUND SCREENING

DEVELOPING A SCREEN TO IDENTIFY MICROBIAL NATURAL PRODUCTS WITH POTENTIAL ANTHELMINTIC ACTIVITY USING *CAENORHABDITIS ELEGANS*

By

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TITLE: Developing a screen to identify microbial natural products with potential anthelmintic activity using C*aenorhabditis elegans*

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LAY ABSTRACT

Helminths are parasitic worms that can cause debilitating diseases in approximately one quarter of the world's population. These infections result in long-term health defects if left untreated but there are currently very few anthelmintic medicines. As such, there is a need to focus research efforts towards the discovery of new treatments. Currently, many medicines that exist in our healthcare system today were derived from natural sources, and we can continue to search here for chemicals that are effective against helminth infections. In this work, we use *Caenorhabditis elegans*, a non-parasitic nematode, to help identify natural products from bacteria and fungi that can kill, paralyze, or affect the development of worms. We developed and optimized a method to assess a collection of natural product extracts for activity against *C. elegans*. This work could uncover new anthelmintic compounds that can be used to treat parasitic worm infections and improve global human health.

ABSTRACT

Parasitic worm infections affect over a quarter of the human population, reducing quality of life and impacting global health. Helminth infections in farm animals are also highly prevalent and threaten the quality and quantity of agricultural resources. Despite the severity and frequency of helminthiases, few treatment options are available and rising levels of resistance to existing anthelmintics necessitates a more active drug discovery strategy. In the past, natural products have proven to be a rich source of clinically relevant therapeutics. There is currently no gold-standard protocol for anthelmintic compound screening, and efforts to look for anthelmintic chemicals from natural sources is also limited. To fill this gap, we developed a screening platform that uses *Caenorhabditis elegans* as a nematode model and optimized the assay conditions for natural product extract screening. Using outputs of motility and image analyses, we are able to identify extracts from environmental microbes that impact worm development. Further characterization confirmed the presence of nematocidal compounds tunicamycin and actinomycin D. We also identified and isolated xanthocillin as an inhibitor of worm growth. This has not been previously reported to have activity against nematodes but preliminary work presented here suggests that it works through heme sequestration. In the field, there has also been some interest in the nematode cuticle as a potential drug target. To assist in discovery efforts here, we also developed a protocol using a

nanoluciferase-based reporter worm strain that responds to cuticle damage. The work presented here contributes to more focused anthelmintic drug discovery efforts. It provides valuable tools and insights that can be leveraged to assist in identifying compounds for the potential treatment of parasitic worm infections.

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encouragement, both on and off the court. Rabia and Monica, thank you for running this marathon with me. It's been awesome growing alongside you. Victoria, our late nights working on group assignments in undergrad turned into early mornings in the lab and there is no one else I would have wanted by my side for every academic milestone. To Anita, you've accomplished everything you set out to do and I've had the best time watching it happen. Thank you for being there for me as a labmate, friend, and roommate. You are going to be an incredible physician. As I reflect on all that I'm grateful for, I'm reminded of a phrase that has quietly guided me: *Non desistas, non exieris*.

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LIST OF ABBREVIATIONS AND SYMBOLS

Abbreviation	Definition
BGC	Biosynthetic gene cluster
BZ	Benzimidazole
CDC	Centers for Disease Control and Prevention
CMCB	Centre for Microbial Chemical Biology
DALY	Disability-adjusted life years
DMSO	Dimethyl sulfoxide
ER	Endoplasmic reticulum
GBD	Global burden of disease
GluCl	Glutamate-gated chloride
MDA	Mass drug administration
nAChRs	Nicotinic acetylcholine receptors
NGM	Nematode growth medium

NTD	Neglected tropical disease
PFL	Prefractionated library
PPIX	Protoporphyrin IX
SNP	Single nucleotide polymorphism
STH	Soil transmitted helminth
WAC	Wright Actinomycete Collection
WHO	World Health Organization
YLD	Years lived with a disability
YLL	Years of life lost from early mortality

DECLARATION OF ACADEMIC ACHIEVEMENT

I have performed all the research in this body of work except for where indicated in each chapter's title page.

CHAPTER ONE – INTRODUCTION

Clinical need for novel anthelmintic compounds

Neglected tropical diseases (NTDs) are life-threatening infections that most commonly affect inhabitants of lower-income countries (Ca et al., 2024; Centers for Disease Control and Prevention [CDC], 2024). The geographic bias of these communicable diseases results from a shortage of medical resources and inadequate sanitation protocols in poorer regions (Houweling et al., 2016). Some of the most prevalent NTDs are caused by parasitic worms (helminths), with over 1.5 billion individuals affected worldwide (World Health Organization [WHO], 2023). Specifically, soil-transmitted helminth (STH) infections are one of the most common parasitic infections, surpassing malaria by over one billion cases (WHO, 2023a, 2024b). Despite the high prevalence, the global burden of helminthiases is often overlooked due to more progressive efforts targeted towards the main diseases affecting low-income countries. Referred to as the "big three", tuberculosis, HIV/AIDS, and malaria have been the primary focus of global research efforts due to their high mortality rates (Feasey et al., 2010; Nakatani, 2016). International funding organizations prioritize research that advances the treatment and prevention of these three diseases and preferentially provide tremendous financial support. For example, the Global Fund, a non-profit organization, has disbursed over $65 billion USD for the big three diseases since 2002 (The Global Fund, 2024). In 2022 alone, $604 million USD went towards malaria research (WHO, 2024a). In comparison,

schistosomiasis, an intravascular infection caused by trematode worms, received only $39 million USD in 2022 despite being the second most devastating parasitic infection behind malaria (CDC, 2024a; GBD 2021 Diseases and Injuries Collaborators, 2024; WHO, 2023b, 2024a). In addition to the high prevalence rates and lack of research, the actual severity of helminthiases is consistently underestimated in global health metrics, contributing to their overall neglect.

The difficulties of quantifying disease burden stem from variations in morbidity and mortality rates, as well as the severity and overall impact on human health. In the Global Burden of Disease (GBD) study conducted in 2021, researchers used disability-adjusted life years (DALYs) as a method of comparing the global burden for over 300 diseases (GBD 2021 Diseases and Injuries Collaborators, 2024). The DALY value is comprised of both the number of years lived with a disability (YLD) and the years of life lost from early mortality (YLL) (GBD 2021 Diseases and Injuries Collaborators, 2024). This metric provides an estimate of the total number of healthy years forfeited due to infection, enabling a more direct comparison of medical conditions and their impacts on health. For parasitic worm infections, the global burden is approximately 8 million DALYs, compared to malaria's 55 million (Table 1). Although there is a striking difference in DALYs between helminthiases and malaria, it should be noted that not all parasitic worm infections were investigated in the study, and the most significant contributor to DALYs for helminth infections is YLD or morbidity. YLD is calculated by multiplying the time spent living with a condition by a

severity or disability weight, which ranges between perfect health (0) to death (1) (GBD 2021 Diseases and Injuries Collaborators, 2024; Grosse et al., 2009). This means that YLD values are heavily dependent on the perceived severity of health conditions, for which there is high variation (Charalampous et al., 2022). For helminthiases, individuals are often left with long-term cognitive and physical impairments due to the chronic nature of the infections. From YLD calculations, a chronic but less disabling condition could be comparable to an acute and short-term illness (Charles H. King, 2015; Hotez et al., 2008a). However, this analysis fails to account for the overall decline in quality of life as a result of physical and social limitations caused by chronic conditions (Grosse et al., 2009). YLD also does not account for comorbidities or treatment access, both of which are significant challenges associated with helminthiases. As such, helminth-related disability is likely underestimated. The limited recognition for helminthiases exacerbates the financial and health burden imposed by these illnesses and necessitates a more aggressive treatment approach.

Table 1: A comparison of the number of disability-adjusted life years (DALY), years lived with a disability (YLD), and years of life lost from early mortality (YLL) of malaria and the parasitic worm infections investigated in the 2021 Global Burden of Disease study (GBD 2021 Diseases and Injuries Collaborators, 2024).

Cause		DALY estimate	YLD estimate	YLL estimate
Malaria		55,174,060.7	2,364,839.0	52,809,221.7
Helminths				
	Schistosomiasis	1,746,333.3	1,245,872.0	500,461.3
	Cysticercosis	1,235,939.0	1,154,653.6	81,285.4
	Cystic echinococcosis	105,071.6	45,549.6	59,521.9
	Lymphatic filariasis	1,314,563.4	1,314,563.4	N/A
	Onchocerciasis	1,262,988.1	1,262,988.1	N/A
	Intestinal nematode infections	1,381,641.0	1,097,345.8	284,295.2
	Food-borne trematodiases	998,028.5	998,028.5	N/A
	Guinea worm disease	0.9	0.9	N/A
Total Helminths		8,044,565.7	7,119,001.9	925,563.8

As with other drugs, decreased treatment efficacy due to resistance is also a concern with anthelmintic compounds. Several studies have already long ago demonstrated an increased tolerance or resistance to medications commonly used to eliminate parasitic worm infections (De Clercq et al., 1997; Ismail et al., 1996; Reynoldson et al., 1997). Resistance in this field can be dangerous, considering the already limited collection of pharmaceutical remedies. Inadequate drug efficacy implicates a rise in the severity and prevalence of human helminth infections, which will ultimately result in a greater disease burden. Therefore, there is an urgent clinical need for novel anthelmintic compounds, and drug discovery efforts should be prioritized.

Helminthiases and agriculture

Parasitic worms commonly infect ruminants such as cattle and sheep, and are among the leading factors that limit production in the agricultural industry (Charlier et al., 2016; Epe & Kaminsky, 2013; Shalaby, 2013; Strydom et al., 2023). Helminthic diseases in livestock often result in reduced weight gain and decreased milk production, ultimately disturbing resource quantity and quality and impeding economic growth (Charlier et al., 2014, 2016; Shalaby, 2013). The pervasiveness of helminth infections provided support for the prophylactic use of anthelmintic compounds to maintain animal health; however, consistent and continued treatment facilitates the development of drug resistance. For many years, the conventional practice was to treat healthy animals once every few months but this quickly led to the rise of resistant worms (Kaplan, 2013). Currently, the control of helminth infections is severely hindered by the high frequency of anthelmintic resistance and a lack of novel therapeutics, which costs some of the major cattle producers billions of dollars each year (Strydom et al., 2023).

Crop damage due to parasitic nematodes is another concern in the agricultural industry. In the 1990s, plant helminths caused an annual reduction of primary fibre and food crops by about 12%, corresponding to a yearly loss of nearly 80 billion USD at the time (Barker et al., 1994; Taylor et al., 2013). However, this value was likely underestimated, as plant damage responses are difficult to assign

to a specific pathogen unless the causative agent can be isolated (Barker et al., 1994).

Previously, pest management in plants involved soil fumigation using methyl bromide, which has a broad spectrum of activity against fungi, insects, and nematodes (F. N. Martin, 2003; Zasada et al., 2010). However, due to the role of methyl bromide in ozone depletion, its use as a pesticide has dramatically decreased, resulting in an increased reliance on the same arsenal of commercially available anthelmintic compounds to which resistance has already developed (F. N. Martin, 2003; Taylor et al., 2013; Zasada et al., 2010).

Current treatment options

Treatment limitations remain a major barrier to the eradication of parasitic worms in plants and animals, including humans. There is likely to be a very low financial return associated with the development of anthelmintic drugs, which decreases the amount of available funding and effort generally required for the drug discovery process (Cheuka et al., 2016; Hotez et al., 2008b; Pedrique et al., 2013). As such, the past four decades have not been particularly fruitful in the realm of discovering new anthelmintic drug classes, thereby increasing reliance on currently available treatments (Kaminsky et al., 2008; Kaplan, 2013). Many existing research routes also prioritize repurposing existing therapeutics, rather than discovery, which is not conducive to novel chemicals reaching the market (Cheuka et al., 2016; Panic

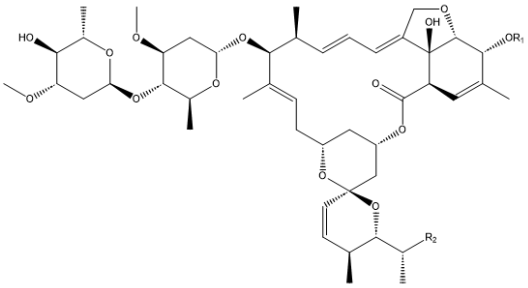
et al., 2014; Weeks et al., 2018). Currently, there are three main classes of anthelmintic agents approved for use in humans: macrocyclic lactones, benzimidazoles, and imidazothiazoles (Kaminsky et al., 2008).

Existing anthelmintic compounds

Macrocyclic Lactones

Background

The avermectins, the first of the macrocyclic lactone class, were isolated as a fermentation product from the *Streptomyces avermitilis* bacteria in the 1970s (Burg et al., 1979; Miller et al., 1979; Õmura, 2008). The crude natural product extract was found to contain four homologous pairs, totaling eight avermectins (Pitterna et al., 2009). Each pair has both a major and minor component, represented as “a” and “b”, respectively (Pitterna et al., 2009). The structures of each of the eight natural products are shown in Figure 1. Avermectin B1, also known as abamectin, is a mixture of avermectin B1a and B1b and is the major fermentation product from the bacteria (Dybas, 1989; Lasota & Dybas, 1990; Pitterna et al., 2009).



Avermectin	X-Y	R_1	R_2
A1a	CH=CH	CH_3	C_2H_5
A1b	CH=CH	CH_3	CH_3
A2a	CH_2-CH(OH)	CH_3	C_2H_5
A2b	CH_2-CH(OH)	CH_3	CH_3
B1a	CH=CH	H	C_2H_5
B1b	CH=CH	H	CH_3
B2a	CH_2-CH(OH)	H	C_2H_5
B2b	CH_2-CH(OH)	H	CH_3

Figure 1: The core 16-membered macrocyclic lactone structure and the respective structures for each of the eight avermectins isolated from S. avermitilis **(**Pitterna et al., 2009; Zhuo et al., 2014).

This was a remarkable discovery by the Merck Institute after a successful collaboration with the Kitasato Institute in Japan, where scientists were working towards the discovery of novel microbial fermentation products (Campbell, 2012). Isolates from actinomycetes bacteria that lacked antibacterial properties were sent to Merck for their anthelmintic screening program, which ultimately led to the discovery of the first avermectin (Campbell, 2012).

Activity and mode of action

Avermectins have been widely used in agriculture for crop protection and treatment of helminth infections in cattle due to their extreme potency against a wide range of nematodes (Benz & Cox, 1989; Dybas, 1989; Egerton et al., 1979; Lasota & Dybas, 1990; Williams et al., 1992). In both human and veterinary medicine, avermectins are relied on for the elimination of parasitic worms (Laing et al., 2017). Since their discovery, a variety of marketable derivatives have been created, with the most successful being ivermectin (Campbell et al., 1984; Õmura,

2008). Ivermectin is a semi-synthetic derivative of avermectin B1 with a higher therapeutic index and increased antiparasitic potency (Õmura, 2008). It was originally thought that avermectin activity was restricted to helminths, arachnids, and insects, and that bacteria, fungi, and protozoa were unaffected (Õmura, 2008; Zhuo et al., 2014). However, some studies have demonstrated the antimycobacterial properties of different avermectins (Lim et al., 2013; Zhuo et al., 2014). Specifically, Lim et al. (2012) showed ivermectin activity against four *Mycobacterium tuberculosis* strains, but inhibitory effects of the drug against Gram-negative or Gram-positive bacterial species remained negligible (Lim et al., 2013).

The anthelmintic activity of avermectins is attributed to their ability to bind to glutamate-gated chloride (GluCl) channels. These ion channels are found only in invertebrates and regulate muscle contractions involved in feeding and movement (Õmura, 2008; Wolstenholme, 2012). Normally, the binding of glutamate produces a rapid channel-opening effect for quick contractile movements (Wolstenholme & Rogers, 2006). Treatment with a macrocyclic lactone compound results in non-competitive binding of the drug to a glutamate-gated ion channel. This interaction maintains the channels in an open configuration, causing a slow flux of chloride ions, thereby preventing further signaling, which induces paralysis (Õmura, 2008; Wolstenholme & Rogers, 2006).

Resistance to macrocyclic lactones like ivermectin has been widely reported in ruminant helminths; however, the exact mechanism or mutant gene has not yet been described in these animals (R. J. Martin et al., 2021). Some studies suggest that an increased frequency of the mutant GluCl channel alleles contribute to resistance (Fissiha & Kinde, 2021; Jayawardene et al., 2021; R. J. Martin et al., 2021). Another study reported that the overexpression of a P-glycoprotein membrane transporter in resistant worms may contribute to ivermectin resistance through drug efflux (Xu et al., 1998). Although exact mechanistic data are lacking in helminths, studies conducted with *Caenorhabditis elegans*, a non-parasitic nematode model, show that mutations in three GluCl channel genes can confer high levels of resistance to ivermectin (Dent et al., 2000). This report validates the target mutation theory, although it is possible that multiple resistance mechanisms contribute to resistance.

Benzimidazoles

Background

The second main class of anthelmintic agents is the benzimidazoles (BZs). In 1961, thiabendazole (Figure 2a) was the first of the BZ class to be used as an anthelmintic (Brown et al., 1961). It was discovered by a Merck research team led by Dr. William Campbell and served as an effective deworming agent and treatment for trichinosis (Brown et al., 1961; Merck & Co., Inc., n.d.). Prior to this discovery,

scientists were interested in the purine-like construction of the BZ core and apparent antimicrobial activity (Woolley, 1944). Since then, there has been widespread interest in medicinal uses of BZ analogs including its potential applications as an antioxidant, anticancer agent, antiviral, and anthelmintic (Banerjee et al., 2023) Currently, albendazole and mebendazole (Figure 2b-c) are more commonly used for the treatment of parasitic infections in humans (Chai et al., 2021).

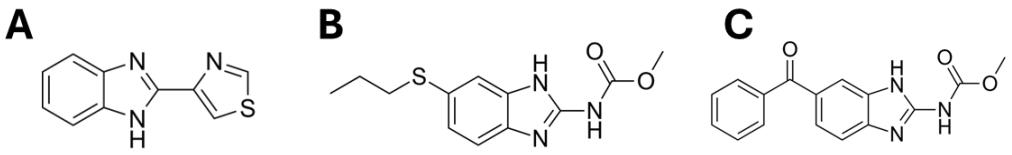


Figure 2: *Examples of benzimidazole anthelmintics***.** A) thiabendazole B) albendazole and C) mebendazole.

Activity and mode of action

Benzimidazoles are popular broad-spectrum anthelmintic compounds with very low host toxicity (Lacey, 1990; McKellar & Scott, 1990). They are the primary treatment for human soil-transmitted helminth (STH) infections and remain heavily relied upon in cases where ivermectin is not effective (Vercruysse et al., 2011). Although first introduced to treat helminth infections, the benzimidazoles are also effective antifungal agents (Allen & Gottlieb, 1970).

The biological target of benzimidazole drugs is microtubules, which play essential roles in the structure, movement, and division of eukaryotic cells (Abongwa et al., 2017; McKellar & Scott, 1990; Nogales, 2000). Microtubules are dynamic cytoskeletal polymers composed of alpha and beta-tubulin subunits (Lacey, 1990). The binding of benzimidazoles to microtubules inhibits proper cell function, especially cell division, and prevents helminth development (McKellar & Scott, 1990; Nogales, 2000). Although microtubules are present in mammalian cells, benzimidazoles have a higher specificity for helminth beta-tubulin and tend to accumulate more readily in worms (Lacey, 1990). This disparity stems from pharmacokinetic variations of the drug in the two organisms rather than a higher affinity for helminth beta-tubulin (Lacey, 1990).

Resistance to benzimidazoles can arise from a single amino acid substitution at residues 167, 198, or 200 in the beta-tubulin gene (Fissiha & Kinde, 2021; Martínez-Valladares et al., 2020; Samson-Himmelstjerna et al., 2007). This observation is consistent with studies conducted in *C. elegans* where mutated beta-tubulin variants result in benzimidazole resistance (Driscoll et al., 1989). Since resistance can easily emerge from a single point mutation, it poses a significant challenge to effective long-term control of infection.

Imidazothiazoles

Background

The imidazothiazoles are less commonly used than the macrocyclic lactones or benzimidazoles (R. J. Martin & Robertson, 2007). Imidazothiazoles were discovered in the 1960s through testing of chemical compounds in helminth-infected chickens and sheep (Raeymaekers et al., 1966; Thienpont et al., 1966). Researchers identified a compound, thiazothienol, that was active as an anthelmintic in chickens and sheep, but not in rats or mice (Raeymaekers et al., 1966; Thienpont et al., 1966). This finding led to a study of the metabolic products excreted by these animals to determine if perhaps thiazothienol was converted to an anthelmintically active metabolite in sheep and chickens, but not murines (Raeymaekers et al., 1966). The group eventually found thiazothielite, a compound excreted by chickens treated with thiazothienol, that was active against worms in all animals they treated, including mice and rats. Chemical modification of thiazothielite to improve its efficacy led to the eventual discovery of an even more potent substance, which was later developed into tetramisole (Figure 3), the first anthelmintic of the imidazothiazole class (Campbell, 2005; Raeymaekers et al., 1966).

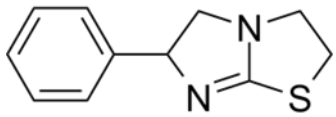


Figure 3: Chemical structure of tetramisole, the first imidazothiazole anthelmintic.

Activity and mode of action

The most frequently employed compound in this class is levamisole, an isomer of tetramisole, which is a broad-spectrum nematicide primarily used in the treatment of intestinal helminths (Wolstenholme et al., 2004). Levamisole's immunomodulatory properties also allowed for its application as an anti-cancer drug; however, detrimental immune effects have limited its use to veterinary settings (Artwohl et al., 2000; Clarke et al., 1997).

Similar to the macrocyclic lactone anthelmintics, imidazothiazoles act by paralyzing worms, thereby facilitating removal by the host immune system (Abongwa et al., 2017). However, rather than interacting with glutamate-gated chloride channels, these cholinergic compounds are agonists for nicotinic acetylcholine receptors (nAChRs) (Selzer, 2009). Binding of an imidazothiazole to an nAChR results in activation of the excitatory receptors and causes a spastic paralysis state (Holden-Dye & Walker, 2005).

Levamisole resistance is facilitated through mutations in nAChRs, where shortened forms of the target receptor are linked to decreased drug sensitivity (Boulin et al., 2011; Fissiha & Kinde, 2021; Sarai et al., 2015). Similarly, in *C. elegans*, nAChR mutants confer resistance to levamisole (Culetto et al., 2004; J. T. Fleming et al., 1997)

Natural products as a source of new anthelmintic agents

Natural products have already proven to be rich sources of various pharmaceutical compounds such as anti-cancer drugs, antibiotics, and antibiotic adjuvants (Clark, 1996; Dias et al., 2012; A. Fleming, 1929; King et al., 2014). Penicillin was an antibiotic discovery that revolutionized the field of medicine and has been a popular example of the success of natural sources since it was isolated from a fungus (A. Fleming, 1929). Then, as resistance to antibiotics emerged as a major clinical concern, natural products again proved to be important sources of compounds that enhance activity in resistant organisms. For example, aspergillomarasmine A, an antibiotic adjuvant, was derived from a natural source (King et al., 2014; Melander & Melander, 2017).

In the field of anthelmintics, the threat of resistance is no less prominent, as helminths are rapidly desensitized to all currently available treatments, especially in agricultural settings (Prichard, 1994; Shalaby, 2013). To combat this problem, more efforts should be targeted towards anthelmintic drug discovery. Specifically, the

bioactive potential of natural products could be further exploited. While mining natural products for anthelmintic activity has been a more prominent research avenue in recent years, there is a heavy bias towards plant natural products (Fahs et al., 2025; Garcia-Bustos et al., 2019; Jayawardene et al., 2021; Liu et al., 2020). Microorganisms, such as bacteria and fungi, also have a long history of producing clinically relevant chemicals (Patridge et al., 2016), and this area should be explored for nematicides. The avermectins offer a prime example of the anthelmintic potential of microbial natural products, underscoring the importance of investing greater efforts into this avenue of discovery.

Using *C. elegans* for compound screening

A major hurdle associated with anthelmintic discovery is the absence of a widely established screening protocol. While there are several published assays exist for determining anthelmintic activity, these assays vary significantly in the type of phenotypic readout. Some potential outputs include motility, egg laying/hatching, and larval development (Herath et al., 2022; Moy et al., 2009; Zamanian & Chan, 2021). There is also no gold standard for primary screening approaches, and studies will vary from *in vivo* animal-based testing to *in vitro* phenotypic methods (Herath et al., 2022; Jayawardene et al., 2021). To remedy this gap, there needs to be an agreement on a screening protocol tha strikes a balance between cost-effectiveness and therapeutic relevance.

Parasitic worms often have very complex life cycles, which can complicate the process of testing potential anthelmintic therapeutics for drug discovery. It is therefore more feasible to conduct compound screens with an established model organism such as the nematode *C. elegans* (Bürglin et al., 1998; Burns et al., 2015a). *C. elegans* are free-living, non-parasitic nematodes, but can serve as a model system in which to detect nematocidal compounds (Burns et al., 2015a). Additionally, their small size, short generation times, and large brood sizes are favourable for high-throughput screening (Burns et al., 2006; O'Reilly et al., 2014). These attributes help reduce experimental costs and reagents, thereby increasing the feasibility and scalability of compound screening.

Despite lacking mechanisms required for parasitism, *C. elegans* shares many structural similarities to parasitic nematodes and belongs to the same phylogenetic clade as several highly prevalent helminths (Coghlan et al., 2019). The majority of anthelmintic compounds are also active against *C. elegans* and these nematodes have already proven to be useful in elucidating the mode of action of currently employed anthelmintics (Burns et al., 2015b; Holden-Dye & Walker, 2018; Kaminsky et al., 2008). Therefore, *C. elegans* is a valuable surrogate for screening and mechanistic studies and should be more widely integrated into early-stage anthelmintic discovery pipelines.

Central hypothesis and summary of study aims

To positively contribute to helminth control, a standardized screening protocol for nematocidal compounds is necessary; preferably, one that can be scaled and optimized for natural product screening. With microbial natural product extracts, it can be challenging to obtain a large supply due to the labour-intensive processes of microoganism cultivation and isolation. The main goal of this project was to establish a liquid-based screening method that minimizes the amount of test material required, thereby accommodating the limited quantities of starting material. We also designed our assay to maximize the information obtainable from the screen, thereby better assessing the different toxic effects against worms. Given the historical success of finding bioactive microbial secondary metabolites and the abundance of these compounds, this work may lead to the discovery of novel therapeutic molecules.

Summary of study

1. Developed a liquid-based assay using *C. elegans* to screen a natural product extract library for potentially nematocidal compounds. Assay data analysis options were compared based on ease of use and the robustness of the information provided (Chapter 2).

2. Followed up with hits from the assay and identified known anthelmintic compounds and a bioactive compound without previously reported activity against worms (Chapter 3).

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CHAPTER TWO – Development and evaluation of motility, image, and luminescence-based screening methods

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Contributions: SC performed experiments, wrote the manuscript, and made the figures.

ABSTRACT

The high global burden of helminth infections necessitates the development of effective anthelmintic drugs. High-throughput screening methods often play a pivotal role in identifying novel compounds; however, there is no gold-standard protocol for the discovery of anthelmintic compounds through screening. Here, we describe two liquid-based assays that use *Caenorhabditis elegans* to identify compounds that affect nematode viability. The first is a phenotypic-based screen that has been optimized for screening natural product extracts to address an untapped niche in the research field. We evaluated three different output methods — motility and both automated and manual image-based scoring — for their sensitivity, efficacy, and reproducibility. Of the three methods, we concluded that manual image scoring was the most sensitive but also the most labour-intensive. We also found that motility readouts are most useful for the rapid identification of extremely toxic compounds that result in lethality or paralysis. Lastly, automated image analysis using computer vision is the quickest method for flagging general abnormalities; however, our current technology may be lacking in sensitivity. Nevertheless, there is a high ceiling for technique development here that could be further exploited. The second screen we describe is a target-based nanoluciferase-dependent reporter assay for cuticle damage. Due to high variability, this was determined to be better suited as a secondary assay or for protocols where test compounds are not limited. Our overall findings suggest that while no single data

acquisition method is universally superior, each offers distinct advantages that can be strategically employed depending on the specific goals of the study.

INTRODUCTION

Helminth infections, caused by parasitic worms, remain a major public health concern, particularly in disadvantaged areas with limited resources (WHO, 2023). Although there are global programs to assist in the eradication of helminthiases, soil-transmitted infections remain a significant concern for approximately a quarter of the world's population (WHO, 2023). The total number of worm infections worldwide is likely much higher, due to limited surveillance and reporting for non-soil-transmitted helminth infections. There is also a high potential for reinfection and sustained infections, which cause long-term cognitive and health defects (Jamison et al., 2006). Despite the severity and prevalence of these diseases, research in the field of novel drug discovery and improving access to existing drugs is lacking, likely because financial return on these investments is expected to be low (Nixon et al., 2020).

Apart from their use in human health, anthelmintics are also widely used in veterinary medicine. Livestock and companion animals depend on the prophylactic administration of anthelmintic compounds, but there is a continued threat that rising levels of resistance will render the already limited arsenal of drugs completely obsolete (Nixon et al., 2020). Another bottleneck is that there is currently no gold-standard approach to screening for nematocidal compounds. For antimicrobial compound screening, organizations have recommended standard laboratory protocols for susceptibility testing (CLSI, 2024; EUCAST, n.d.). This is not the same

case for testing in nematodes. Traditionally, discoveries were made through *in vivo* screening of small animal infection models, which presented several challenges (Nixon et al., 2020; Zamanian & Chan, 2021). Most notably, these assays were very low-throughput and labour-intensive, and parasitic worms can be difficult to work with due to the need for a host. More recent work in the field has involved the use of nematode models, such as *Caenorhabditis elegans*, to improve screening throughput (Holden-Dye & Walker, 2018; Partridge et al., 2020).

There are two main approaches to screening, both of which could be leveraged for anthelmintic discovery: target-based and phenotypic/empirical screening. Target-based assays require an informed selection of a specific biological target or process for which the goal is to identify a particular inhibitor or activator. Phenotypic screens focus more on an overall desirable response to compounds, and a specific target and mechanism are elucidated later. Both methods can be beneficial; however, there is currently a lack of a universally accepted screening process for anthelmintic discovery that must be addressed. This is proving to be a challenge as research groups will spend more time on assay development and optimization rather than screening for bioactive compounds.

Since parasitic worms often have complex life cycles that can complicate assay development, *Caenorhabditis elegans*, a well-characterized and non-pathogenic nematode, can be used as a model to facilitate drug discovery (Burns et

al., 2015). Despite lacking mechanisms required for parasitism, *C. elegans* shares many structural similarities to parasitic nematodes and belongs to the same phylogenetic clade as several highly prevalent helminths (Coghlan et al., 2019). In addition, *C. elegans* have relatively short generation times and large brood sizes, making them ideal for high-throughput screening purposes.

C. elegans shares main features of the nematode body plan, such as the cuticle, with parasitic relatives (Hahnel et al., 2020). The cuticle is an essential component that serves as a physical barrier to the outside environment and directly affects locomotion (Johnstone, 1994). In nematodes, this structure is also shed and replaced between larval stages through a process called moulting (Lažetić & Fay, 2017). The enzymes responsible for the degradation and resynthesis of the cuticle during this process could serve as potentially viable drug targets. Previous work has demonstrated that disruptions to the cuticle structure or the enzymes responsible for its assembly often result in severe morphological defects or lethality (Barbazuk et al., 1994; Page et al., 2014; Peters et al., 1991). Given its crucial role in structural integrity and viability, the cuticle is a compelling target for anthelmintic compounds. This exoskeletal structure is also lacking in mammals such as humans, suggesting that off-target effects would be minimized. Taken together, this reinforces the potential for target-based discovery efforts specific to the nematode cuticle.

In this chapter, we present two complementary assays that can facilitate the discovery of anthelmintic drugs. One is a phenotypic screen that can be used to assess overall worm motility and viability in response to different compounds, either by quantifying movement or acquiring microscopic images of worms. After evaluating this assay and the various readouts for throughput, ease of automation, feasibility, and the number of hits generated from screening, we believe that an image-based phenotypic assay should be the standard for identifying general inhibitors of worm development. Our second cuticle damage reporter assay, which relies on luminescence, requires more starting material and should be reserved for use when test compounds are readily available or as a secondary screen to assist with mechanistic determination.

METHODS

C. elegans maintenance

The *C. elegans* strain used in the motility assay is the wild-type Bristol (N2) strain, obtained from the *Caenorhabditis* Genetics Center (CGC). For the luminescence-based assay, we used the PHX5152 *(sybIs5152[nlp-29p::NLuc]; unc-119(ed3))* strain, constructed by SunyBiotech. General maintenance protocols were followed as previously described (Stiernagle, 2006). Briefly, worms are maintained under monoxenic conditions on plates of solid nematode growth media (NGM)

seeded with *Escherichia coli* OP50 as a food source, as described by Sydney Brenner (Brenner, 1974). Populations are propagated at 20 °C by transferring worms onto new, seeded plates when *E. coli* OP50 is depleted.

Synchronizing C. elegans Population

C. elegans were grown on NGM until adulthood, and a standard bleaching procedure was used to collect *C. elegans* eggs (Stiernagle, 2006). To synchronize the population, eggs were resuspended in 7 mL of M9 buffer (Table 1) and incubated overnight at 20 °C on a rocker to allow the eggs to hatch and synchronize the population to the first larval stage (L1).

Natural product extract library

For our screen, we used an in-house natural product extract library called the pre-fractionated library (PFL). This is composed of crude and semi-pure (fractionated) DMSO extracts from microbes isolated from various soil samples (Cook et al., 2023). Extracts were pre-aliquoted into 96-well plates for high-throughput screening. Each plate contains extracts from 8 strains arranged in separate rows. Solid and liquid-media extracts are in columns 2 and 3, respectively, and semi-pure extracts are arranged in rows 4-11 in order of increasing hydrophobicity from left to right (Figure 1). Columns 1 and 12 were left empty for controls.

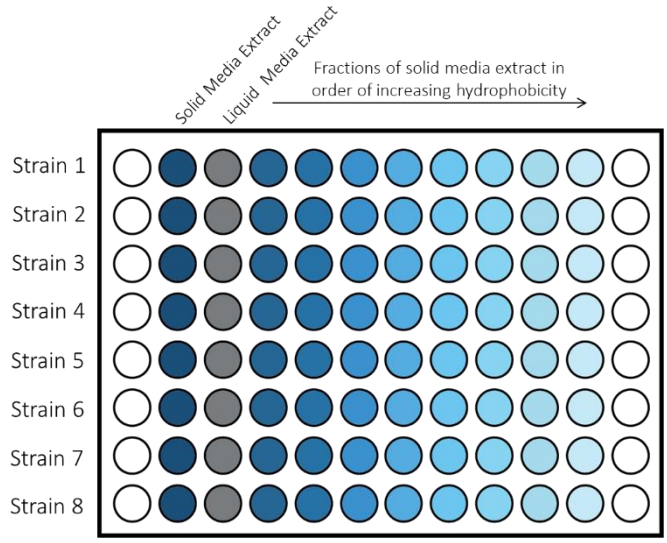

Figure 1: Outline of the arrangement of extracts in the pre-fractionated library tested in this assay.

Motility assay set-up

The screening conditions and set-up for this study have been carefully optimized for screening natural product extracts. The step-by-step protocol is detailed below, beginning with *C. elegans* population synchronization. Prior to this stage, worms were developed on NGM agar seeded with *E. coli* OP50, as outlined above, until they were gravid adults, which takes approximately three days from eggs at 20 °C. The protocol uses automation and machine liquid-handlers in the Centre for Microbial Chemical Biology (CMCB) at McMaster University. However, note that manual pipetting is also possible, although this approach will be more time-consuming and introduce additional variability.

Day 1

1. Bleach gravid N2 adult worms and resuspend eggs in M9 to hatch overnight on a rocker.
2. Prepare an *E. coli* OP50 culture by inoculating LB with a single colony. Grow overnight at 37 °C, 250 rpm.

Day 2

1. Calculate the number of hatched worms in the sample using the equation below. First, pipette 5 µL of the sample onto an agar plate and count the number of worms.

$$\text{total \# of worms} = \text{\# of worms in 5 µL} \times \frac{\text{total sample volume in µL}}{5}$$

2. Centrifuge hatched L1 worms at 1200 rpm for 2 minutes. Remove M9 buffer and resuspend worms in S-basal (Table 1) to a concentration of 1 worm/µL, as this is the sample concentration that provides optimal Worm Sorter performance.
3. Centrifuge the *E. coli* OP50 culture at 3900 rpm for 20 minutes. Remove the supernatant and concentrate in S-basal to an OD_{600} of 5.
4. Into each well of a 96-well clear flat-bottom plate, dispense 60 worms (~ 60 µL) using the COPAS worm sorter. Note that natural product extracts were tested in duplicate so two plates were needed for each PFL plate tested.

5. Add 89 µL of the concentrated *E. coli* OP50 solution with the Tempest automated liquid handler (Formulatrix).
6. Using a Mosquito low volume liquid handler (SPT Labtech), add 1 µL of PFL extract (0.6% v/v) to the designated wells, and add 1 µL of DMSO to columns 1 and 12 as a solvent control.
7. Cover the plates with a gas-permeable seal to prevent evaporation and contamination between wells. Incubate the plates at 20 °C and 150 rpm for six days.

Day 8

1. Read each plate for 30 minutes using the Wmicrotracker motility tracker (described below).
2. Image every well using an automated Nikon AZ1000M microscope (described below).

Table 1: Recipes for M9 buffer and S-basal.

Media	Ingredient	Amount
M9 buffer	Potassium phosphate monobasic (KH_2PO_4)	3 g
	Sodium phosphate dibasic (Na_2HPO_4)	6 g
	Sodium Chloride (NaCl)	5 g
	ddH_2O	to 1 L
	After autoclaving: Magnesium sulphate ($MgSO_4$), 1 M	1 mL
S-basal	Sodium Chloride (NaCl)	5.85 g
	Potassium phosphate dibasic (K_2HPO_4)	1 g
	Potassium phosphate monobasic (KH_2PO_4)	6 g
	ddH_2O	to 1 L
	After autoclaving: cholesterol, 5 mg/mL in ethanol	1 mL

Phenotypic analysis methods

Dead and paralyzed *C. elegans* have very similar phenotypes. Both result in worm rigidification and, ultimately, a lack of movement. The WMicrotracker One (NemaMetrix), which was developed to measure thrashing activity of *C. elegans* in liquid media, was used in this screen as a method of identifying wells within a 96-well plate with no *C. elegans* movement. This device works by measuring light scattering when an object passes through one of two infrared microbeams that span each well of a 96-well plate (Golombek & Simonetta, 2010). The tracker monitored movement within wells for 30 minutes, and hits were confirmed by eye to eliminate false positives.

A Nikon AZ100M microscope was used to take images of all screening plates. Using the microscope's NIS-Elements program, the microscope was programmed to capture a single brightfield image for each well of a 96-well plate using the AZ Plan Fluor 2X objective lens. Software parameters were set so the microscope would autofocus on the center of each well and take pictures of wells in a serpentine manner. With these settings, each plate took approximately 20-25 minutes to image.

This image dataset was used to develop and train a computer vision model for image classification. This was done in collaboration with the Moradi Lab at McMaster University (Wang et al., 2025).

Nanoluciferase assay set-up

PHX5152 worms were used for this assay as they contain the nanoluciferase gene responsible for luminescence. The assay set-up is the same as the motility assay except for the incubation period (3 days instead of 6). The steps for Days 1 and 2 from above can be followed exactly, and the protocol for the nanoluciferase assay continues below:

Day 5

1. Add 5 silicon carbide beads (1 mm diameter) into each well using a bead loader (Biospec Products).
2. Firmly seal plates with tape and vortex for 5 minutes to lyse worms.
3. Centrifuge each plate at 3900 rpm for 2 minutes to pellet lysate.
4. Transfer 25 µL of supernatant from each well into a new white clear-bottom 96-well plate. Add 25 µL of NanoGlo reagent (Promega N1110) in the same new plate and combine well by pipetting, taking care to avoid introducing bubbles.
5. Measure luminescence using a Biotek Synergy Neo plate reader.

RESULTS AND DISCUSSION

We developed two different approaches to facilitate the discovery of nematocidal compounds (Figure 2). The first is an image/motility-based assay designed to acquire information about overall worm viability. Image analysis can be conducted either manually or with a computer vision program that was developed. The second is a target-specific, luminescence-based assay that uses a nanoluciferase reporter to identify cuticle damage in worms. These can be used in tandem to provide a more comprehensive evaluation of compound activity or independently to address specific research questions.

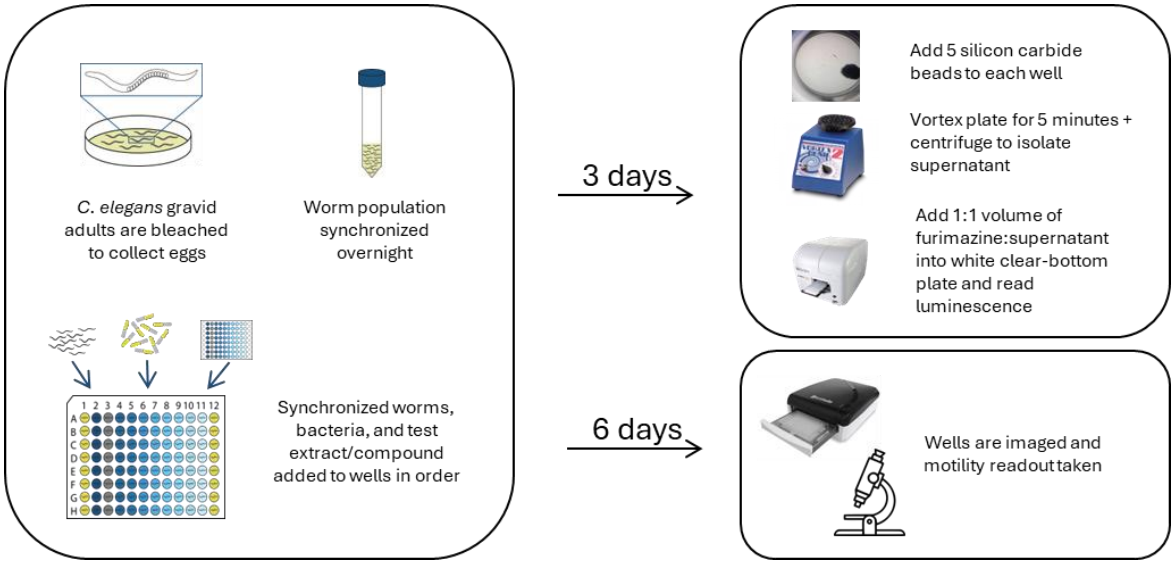

Figure 2: *Motility and luminescence assay set up.* The worm population is first synchronized, and wells in assay plates consist of worms, *E. coli* OP50, and test extract or DMSO control. For the motility assay, plates are incubated for six days and then scored for viability using a motility tracker. Wells are also imaged to facilitate phenotypic assessments. For the luminescence assay, plates are incubated for

three days, and worms are lysed by vortexing with silicon carbide beads. The substrate for nanoluciferase, furimazine, was added in a 1:1 ratio with the worm lysate supernatant, and luminescence was measured in a luminometer.

An analysis of the value of motility data versus image analyses for a phenotypic screen

For the phenotype-based assay, we first used motility as an indicator of worm viability and initially tested 384 crude and 3,072 semi-pure extracts (Supplementary Figure 1). Motility scores ranged from 0 to > 200 and represented the number of times an infrared beam passing through the well was interrupted. For extracts that caused worm death or paralysis, we were expecting to see a motility score of zero or close to zero. We set an arbitrary maximum threshold of 10, which resulted in 13 hits that reproducibly limited worm movement across screening duplicates (Table 2).

All wells of the screening plates were also imaged to manually identify any growth defects. Compounds with anthelmintic activity are known to impair worm development at sublethal concentrations (Jensen et al., 2007; Shaver et al., 2023). As such, wells with few to no offspring, unhatched eggs, asynchronous worm development, and rigidified worms were noted as containing potentially toxic compounds (Figure 3). Based on the way the PFL was assembled, there is a higher likelihood that extracts on the same plate are duplicates or more similar to one another. This is because microbial strains isolated from the same soil sample were assigned adjacent labels, resulting in their extracts being physically arrayed together

in the PFL. Therefore, for screening plates with more than five hits, we chose to exclude all of them to eliminate any potential for duplication. Once these were filtered out, we were left with a list of 12 hits, 9 of which were already identified from the motility results (Table 2). The high level of overlap is consistent with expectations as low motility values are reflective of little to no movement in wells, which would mean worms are likely dead or paralysed, and this would be easily confirmed upon visual inspection.

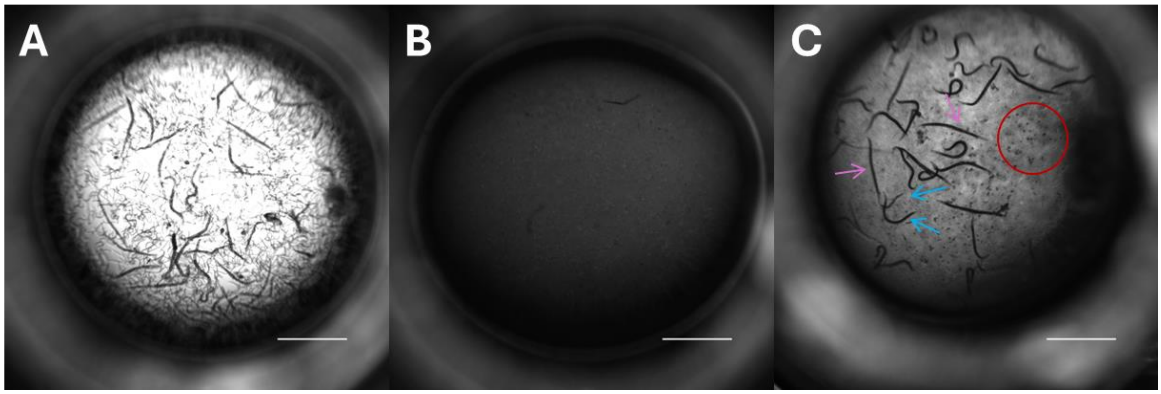

Figure 3: *Representative well images of a negative control (A), positive control or hit (B), and intermediate phenotype (C).* Annotations in C highlight relevant phenotypes that could indicate the presence of a sublethal concentration of nematocidal compounds. Unhatched eggs are featured in the red circle, pink arrows are pointing to worms that have rigidified, and blue arrows are pointing to asynchronously developing worms.

While the hits identified between motility scoring and manual image analysis are not identical, each method can provide insight into toxicity levels within wells. Low motility scores enable the immediate identification of highly toxic substances within the well, which is useful for prioritizing extracts or compounds with extremely

potent nematocidal activity. However, phenotypes that reflect mild toxicity to worms cannot be easily identified from motility scores alone. This is where manual inspection of images can be beneficial. Targeting wells where worm development appears to be impacted can reveal chemicals that affect growth or reproduction without causing immediate lethality or paralysis, and these may still hold therapeutic potential. Additionally, another factor to consider is that we are screening an extract library, and moderate effects on the worms may indicate that toxic compounds are present in sublethal concentrations. This is a significant advantage of manually scoring images, as it enables higher sensitivity and a broader range of detection for potentially toxic compounds. However, a major disadvantage of manually evaluating worms is that it can be extremely time consuming, especially when trying to score several phenotypes.

Due to the labour-intensiveness of manually scoring worms, we contacted the Moradi Lab at McMaster University to assist with generating an automated binary scoring system to quickly identify wells that were abnormal versus normal (Wang et al., 2025). Upon identifying wells that were scored as abnormal with 90% confidence and then eliminating all wells from plates with more than five hits, we identified an additional 22 hits, most of which did not correspond to positive hits from the other two scoring methods (Table 2). Despite this, manual inspection of the images flagged by the computer vision software confirms that wells contain abnormal worm phenotypes, indicating high accuracy. Lowering the confidence

threshold would result in a greater number of hits; however, it can sometimes be difficult to manually distinguish between an abnormal well with a confidence score of 90% and one with a score of 75%. Therefore, a higher score may not directly correlate with poorer worm conditions. Since the model is currently only trained to provide a binary scoring system, the discrepancy and lack of overlap in hits compared to other scoring methods may be a result of the program flagging visually distinct but less biologically significant changes. For example, some wells may have a greater number of curved or coiled worms due to their positioning at the time of imaging. The algorithm may classify these wells as “abnormal” based on the shape deviation from worms in control wells; however, these deviations would be considered insignificant in a manual inspection, as live worms are naturally curved. Regardless, this method can still be effective as a first pass to sift through large quantities of images. This automated image analysis pipeline is relatively new, and further work can be done to train the algorithm to enhance its precision and expand its capabilities.

Table 2: *Hits obtained from motility and image-based readouts.* ***Cells*** highlighted in green represent the extracts that were classified as hits from the different readouts. Strain # corresponds to a strain in our in-house soil microbe collection. Motility score was cut off at 10 to select for extracts that caused worm death or paralysis. For manual and automated image scoring, screening plates with >5 hits were completely excluded from the dataset.

Strain #	Motility <10	Hit from manual scoring	Hit from computer vision
1325			
1490			
8452			
8518			
8103			
8117			
8348			
8360			
8472			
8478			
10980			
10981			
10988			
10991			
10992			
10993			
10994			
10995			
11018			
11019			
11020			
11021			
11026			
11153			
11171			
11192			
11193			
11194			
11195			
11203			
11216			
11217			
11221			
11258			

Overall, each method of data acquisition and analysis has its own advantages and disadvantages, summarized in Table 3. Depending on the study goals, one method may be preferred over another, or a combination of methods could be used. For example, if looking for inhibitors of reproduction, an image-based analysis method may be preferred. Automated scoring can help refine a large dataset, and manual inspection can help identify which wells to prioritize for further investigation. For our follow-up work, since the automated scoring method was still under development, we prioritized motility and manual scoring results.

Table 3: A summary of the data acquisition and analysis methods, their pros and cons, and recommended applications.

Data acquired	Motility	Images – manual scoring	Images – computer vision (automated scoring
Pros	- Quick identification of dead/paralysed worms - Not labour-intensive; easy set-up	- Allows for the identification of compounds that affect development and reproduction, or sublethal concentrations of toxic compounds	- Generates results rapidly from a large dataset - Not labour-intensive
Cons	- Time consuming (30 minute read time) - Not sensitive enough to identify intermediate phenotypes	- Time consuming - Labour-intensive	- Binary scoring system; unable to identify intermediate phenotypes and confidence score doesn't correlate with severity of phenotypes
Recommended applications	Best for identifying very toxic compounds/extracts that induce death or paralysis	Best for identifying intermediate phenotypes and validating computer vision results	Best when working with large image datasets

Target-based luminescence assay

We also developed a luminescence-based assay that uses a transcriptional reporter worm strain to detect cuticle damage. The antimicrobial peptide, NLP-29, is produced by *C. elegans* in response to tissue damage, moulting defects, and developmental defects (Dodd et al., 2018; Pujol et al., 2008). We cloned the nanoluciferase gene downstream of the *nlp-29* promoter to generate a transgenic worm strain (PHX5152) that expresses nanoluciferase in response to cuticle damage. One disadvantage of this screening method is that worms must be lysed to release the nanoluciferase enzyme, as its substrate, furimazine, cannot penetrate worm tissues (Sfarcic et al., 2019). Lysis can be accomplished by vortexing the worms with silicon carbide beads; however, this introduces an additional challenge as luminescence intensity can be dependent on vortex time, and there are high levels of variability in lysis completion (Figure 4A). An explanation for this variability is that increased vortex times generate excess heat, which cannot be withstood by the nanoluciferase enzyme.

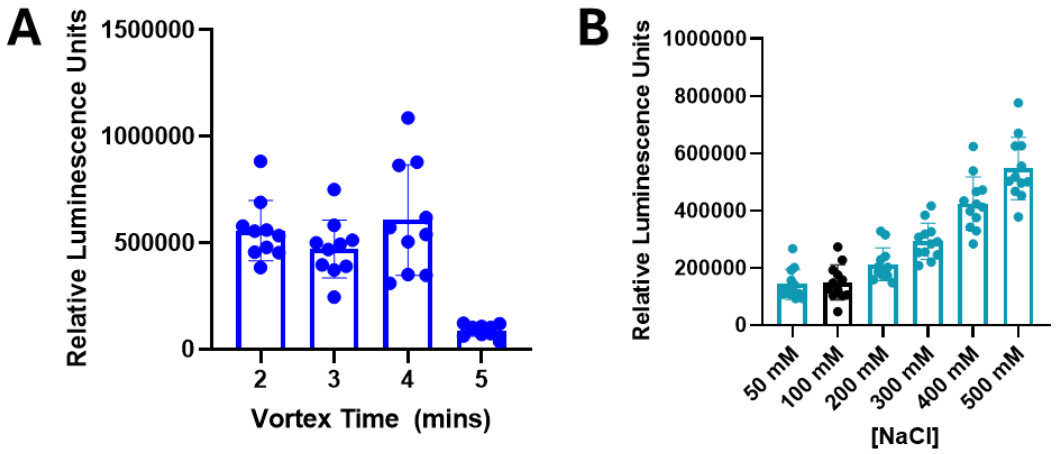

Figure 4: *Nanoluciferase assay optimization and test results*. **A**) Optimal vortex time for lysing worms in 96-well plates. B) Induction of nanoluciferase production by the *nlp-29* promoter as represented by relative luminescence units in response to varying salt concentrations. 100 mM of salt is the concentration in standard S-basal media. Each dot represents one well containing approximately 60 worms.

To assess the sensitivity of our nanoluciferase system, we tested a compound known to induce *nlp-29* expression via osmotic stress: sodium chloride. We can observe differences in high versus low luminescence levels; however, with higher induction levels, there is also an increase in variability (Figure 4B). The challenges and inconsistencies associated with worm lysis and the increased variability from promoter induction led us to conclude that this assay would not be suitable for preliminary high-throughput anthelmintic screening, especially for natural product screening when test extracts are scarce. However, since extra replicates allow for clearer distinctions between positive and negative results, we believe this can be a valuable secondary screen that may help with elucidating

mechanisms of action for identified compounds. It may also prove useful in cases where test material is in high abundance.

To summarize, we developed two distinct screening methods. While the intention was to use both methods to screen the PFL, we found that the variability associated with the nanoluciferase assay was not ideal for screening natural product extracts. Nevertheless, we have already troubleshooted and optimized the lysis method to the best of our abilities, and the assay can still be used in mechanistic studies upon identification of a hit compound. For the phenotypic assay, we suggest that image-based analysis methods can generate the greatest number of potential hits for follow-up.

SUPPLEMENTARY MATERIAL

Supplementary Figure 1: Motility data of microbial natural product extracts screened against C. elegans. Crude and semi-pure extracts were arranged in 96-well plates, and each plate was screened in duplicate. Motility output was measured using the WMicrotracker from Phylum Tech with a read time of 30 minutes. Cells are shaded in blue with darker shades indicating higher numbers.

Screening plate 1

201	206	190	204	171	202	158	132	216	188	200	206
202	138	122	208	168	136	177	170	165	188	160	184
168	170	83	176	170	165	168	194	204	155	168	162
165	181	184	186	140	152	138	154	169	200	145	150
167	188	200	192	163	192	148	196	197	204	151	150
161	201	164	142	154	114	164	177	168	168	174	164
174	188	184	172	141	184	154	190	208	174	130	166
218	208	182	209	147	155	176	199	188	169	164	188
194	204	202	216	182	226	190	210	231	212	184	199
204	202	211	204	236	181	178	162	194	154	151	186
210	196	213	210	162	161	160	184	185	161	134	89
203	202	147	237	160	143	129	158	146	188	132	108
158	204	196	180	120	174	106	210	228	148	160	86
154	204	164	191	122	106	158	161	161	206	136	80
156	160	184	154	180	186	162	188	201	175	99	198
200	184	205	204	166	106	140	184	144	152	144	182

Screening plate 2

183	187	104	204	137	140	136	150	182	174	182	176
168	165	75	126	170	38	158	140	128	162	192	168
138	208	46	180	161	180	112	106	167	120	112	118
156	192	68	126	88	87	122	144	116	136	93	176
111	150	118	117	105	216	168	154	148	147	104	97
103	125	56	156	106	90	174	116	161	189	154	86
150	110	101	126	74	162	62	146	120	134	126	126
206	144	99	166	48	91	128	138	136	140	148	181
202	210	222	197	171	210	126	120	192	176	218	202
208	185	140	155	218	96	139	162	145	192	160	200
198	221	176	152	160	211	92	200	154	168	164	150
198	154	122	177	163	130	66	146	168	182	138	176
160	128	156	184	183	193	146	134	164	190	142	168
124	176	148	182	130	47	122	136	180	190	166	95
154	136	140	153	92	170	98	182	177	179	150	168
232	198	200	210	133	154	186	170	164	192	217	200

Screening plate 3

188	196	124	232	161	178	142	188	214	182	168	192
218	151	88	172	140	50	128	140	119	139	135	178
170	150	165	168	148	179	148	122	146	154	173	159
207	173	66	122	76	105	97	156	160	181	154	171
174	132	18	52	137	169	210	194	150	155	178	200
168	177	0	148	96	64	154	164	145	188	174	182
212	160	29	52	122	176	124	156	152	183	96	170
120	182	144	139	88	132	173	144	132	213	208	195
220	209	204	202	206	181	129	162	206	186	154	192
208	176	156	160	193	48	165	120	159	192	113	170
192	177	168	109	118	202	144	152	138	176	134	104
221	196	128	124	144	124	146	148	158	148	148	188
174	154	58	146	134	198	162	198	204	179	189	192
84	148	167	238	128	54	110	176	154	195	184	200
100	144	14	153	148	142	116	199	214	188	156	94
180	172	194	213	87	88	140	171	128	186	129	172

Screening plate 4

174	155	51	176	131	158	82	152	200	204	135	198
186	156	153	162	127	100	72	102	172	174	170	156
110	182	149	150	137	146	78	104	164	196	150	99
81	180	0	39	117	106	54	162	180	168	149	198
100	156	221	88	104	135	120	180	152	172	159	118
94	138	125	194	72	96	132	152	189	172	158	137
120	106	86	114	110	119	146	148	190	206	161	148
162	196	106	172	52	96	184	202	174	192	166	182
156	211	212	146	147	194	76	183	182	149	140	198
196	136	97	139	149	52	132	130	171	178	134	146
158	152	150	164	126	196	53	148	162	176	170	204
168	175	134	189	76	142	86	190	136	171	98	138
134	212	246	183	186	218	158	124	158	158	134	112
98	156	42	157	77	94	82	106	146	172	136	122
95	153	62	124	85	92	144	158	151	147	142	94
182	174	120	148	48	84	120	191	166	196	134	168

Screening plate 5

202	201	210	198	208	218	166	202	200	198	169	208
190	0	198	196	210	189	166	164	0	203	167	174
196	192	176	204	185	172	170	196	192	216	166	171
179	0	104	188	182	131	120	160	0	178	186	184
158	210	205	211	158	185	134	174	138	197	182	185
122	198	176	178	122	142	150	164	192	172	182	180
142	172	191	198	186	156	162	170	178	185	186	192
197	208	193	198	166	152	160	196	201	206	198	184
201	194	208	189	202	186	177	142	176	186	194	215
140	0	125	142	222	138	180	100	0	186	148	157
146	222	75	163	208	190	136	184	159	132	126	151
103	0	78	230	142	122	86	90	0	178	116	132
88	152	117	214	162	206	141	160	186	182	118	106
88	144	74	168	138	85	102	130	140	197	158	178
92	122	152	224	118	140	97	100	136	140	138	60
136	186	210	156	118	138	158	168	154	166	161	218

Screening plate 6

200	213	194	170	196	198	166	182	175	180	192	182
199	130	117	114	190	152	152	142	185	202	164	175
172	180	156	165	192	204	120	169	184	176	174	182
170	135	155	216	130	108	100	142	158	165	149	203
115	191	77	192	114	194	79	166	187	171	196	171
88	174	98	108	116	104	101	154	174	196	182	188
88	111	146	134	97	148	140	154	165	172	160	174
175	214	210	124	93	88	182	190	171	191	208	196
212	203	187	240	220	210	180	206	210	211	181	195
206	177	160	191	200	137	159	150	174	176	187	172
182	188	121	180	184	188	104	173	178	178	138	146
182	188	92	203	156	148	136	164	150	201	165	166
137	184	146	179	178	170	62	176	178	188	175	158
118	164	70	86	99	85	130	130	172	178	140	140
132	124	140	184	139	137	140	208	80	179	183	176
180	188	211	228	142	80	170	196	162	194	186	185

Screening plate 7

196	186	203	222	208	206	192	200	235	218	203	200
202	170	137	218	223	194	213	194	204	213	188	205
200	210	198	232	190	206	196	208	196	201	196	168
198	182	240	204	195	181	150	191	205	220	172	192
182	176	228	220	174	195	178	194	200	190	198	186
172	202	171	213	190	173	168	214	213	211	178	190
162	220	206	206	184	194	195	211	192	194	182	200
180	209	206	220	166	155	202	164	185	214	194	203
200	177	204	224	188	204	162	180	220	184	183	200
187	184	208	204	223	178	208	182	180	200	189	192
206	204	206	200	204	198	204	197	208	177	188	187
195	214	186	240	202	200	128	179	190	192	182	191
180	210	216	223	214	190	162	190	201	210	178	187
160	210	186	215	201	188	190	185	194	214	199	162
182	202	195	206	196	207	158	192	216	206	202	184
184	184	199	200	176	184	203	216	180	226	200	194

Screening plate 8

194	192	192	203	191	226	154	176	214	192	193	192
210	215	196	197	233	194	194	180	168	214	168	184
202	212	210	216	214	188	172	188	196	198	186	188
189	216	186	244	190	200	153	171	189	200	184	184
178	220	212	244	197	192	140	189	213	199	194	174
180	214	170	200	177	182	182	180	196	210	190	172
165	182	216	220	182	194	192	199	198	206	204	172
201	214	220	216	152	165	208	210	202	198	197	194
180	197	201	218	204	214	193	173	199	200	218	208
201	206	174	208	230	210	219	190	174	192	190	198
213	203	188	210	231	222	206	182	198	188	206	192
187	218	182	217	214	212	182	170	174	175	193	200
172	206	196	224	226	231	194	197	204	184	194	204
188	216	159	213	198	190	195	190	207	220	208	178
172	208	198	205	212	217	196	214	203	191	191	195
206	210	216	220	156	224	229	205	196	188	208	224

Screening plate 9

186	180	181	192	180	227	151	178	203	176	181	194
206	192	198	190	215	166	202	158	170	186	190	184
192	210	200	225	204	209	171	174	174	180	194	193
187	222	188	218	204	203	181	168	186	186	187	200
164	204	211	230	190	197	187	200	190	194	200	180
184	210	167	212	190	158	170	186	186	216	194	198
161	198	199	192	178	207	178	207	194	194	178	183
182	202	202	218	164	157	206	216	162	186	178	200
194	170	203	220	197	210	164	171	211	182	170	184
187	184	186	214	209	168	184	174	181	191	183	166
210	214	202	221	178	210	202	192	206	206	192	180
185	223	186	216	206	202	176	172	206	208	170	182
180	204	214	211	182	206	172	187	191	186	174	172
168	211	168	224	162	148	184	192	202	218	200	185
178	188	212	215	186	186	190	184	225	196	183	174
192	198	194	223	151	160	196	166	172	194	187	165

Screening plate 10

173	136	157	214	216	218	160	188	192	170	186	208
202	183	176	190	214	183	215	172	193	206	194	207
200	206	188	173	226	221	208	172	198	184	204	220
180	209	184	198	224	215	170	200	164	162	170	216
182	200	188	224	210	237	135	181	213	186	219	218
168	206	176	194	209	177	233	166	188	222	208	179
178	196	192	220	197	200	198	184	189	173	208	190
182	190	204	206	180	192	215	196	160	178	190	187
184	191	181	196	206	213	160	160	197	176	185	216
196	178	170	179	211	190	212	182	154	194	196	206
208	192	188	200	210	208	192	171	198	192	178	184
178	200	186	214	220	196	154	183	160	134	190	214
190	211	204	226	192	222	162	180	182	188	206	199
183	200	140	187	217	198	189	198	185	220	176	213
172	195	182	199	186	200	178	170	176	166	169	182
188	196	215	208	166	164	192	182	169	200	194	187

Screening plate 11

180	188	194	203	183	216	150	151	226	178	166	188
186	194	179	189	214	178	207	176	174	181	164	186
199	219	194	211	198	194	197	194	190	174	160	184
186	189	190	225	204	204	170	174	178	183	184	182
174	202	214	229	184	187	162	190	180	194	201	209
188	188	159	204	181	162	162	148	157	222	188	191
163	176	192	210	172	209	186	184	200	179	196	194
187	210	208	216	164	172	196	200	180	182	188	172
192	209	214	226	205	220	174	184	210	188	171	211
204	190	188	220	238	172	216	178	190	206	190	190
198	206	188	220	204	200	205	187	194	186	198	198
190	204	176	216	204	184	174	190	203	201	182	200
179	209	215	223	192	193	162	184	206	212	196	193
162	214	188	209	184	158	191	132	214	215	188	189
180	177	215	232	201	184	193	219	210	206	184	179
188	198	204	212	152	166	214	198	196	192	171	188

Screening plate 12

180	134	198	235	218	210	163	202	224	208	204	202
192	214	198	202	223	154	171	202	179	204	198	206
211	222	185	182	192	220	178	189	210	180	170	173
193	225	195	205	191	201	154	192	194	222	176	187
171	193	214	208	174	170	144	186	197	157	182	188
172	171	170	204	176	148	176	198	215	214	190	193
184	188	227	206	187	184	216	215	216	208	200	174
180	192	204	223	155	172	208	214	197	214	182	194
172	186	202	236	194	201	184	196	234	206	176	188
188	196	205	193	205	170	170	199	204	218	203	158
192	194	192	210	166	170	162	198	204	205	190	199
202	222	206	218	145	146	160	192	197	212	184	200
173	192	224	188	164	166	143	191	195	202	164	162
171	180	192	190	143	130	172	180	188	212	189	190
165	203	226	161	151	158	150	204	194	186	199	172
182	212	178	188	144	168	206	212	184	199	181	184

Screening plate 13

190	190	198	199	205	212	164	168	219	178	174	208
214	158	199	194	232	180	196	186	170	201	194	192
204	223	194	0	198	207	181	204	188	179	205	188
200	178	171	0	198	200	188	176	178	210	200	182
166	214	224	182	216	194	163	165	191	204	196	174
192	207	174	186	188	188	178	190	186	228	190	199
160	212	197	105	198	197	181	213	139	192	201	175
212	210	223	218	164	179	192	194	152	200	184	190
184	228	200	218	196	207	196	168	210	200	170	190
218	165	142	195	206	138	188	170	182	222	187	178
208	199	168	0	123	196	135	170	176	198	196	189
188	151	166	0	155	156	186	201	174	216	172	192
186	211	202	159	132	138	184	196	214	208	212	200
165	209	168	148	150	120	165	208	184	217	198	196
168	202	228	74	132	184	174	210	202	210	206	166
196	203	218	184	160	146	186	218	192	212	204	176

Screening plate 14

192	202	195	111	188	202	179	208	186	184	178	178
146	146	154	16	179	180	208	148	171	194	188	176
164	213	116	117	157	168	142	166	173	191	188	190
178	107	138	12	158	130	123	164	163	222	184	214
138	202	174	1	170	181	144	144	188	206	196	140
144	227	104	95	122	102	122	156	186	216	194	139
146	160	200	0	106	156	133	170	177	153	158	202
150	145	116	0	115	130	183	205	196	194	164	176
202	184	206	133	204	240	178	211	214	216	234	208
220	180	216	6	237	129	157	178	194	194	190	164
181	212	139	69	202	184	156	188	172	200	218	188
202	170	202	0	184	168	152	192	161	168	202	210
158	220	164	13	192	200	174	151	188	202	188	204
145	196	112	99	190	143	156	186	121	221	223	210
162	172	207	11	194	210	188	184	189	197	210	224
229	222	220	81	192	246	234	218	202	244	214	224

Screening plate 15

206	206	216	246	229	238	184	184	232	223	217	206
206	202	224	43	245	178	188	166	184	186	194	174
228	215	228	148	180	223	133	182	209	216	200	222
208	224	207	244	199	182	202	154	202	218	196	224
209	250	231	208	202	205	162	194	210	199	194	198
182	229	178	159	194	185	165	202	209	228	208	186
172	220	248	171	214	232	204	222	214	244	198	197
234	227	232	224	166	198	202	233	210	194	205	196
202	188	196	224	194	239	130	193	208	226	180	208
216	186	198	90	200	172	167	164	182	220	208	210
226	208	174	183	208	193	194	152	170	176	204	214
216	236	178	188	172	216	130	132	162	155	204	218
192	217	164	175	170	164	174	192	173	177	195	202
182	222	141	224	173	168	206	174	164	168	202	192
165	204	188	125	156	193	196	180	180	148	182	188
186	222	218	219	173	192	196	211	165	207	208	223

Screening plate 16

178	171	198	218	198	234	176	172	220	194	188	214
213	197	222	210	246	145	207	178	168	208	154	222
204	221	225	184	213	208	164	194	200	168	172	204
224	229	202	218	198	190	172	177	199	210	178	206
173	250	241	211	217	197	176	198	212	206	196	204
177	224	188	210	198	164	169	188	210	223	180	198
181	187	215	220	210	206	186	196	216	180	191	202
197	219	229	236	165	175	178	209	198	194	198	202
214	212	204	220	212	229	202	202	223	222	222	192
221	220	204	178	238	198	218	190	204	232	216	192
216	224	162	63	218	220	175	182	216	202	198	210
208	196	176	248	181	216	186	168	187	173	206	190
197	228	197	211	206	229	196	145	218	221	197	224
190	230	179	236	184	170	130	193	200	207	167	202
193	197	210	214	168	206	214	190	212	217	208	192
206	192	242	106	171	225	146	210	210	224	208	222

Screening plate 17

222	201	196	207	214	223	166	204	228	188	218	191
206	180	214	6	210	188	154	198	211	208	192	200
200	173	206	196	182	73	184	204	180	189	164	200
187	160	176	0	176	208	180	182	170	204	178	222
198	174	176	102	184	214	156	189	221	184	164	180
158	215	107	165	187	197	178	198	192	177	194	191
182	185	190	156	176	200	172	154	181	148	200	198
208	207	230	107	134	188	224	180	192	210	188	223
222	200	212	209	108	234	184	202	216	209	174	202
219	191	188	9	204	213	222	196	203	227	194	208
201	167	188	231	220	28	150	212	175	174	209	196
226	132	176	0	172	160	158	180	157	188	192	202
198	186	150	85	152	200	173	176	217	200	212	188
154	205	78	158	128	142	178	173	162	214	199	189
178	157	192	158	149	194	137	172	164	151	197	206
199	223	231	62	152	186	200	203	199	216	167	192

Screening plate 18

202	212	214	232	190	238	134	184	214	166	192	192
225	174	138	147	226	162	189	142	200	217	176	140
198	210	184	204	180	202	126	192	195	216	159	192
223	221	170	167	217	224	152	112	187	209	210	236
186	226	178	218	204	214	128	162	152	220	194	186
185	144	171	150	172	176	211	156	202	140	165	186
172	212	200	175	163	196	174	180	174	192	195	202
214	212	218	251	180	197	200	198	195	208	218	195
223	212	194	226	180	211	164	186	202	226	196	202
196	204	97	182	236	180	200	118	180	192	198	181
176	200	130	187	188	189	174	142	176	177	186	202
164	199	180	4	162	198	184	194	176	204	197	209
202	150	190	205	176	214	160	190	200	199	220	182
178	185	86	160	152	168	194	190	190	190	204	220
168	176	228	229	178	190	158	177	182	210	184	210
208	220	226	216	172	174	207	219	211	196	222	202

Screening plate 19

218	203	210	120	221	211	186	236	184	224	172	197
241	218	218	140	218	190	202	216	180	226	202	201
194	240	195	216	174	208	168	208	197	212	200	203
208	232	235	239	203	186	172	190	164	218	168	204
194	220	226	124	194	218	160	192	210	221	210	198
170	231	194	50	190	161	164	160	230	226	206	188
184	180	224	73	182	199	210	221	210	207	202	198
222	210	224	220	154	175	224	230	206	230	215	212
196	206	218	89	200	219	187	198	219	206	171	219
218	191	202	214	232	194	180	200	191	218	204	180
217	218	216	222	214	196	211	164	204	194	208	215
221	203	199	244	216	184	178	151	188	180	162	211
184	242	250	223	186	204	182	188	214	218	214	174
181	238	182	74	192	186	190	198	208	226	188	193
178	194	216	164	191	185	194	168	218	214	174	176
211	203	210	234	173	158	194	211	192	212	197	204

Screening plate 20

166	188	168	138	144	206	144	162	190	146	195	194
178	166	162	178	212	196	206	185	174	170	202	198
209	220	185	201	210	207	186	190	188	178	188	193
180	205	184	204	200	206	161	178	162	186	182	172
171	194	196	220	190	222	188	181	182	188	156	177
177	212	188	186	188	178	168	196	174	189	170	148
162	198	217	208	202	230	184	192	188	176	225	178
174	212	208	202	168	215	206	158	159	182	190	172
134	168	198	216	201	207	148	176	187	162	182	206
190	214	196	204	214	205	216	173	182	202	218	212
187	202	190	212	218	201	200	180	194	188	200	214
156	218	198	208	222	202	196	184	192	216	217	197
172	206	209	225	216	232	196	195	134	202	222	214
129	198	166	208	220	174	192	180	206	212	213	207
177	213	213	217	204	234	212	210	207	190	209	206
172	212	198	212	179	184	209	160	170	199	224	180

Screening plate 21

158	192	205	198	202	222	174	176	198	194	183	204
176	198	178	226	236	130	208	198	200	206	195	216
194	198	177	176	238	200	208	182	172	188	198	198
184	208	174	210	209	121	164	177	173	196	194	220
198	210	150	174	228	191	190	175	194	180	216	208
169	204	106	152	210	130	192	184	206	216	225	225
172	203	184	170	195	198	188	212	184	210	214	196
186	162	208	194	146	188	220	194	174	210	200	180
180	178	204	204	204	204	170	152	211	191	195	203
176	180	201	194	242	162	200	160	174	210	200	210
170	219	168	182	231	196	160	152	183	198	196	216
181	200	153	216	198	124	190	160	136	204	190	221
157	166	214	200	171	209	185	202	181	200	182	211
164	218	148	181	183	98	164	174	180	196	206	215
170	196	185	184	182	152	172	171	187	202	181	210
173	176	232	230	173	198	222	209	191	209	200	184

Screening plate 22

205	182	205	207	178	222	162	186	212	172	165	202
216	200	182	199	204	146	179	174	184	210	172	182
212	212	190	206	158	192	134	171	152	198	189	188
190	212	187	2	165	173	165	172	168	196	198	194
165	212	179	144	162	205	144	191	156	192	212	184
160	197	171	126	151	128	146	176	176	206	196	195
164	188	201	157	140	156	140	155	221	197	196	176
191	189	210	226	134	153	188	212	177	196	179	206
204	194	210	213	195	220	134	198	218	189	148	211
200	198	120	43	110	89	63	99	166	186	172	178
167	164	128	147	155	164	132	117	144	148	141	123
174	132	133	6	140	121	134	159	139	150	120	130
164	145	108	104	138	170	164	171	122	154	136	129
143	143	78	34	166	92	161	188	178	142	117	136
156	188	92	102	102	154	148	140	176	107	118	150
206	200	174	185	125	128	90	113	173	196	182	182

Screening plate 23

198	188	203	137	194	209	136	194	192	170	202	197
220	177	195	168	213	160	190	194	156	192	155	184
226	186	182	190	142	156	162	196	187	203	202	182
210	208	140	183	159	147	152	140	164	194	168	187
165	206	166	120	153	178	142	193	175	179	186	174
174	208	128	186	139	136	160	174	172	216	185	204
162	197	174	146	136	158	155	192	182	201	184	172
212	190	200	77	178	168	182	212	178	216	194	191
194	187	190	168	186	216	138	140	187	115	192	206
203	181	200	177	226	170	190	178	193	178	164	196
191	198	176	202	208	193	176	213	178	192	194	185
203	186	181	200	199	193	172	195	170	190	172	201
174	195	220	174	190	186	194	196	211	212	218	182
186	200	178	215	177	150	190	197	197	200	166	202
176	190	206	213	180	208	178	189	205	192	187	179
200	196	216	204	154	190	204	209	172	202	206	180

Screening plate 24

193	200	201	213	198	240	180	196	222	202	193	192
201	189	165	14	214	189	182	186	161	192	206	188
187	208	183	130	208	130	174	142	201	198	214	232
178	180	138	16	180	172	184	150	149	204	199	196
152	196	150	164	172	201	180	175	152	180	200	194
166	180	126	154	172	151	70	155	168	192	199	196
174	170	41	159	158	203	162	182	166	198	202	196
198	168	205	194	163	174	186	218	186	208	210	224
198	180	190	202	193	194	137	174	204	180	175	174
205	210	161	34	200	186	204	178	188	210	190	166
188	190	168	70	166	236	186	144	174	184	198	182
183	138	183	76	192	184	173	160	174	191	198	202
171	212	29	151	174	158	157	172	184	180	200	198
158	200	132	151	186	146	158	139	170	158	194	200
188	178	165	150	152	156	154	144	134	190	166	180
188	195	196	214	164	188	208	210	178	210	194	190

Screening plate 25

188	196	190	200	188	218	168	182	222	198	139	227
196	200	198	40	197	171	171	114	178	201	192	194
195	196	194	180	176	139	167	180	178	175	200	181
188	184	190	146	148	188	166	184	162	189	173	207
150	116	160	17	152	166	196	198	172	166	188	196
166	200	160	168	164	146	178	160	164	193	188	200
170	188	155	108	150	152	146	138	188	186	194	186
204	202	194	218	169	198	218	202	204	217	192	201
184	200	179	214	187	206	167	168	191	164	191	190
198	172	184	181	232	184	186	172	165	210	199	194
204	202	172	162	138	206	172	179	185	194	210	194
170	158	109	135	183	189	160	112	132	203	196	210
181	107	153	50	158	198	196	174	146	193	194	186
176	202	138	154	136	120	180	166	162	167	184	182
182	192	167	127	24	138	106	148	189	203	180	174
202	220	152	158	172	164	202	208	168	192	169	207

Screening plate 26

188	208	188	138	201	218	158	176	209	202	208	214
222	194	186	122	210	200	208	194	192	194	192	196
218	222	190	212	197	202	183	184	188	176	184	204
204	178	178	235	166	170	146	162	162	171	154	198
207	202	206	202	158	192	152	186	168	188	189	193
187	202	144	173	186	156	180	158	164	138	98	172
201	190	218	200	160	178	175	174	196	157	200	202
192	207	222	242	171	154	206	176	198	210	192	236
173	178	200	218	178	213	154	207	230	190	180	192
172	186	207	92	214	192	212	150	179	218	208	190
180	206	197	182	187	202	192	192	200	189	210	190
188	174	152	194	179	214	157	174	178	181	182	180
174	199	168	187	158	196	164	166	124	185	152	194
168	146	144	151	148	161	173	152	176	188	154	192
163	184	197	188	163	168	183	153	180	111	186	196
178	188	214	222	167	174	204	194	180	220	187	200

Screening plate 27

160	154	190	178	210	210	183	199	214	201	200	196
178	170	187	178	232	176	206	161	181	222	192	218
188	222	196	146	186	212	150	170	179	186	236	196
183	226	194	196	205	190	164	156	188	226	161	194
178	188	201	204	181	210	155	165	133	204	186	186
188	197	158	0	179	162	160	178	194	210	186	211
170	190	202	212	188	192	192	182	192	192	190	204
202	189	222	214	178	190	224	205	180	210	215	208
163	154	177	200	200	209	163	193	210	204	204	210
182	162	173	195	220	192	218	152	104	200	152	187
170	205	202	150	195	204	194	125	191	214	163	198
172	200	187	188	190	178	147	178	184	186	186	190
159	178	196	225	192	188	184	176	177	190	204	190
151	203	138	0	192	158	184	150	182	185	160	204
175	193	216	193	171	195	184	170	198	184	188	186
193	186	203	215	182	173	201	209	183	188	214	192

Screening plate 28

176	198	218	217	203	208	157	178	218	213	212	207
222	162	216	215	248	198	211	187	174	206	220	206
230	222	228	222	208	216	200	194	186	202	210	209
198	218	174	208	219	200	168	164	174	194	206	220
172	228	219	206	207	236	170	186	188	200	194	216
180	212	178	206	204	179	176	192	178	214	212	210
174	194	214	225	194	192	194	210	222	213	208	204
208	217	249	226	178	190	234	214	216	222	225	230
182	207	204	193	206	226	176	186	191	216	255	208
212	210	197	207	232	218	215	200	179	202	198	188
210	186	216	172	221	222	204	210	192	183	207	207
220	107	188	208	204	209	164	182	187	199	220	212
175	227	226	206	208	188	180	185	182	192	210	212
196	221	166	207	188	210	185	174	214	224	206	210
184	182	220	224	196	222	186	208	220	213	195	193
230	200	170	238	180	196	204	238	210	215	206	242

Screening plate 29

191	192	215	210	216	230	175	199	209	218	222	210
194	194	218	214	244	189	214	210	176	232	206	186
213	240	202	224	220	222	161	202	205	173	217	208
216	235	198	245	206	198	179	184	186	204	202	216
171	221	194	168	209	226	169	191	194	198	205	200
202	214	176	174	215	186	188	162	159	192	202	197
194	186	199	68	191	220	211	211	193	192	187	187
216	220	250	71	171	193	226	216	212	201	210	230
206	226	216	214	228	240	166	196	212	220	211	215
226	189	201	144	212	194	188	144	203	205	192	198
204	210	209	101	196	166	176	131	154	157	170	206
208	217	174	186	153	189	124	169	172	222	199	208
184	124	87	48	200	174	156	184	164	160	176	206
167	164	73	182	202	112	162	128	150	166	160	192
198	127	172	30	122	186	178	184	116	144	194	188
220	160	170	126	176	189	212	224	200	190	220	220

Screening plate 30

214	192	208	208	220	219	192	196	205	203	187	207
190	197	204	210	246	228	240	206	208	227	220	202
206	224	203	222	219	216	199	202	202	203	197	201
212	241	192	242	207	210	184	186	192	222	196	220
178	216	232	218	206	213	174	184	215	206	207	207
178	215	192	234	207	191	156	188	184	210	210	210
178	196	219	228	189	171	186	198	128	164	199	188
198	196	232	230	196	192	210	232	191	220	216	218
180	182	215	205	212	218	191	168	202	176	168	192
201	215	196	198	230	219	224	192	200	206	194	213
206	216	201	224	236	230	212	207	214	199	231	207
206	220	206	234	236	230	190	196	178	203	226	216
176	216	222	234	200	213	180	187	206	195	214	216
165	225	182	209	222	192	196	209	210	218	210	222
158	202	210	230	209	216	209	200	190	214	214	226
208	192	238	223	182	186	219	213	193	224	212	202

Screening plate 31

164	194	219	206	192	220	182	192	186	182	199	175
180	183	218	222	228	192	230	165	171	203	202	173
202	210	216	198	229	216	190	182	184	170	188	206
208	216	184	224	196	208	176	178	192	197	192	225
188	225	207	212	184	210	152	204	192	203	188	188
182	208	148	178	196	169	162	200	193	219	178	215
161	192	222	200	193	196	192	206	196	198	194	200
210	200	206	222	160	180	207	206	188	186	181	184
163	166	202	221	206	206	164	194	192	204	200	228
188	192	209	214	245	193	216	187	172	205	200	196
208	197	212	160	237	215	196	207	192	188	194	204
194	219	180	237	192	189	164	180	180	214	181	209
188	206	216	214	200	228	191	203	186	188	194	210
182	190	168	188	185	174	181	188	186	201	196	200
168	181	210	203	204	214	187	204	182	187	196	202
200	200	220	216	181	192	212	202	192	205	205	218

Screening plate 32

124	180	194	207	186	220	174	189	186	196	188	184
168	172	186	210	236	176	228	192	182	208	188	198
178	210	205	239	224	232	200	172	194	202	194	199
152	204	195	229	198	202	190	171	198	202	180	205
138	205	225	220	219	230	164	200	198	154	208	209
132	210	186	205	193	174	178	172	207	224	182	196
132	192	191	216	199	230	200	191	202	192	202	187
176	195	202	216	163	184	209	204	169	171	186	184
165	181	174	170	200	204	130	178	183	172	200	204
178	175	159	184	227	196	225	177	166	203	201	182
194	210	208	202	226	200	182	190	179	184	188	208
164	193	206	216	204	210	190	194	164	207	206	190
124	196	210	210	208	228	162	194	206	198	180	194
182	188	167	210	201	180	166	188	178	232	186	205
141	184	207	210	222	228	200	210	178	199	196	176
182	190	221	209	174	206	191	205	176	199	212	172

Screening plate 33

183	178	202	213	205	204	167	188	212	200	180	216
201	194	200	198	236	192	216	193	174	204	206	223
206	210	207	222	210	202	177	207	192	204	194	195
188	208	188	210	220	194	174	184	192	202	186	194
190	217	219	230	206	218	144	194	194	200	188	202
150	217	165	200	210	154	191	198	190	214	190	198
168	176	216	211	183	220	196	206	198	188	192	198
197	210	209	200	154	186	222	215	190	202	196	206
179	164	178	210	198	218	160	178	190	196	184	195
170	196	184	206	224	179	194	142	184	193	188	199
200	206	208	216	214	160	180	192	183	184	190	207
196	199	176	239	207	186	158	177	196	200	204	214
164	212	216	217	194	200	170	197	200	188	199	208
178	218	168	208	199	188	190	194	209	214	192	198
154	188	192	228	194	195	178	172	211	208	188	188
186	207	212	208	158	189	216	195	190	197	188	172

Screening plate 34

178	184	200	212	189	222	194	186	190	198	206	207
186	188	215	204	224	199	196	170	191	198	160	222
200	220	200	228	192	201	175	204	185	178	192	199
205	209	180	0	204	194	162	171	156	194	195	211
174	206	216	244	192	210	178	172	156	188	190	202
180	210	181	197	202	181	178	178	192	214	196	176
169	202	209	210	208	192	194	198	172	199	188	190
198	214	202	204	171	188	206	210	183	204	184	202
142	161	167	220	179	192	174	208	210	216	201	200
167	185	199	192	224	168	204	192	194	218	172	202
184	193	202	230	185	220	178	142	174	193	192	201
165	202	182	0	209	198	160	179	178	195	183	207
134	213	216	230	184	216	188	186	184	212	208	210
144	210	157	212	207	166	184	201	193	213	222	193
150	210	202	216	186	202	191	200	192	180	181	198
195	203	212	216	178	196	218	222	186	208	178	200

Screening plate 35

195	205	228	235	236	242	180	189	219	219	215	228
226	215	165	204	260	192	212	190	210	225	194	226
223	210	203	194	229	223	212	194	198	202	214	220
202	228	190	214	186	200	180	170	187	209	234	207
173	206	211	198	206	240	212	200	171	215	218	204
196	222	167	192	186	170	190	164	201	218	212	200
190	219	220	182	211	238	218	160	181	194	207	223
214	221	218	230	192	200	226	212	190	202	216	228
189	198	206	220	210	231	196	193	224	208	200	227
217	218	213	250	243	197	216	220	204	238	214	218
214	239	220	237	213	240	184	197	198	137	218	241
207	234	204	208	197	197	169	202	207	217	212	226
188	222	213	227	204	242	188	180	168	228	212	198
202	219	178	204	180	166	176	181	194	218	205	219
200	202	216	209	164	198	206	184	170	223	225	212
216	201	243	242	168	182	238	220	190	199	149	224

Screening plate 36

209	208	216	226	176	217	184	217	226	230	218	216
156	173	146	222	202	186	206	201	208	218	181	214
169	198	160	219	200	195	148	172	178	168	198	220
146	220	138	190	198	208	166	171	177	200	200	204
146	184	107	126	202	214	201	187	118	166	208	188
136	194	46	156	168	158	211	183	192	190	208	212
182	176	143	140	208	212	216	205	210	203	192	184
204	219	246	218	204	220	228	213	221	226	226	245
212	219	208	224	216	228	198	203	228	216	214	223
224	209	184	233	210	190	210	196	203	204	222	200
202	220	190	158	186	218	200	176	198	195	213	227
226	202	147	183	197	189	166	164	172	196	208	226
148	189	150	182	174	229	202	196	192	196	219	213
182	218	168	162	177	182	184	197	161	190	194	212
187	214	178	114	193	208	181	193	206	194	204	218
218	240	218	224	185	208	229	216	198	236	196	215

Screening plate 37

186	190	226	207	194	208	187	198	208	221	208	235
163	210	214	205	219	216	224	228	194	224	208	212
188	224	213	233	237	233	215	216	214	202	206	203
196	222	186	225	239	229	182	164	160	224	189	220
174	234	209	213	217	226	172	198	191	204	212	220
181	216	162	210	217	170	182	193	214	219	219	214
170	201	208	236	202	221	200	217	146	212	222	202
199	216	214	240	184	195	235	216	198	216	215	220
197	214	212	219	219	232	172	206	213	192	212	206
189	206	206	234	257	198	216	222	205	233	200	218
198	225	213	232	230	224	184	217	192	194	202	206
206	216	210	227	201	202	176	186	187	219	215	230
171	222	228	206	210	228	178	186	185	211	188	198
179	208	173	224	200	178	184	202	205	222	178	201
165	208	218	239	177	222	177	220	194	200	206	208
192	204	216	222	198	210	222	230	184	224	202	206

Screening plate 38

164	184	214	225	185	231	171	189	220	187	220	179
183	183	196	203	236	216	228	205	186	224	216	222
215	209	210	236	224	228	190	224	194	209	220	215
184	230	202	238	190	211	165	192	204	228	200	230
165	230	228	218	221	229	169	186	204	208	210	175
166	232	158	220	212	192	184	178	202	248	206	226
164	210	212	238	215	220	211	216	202	230	216	188
208	226	216	224	190	244	228	200	210	212	227	211
205	172	218	207	220	236	201	196	232	204	208	209
192	198	202	214	242	199	213	218	206	224	202	226
221	212	214	246	227	232	203	202	218	211	233	230
212	229	204	241	204	214	178	198	194	212	214	226
181	235	229	226	218	223	170	190	218	212	208	244
172	217	178	218	208	187	206	192	200	214	208	215
186	214	216	252	213	240	212	209	195	203	205	188
217	217	228	240	204	218	242	212	211	223	228	206

Screening plate 39

154	188	190	220	185	198	164	170	208	180	212	208
186	200	208	224	232	180	210	208	182	218	187	216
190	204	224	213	242	244	198	201	196	197	208	201
204	232	203	235	211	240	186	188	179	208	220	191
161	216	226	252	218	220	190	206	213	220	227	200
184	214	184	218	202	206	204	194	223	256	200	186
156	205	223	229	216	238	206	202	230	211	230	192
177	194	220	204	124	198	234	192	171	206	215	193
165	196	188	222	182	229	170	190	206	198	226	224
201	203	206	187	246	205	224	195	194	220	210	229
192	208	218	217	240	244	194	206	198	198	213	226
184	232	203	248	227	234	194	206	186	213	196	199
171	222	216	246	234	244	193	203	202	220	201	194
182	230	182	214	216	210	205	196	200	213	208	194
186	218	214	232	224	226	209	220	217	196	234	188
188	198	220	222	172	200	212	208	180	188	222	196

Screening plate 40

159	160	172	212	178	193	161	179	194	206	215	216
186	178	206	175	192	202	221	200	187	214	204	239
194	216	201	216	217	234	200	204	216	184	220	212
196	224	199	236	221	228	206	184	200	187	215	199
168	226	226	216	222	213	174	198	214	208	224	218
186	222	179	219	216	210	217	192	202	220	220	210
148	203	223	211	208	230	230	198	223	218	224	158
167	202	214	226	152	201	219	199	204	188	204	186
170	148	202	226	172	214	166	208	210	218	200	223
182	180	197	196	224	212	233	188	194	212	226	224
189	205	217	220	248	250	195	204	210	210	228	212
186	218	176	240	226	225	196	195	194	215	227	203
166	214	220	234	237	239	198	184	206	212	234	220
162	206	180	216	216	202	227	204	204	215	214	206
170	194	216	226	214	229	231	218	235	197	234	188
202	194	218	205	176	212	224	182	200	204	196	206

Screening plate 41

204	210	198	232	204	221	190	204	226	212	184	220
229	202	190	202	244	182	214	206	205	207	204	202
207	223	214	230	214	236	210	193	223	210	220	203
217	226	187	258	230	214	197	186	202	206	197	220
196	208	184	213	198	228	193	208	216	226	205	204
188	242	156	243	200	182	206	220	204	230	202	232
200	200	204	238	202	209	186	232	202	208	228	209
213	228	230	242	162	178	234	230	189	200	189	224
210	181	210	220	202	226	204	186	210	216	226	230
208	191	205	230	270	195	216	206	205	228	215	230
226	228	226	216	224	231	202	204	208	212	206	220
196	228	198	252	222	225	186	180	204	222	204	221
200	214	226	203	206	252	168	172	222	202	196	238
186	202	182	200	189	190	200	212	214	231	204	215
188	216	190	246	221	218	208	218	216	200	220	234
220	212	213	248	190	226	240	221	202	206	224	244

Screening plate 42

211	196	210	225	208	246	178	213	225	230	221	226
204	190	222	227	260	191	197	205	206	232	188	208
223	219	225	226	217	220	188	218	219	222	228	232
218	225	201	239	193	187	178	202	211	214	208	202
192	246	206	232	206	204	185	204	217	208	202	208
196	218	172	194	213	178	197	208	200	220	190	202
198	198	224	248	208	216	198	194	218	232	208	216
232	212	210	228	194	212	234	218	214	214	213	236
210	206	214	238	213	222	184	190	230	227	218	210
227	216	203	238	244	212	244	214	226	223	198	208
212	227	223	218	262	232	220	208	208	218	246	234
208	235	185	249	225	206	168	193	196	219	214	222
194	232	215	232	218	231	188	205	222	200	224	244
204	222	178	210	232	186	215	177	212	218	214	216
178	214	210	226	225	236	209	208	212	208	240	216
218	210	227	230	206	234	253	195	205	238	222	230

Screening plate 43

194	207	224	234	229	246	203	226	212	202	208	208
231	220	198	180	270	218	237	208	205	230	224	224
224	218	234	222	211	216	181	218	204	196	230	214
211	216	214	230	206	224	182	192	193	230	228	208
188	209	256	216	193	206	174	209	228	227	189	203
182	220	190	215	214	189	172	206	208	225	205	229
186	211	224	197	204	204	200	229	206	222	222	228
204	204	220	225	182	197	231	222	220	212	210	208
197	204	212	234	191	238	190	182	214	193	224	244
213	204	206	210	260	213	228	202	188	215	202	236
232	206	204	198	248	221	209	216	200	214	213	202
194	197	197	234	222	211	180	202	204	180	198	210
202	206	212	209	209	205	157	182	220	214	204	201
146	216	192	210	209	204	192	224	206	214	217	212
188	210	222	232	202	214	218	212	218	208	222	212
217	224	221	236	184	212	232	213	209	212	223	222

Screening plate 44

194	200	214	225	216	226	184	208	226	220	214	222
224	188	216	207	251	217	224	204	214	222	198	223
234	210	224	214	229	228	190	225	187	205	199	220
226	213	184	230	208	211	186	202	192	224	190	224
182	202	215	234	209	248	214	198	209	212	220	225
206	194	167	212	214	203	210	210	230	209	204	226
190	198	212	224	232	238	206	228	200	194	217	207
214	237	236	238	188	214	248	219	194	221	234	235
195	219	208	214	214	252	162	211	206	194	204	204
217	186	204	202	255	240	236	204	196	216	197	220
239	212	224	222	228	210	196	210	234	192	205	212
158	232	194	218	208	212	199	189	178	223	216	226
190	200	220	224	205	248	174	198	206	189	190	200
217	187	168	228	207	173	190	200	185	233	198	218
192	224	217	224	222	227	204	202	217	215	222	206
204	220	219	235	188	200	220	208	198	200	226	217

Screening plate 45

210	204	232	223	208	248	170	189	208	214	202	213
206	214	204	202	245	196	228	202	204	233	214	229
214	208	214	224	246	241	211	214	212	212	204	232
203	240	210	240	234	220	199	191	194	237	190	220
207	208	214	226	222	250	182	194	232	216	212	210
208	225	182	206	220	191	200	217	219	230	192	226
198	196	206	214	211	236	226	224	219	200	198	197
216	214	225	227	182	190	232	215	202	217	194	215
195	198	226	229	192	250	213	208	214	206	208	222
214	212	202	212	245	223	230	203	214	218	200	226
226	224	212	224	227	232	206	214	234	208	200	220
166	232	206	226	221	210	200	208	216	230	204	229
174	214	230	244	217	222	196	214	224	231	203	220
168	199	194	216	214	190	206	206	212	229	204	212
178	208	210	223	210	230	208	204	219	204	206	196
207	223	212	222	192	192	230	216	196	214	218	196

Screening plate 46

183	189	208	260	215	229	204	216	228	219	200	219
233	203	207	226	254	199	222	196	174	218	218	210
218	235	206	228	240	230	208	222	201	208	217	206
204	214	208	240	218	212	206	195	198	208	216	218
205	224	246	238	210	236	183	218	216	209	205	194
182	200	197	212	208	193	200	218	215	221	204	204
174	228	234	236	216	228	200	215	209	210	212	204
218	196	226	229	181	174	226	230	218	228	192	219
215	198	205	236	234	246	199	212	234	235	232	204
212	223	207	197	240	190	218	200	204	238	180	216
195	241	214	202	234	235	202	194	186	202	184	208
188	215	193	161	215	206	134	194	142	203	192	214
161	176	230	186	194	206	222	194	191	198	188	212
186	220	201	172	202	163	188	182	170	214	223	230
184	205	218	150	180	183	210	205	194	184	211	200
212	220	244	242	192	216	242	219	214	233	215	244

Screening plate 47

210	202	204	246	233	239	198	228	228	231	218	218
218	214	172	218	234	176	222	208	203	222	224	223
212	223	204	218	184	199	190	189	194	210	208	213
212	164	144	191	194	177	168	173	146	184	180	218
202	206	186	196	179	184	229	206	180	192	192	206
190	186	182	166	189	160	200	179	198	215	197	208
168	189	210	184	168	211	204	210	207	204	209	193
224	231	212	214	201	208	247	244	214	228	201	248
208	228	218	244	238	228	212	213	230	213	190	196
217	190	214	226	234	214	222	232	194	218	195	220
236	232	208	202	226	204	188	224	222	204	222	217
206	226	201	194	169	190	194	202	219	240	216	204
188	219	216	216	186	204	180	198	235	201	212	184
168	218	190	172	208	141	187	197	220	220	213	218
178	212	218	203	188	209	184	200	218	207	206	189
202	196	224	224	156	182	232	228	214	216	208	194

Screening plate 48

202	192	205	235	228	229	206	197	212	205	210	214
238	219	200	208	243	206	222	221	198	206	204	212
230	224	197	216	242	239	218	219	208	204	216	220
222	218	190	214	208	204	182	202	202	207	200	215
202	234	210	196	201	219	165	216	218	210	229	222
188	218	174	216	193	184	196	206	222	228	208	188
180	192	192	210	170	206	200	220	216	229	208	193
214	218	222	233	184	186	218	224	184	213	207	224
206	199	214	232	204	211	178	198	218	203	192	222
202	214	181	223	242	202	238	182	202	205	214	207
213	209	210	218	214	216	200	213	210	202	192	202
205	214	168	252	202	222	182	210	220	215	214	200
192	214	208	232	228	218	191	204	220	224	196	200
210	222	180	222	218	183	198	220	198	234	200	200
208	206	206	206	202	223	205	221	230	234	202	194
210	218	234	238	196	190	208	216	206	212	202	208

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CHAPTER THREE – A screening method for the discovery of natural products with anthelmintic activity using *Caenorhabditis elegans* as a nematode model

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Declaration: Research presented as part of this chapter will be prepared for publication.

Contributions: SC performed experiments, wrote the manuscript, and made the figures. WW developed the natural product purification protocol and conducted NMR analyses.

ABSTRACT

Parasitic worm infections affect over a quarter of the human population, reducing quality of life and impacting overall global health. Helminth infections in farm animals are also prevalent and threaten the quality and quantity of agricultural resources. Despite the severity and frequency of helminthiases, few treatment options are available and research efforts targeted towards novel drug discovery are limited. However, the rising levels of resistance to existing anthelmintics necessitates a more active drug discovery strategy. In the past, natural products have proven to be a rich source of clinically relevant therapeutics but research in the field is bottlenecked by the labour-intensive process of working with natural sources. As such, we have developed and optimized a liquid-based assay using *Caenorhabditis elegans* specifically to uncover natural compounds with anthelmintic activity. As proof of concept, we screened a unique natural product extract library composed of both crude and semi-pure extracts and identified two well-known bioactive compounds: tunicamycin and actinomycin D. We also found xanthocillin Y1 and xanthocillin X, compounds with strong nematocidal activity with a previously unidentified mechanism and propose that it acts through rapid heme depletion in *C. elegans*. Our findings highlight the utility of mining natural sources for compounds with potential anthelmintic activity.

INTRODUCTION

Helminths are parasitic worms capable of infecting a variety of organisms including humans, plants, and animals. Approximately a quarter of the world's population suffers from at least one helminthiasis, which often results in long-term cognitive and physical impairments (Hotez et al., 2008; Pullan et al., 2014). Parasitic worms are also extremely detrimental to plants and livestock animals, posing a significant threat to the economic success of the agricultural industry. The low financial return associated with anthelmintic drug development may also be a deterrent to supporting research in the field, limiting the financial and human resources generally required for the drug discovery process. Moreover, many existing research efforts have abandoned discovery campaigns altogether in favour of repurposing existing therapeutics, but this approach does not support the discovery of novel chemicals that could possess more favourable anthelmintic activity (Cheuka et al., 2016; Panic et al., 2014; Weeks et al., 2018). As such, the past three decades have not been particularly fruitful in the realm of novel anthelmintic drug class discovery, with no new approved drugs for the treatment of human helminth infections (Nixon et al., 2020).

As with other drugs for the treatment of infections, decreased treatment efficacy due to resistance from overuse is a concern with anthelmintic compounds. There are several comprehensive reviews and meta-analyses that document the rising levels of resistance to medications commonly used to eliminate parasitic

worm infections (Ae, 2018; Baiak et al., 2019; Papadopoulos et al., 2012; Rose Vineer et al., 2020). This rise of resistance is concerning, considering the already limited arsenal of pharmaceutical remedies. Therefore, there is an urgent clinical need for novel anthelmintic compounds and drug discovery efforts should be prioritized.

Natural products have yielded a diverse array of clinically-significant pharmaceutical compounds such as anti-cancer drugs, antibiotics, and antibiotic adjuvants (Clark, 1996; Dias et al., 2012; Fleming, 1929; King et al., 2014). The bioactive potential of natural products could be further exploited in the search for novel anthelmintic compounds. Bacteria and fungi are well-known producers of secondary metabolites and are known to produce molecules with anthelmintic activity (Genilloud, 2017; McKellar & Scott, 1990; Miller et al., 1979; Schueffler & Anke, 2014). To address this gap in the field, we developed an assay that better accommodates natural product extract screening. We screened over 300 crude and 3,000 semi-pure bacterial and fungal extracts from the Wright Lab's pre-fractionated library (PFL) (Cook et al., 2023) and identified thirteen hits thus far. Preliminary work on hit follow-up revealed two compounds known to be potent inhibitors of worm development, tunicamycin and actinomycin D, and one compound without previously reported nematocidal activity: xanthocillin. Here, we highlight the success of our assay in identifying nematocidal compounds from natural sources.

MATERIALS AND METHODS

C. elegans Maintenance and Strain Information

C. elegans Bristol (N2) was used as wild-type strain in the screen is the Bristol (N2) strain. SJ4005 (*zcls4[hsp-4::GFP; lin-15(n765)]*) was used as a reporter for endoplasmic reticulum (ER) stress. Both strains were obtained from the *Caenorhabditis* Genetics Center (CGC). *C. elegans* general maintenance protocols were followed as previously described (Stiernagle, 2006). Briefly, worms are maintained on plates of solid nematode growth media (NGM) seeded with *Escherichia coli* OP50 as a food source (Brenner, 1974). Populations are propagated at 20 °C by transferring worms onto new, seeded plates when *E. coli* OP50 is depleted.

Motility Assay

The Wright Lab houses a collection of over 11,000 bacterial and fungal strains isolated from environmental samples, called the Wright Actinomycete Collection (WAC). The PFL consists of both crude and semi-pure extracts made from these WAC strains. Details of library preparation and composition have been published (Cook et al., 2023). This is the extract library used for our screen. The screening conditions and set-up for this study have been optimized for screening natural products, and the protocol is detailed below, beginning with *C. elegans* population synchronization. Prior to this stage, worms developed on NGM agar seeded with *E.*

coli OP50, as outlined above, until they were gravid adults, which takes approximately three days from eggs. Each well contained a total volume of 150 µL: 60 L1 *C. elegans* (60 µL), 89 µL of concentrated *E. coli* OP50, and 1 µL (0.6% v/v) of either an extract or the negative control, dimethyl sulfoxide (DMSO). To ensure an accurate and consistent worm count in each well of a 96-well plate, Union Biometrica's COPAS Flow Pilot worm sorter was used.

C. elegans were grown on NGM seeded with *E. coli* OP50 until adulthood, and a standard bleaching procedure was used to collect *C. elegans* eggs (Stiernagle, 2006). To synchronize the population, eggs were resuspended in 7 mL of M9 buffer (3 g KH_2PO_4, 6 g Na_2HPO_4, 5 g NaCl, 1 mL 1 M $MgSO_4$, 1 L H_2O) and left on a rocker overnight at 20°C to allow eggs to hatch and the population to be synchronized to the first larval stage (L1). L1 worms were resuspended in S-Basal (5.85 g NaCl, 1 g K_2HPO_4, 6 g KH_2PO_4, 1 mL cholesterol (5 mg/mL in ethanol), 1 L H_2O) to reach a concentration of 1 worm/µL. 60 L1 worms were sorted into each well of a 96-well clear flat-bottom plate using the COPAS Flow Pilot worm sorter. *E. coli* OP50 was resuspended in S-basal to an OD_{600} of 5, and 89 µL was added to each well with the Tempest automated liquid handler (Formulatrix), followed by the addition of 1 µL of PFL extract (0.6% v/v) using a Mosquito low-volume liquid handler (SPT Labtech). DMSO was added to columns 1 and 12 as a negative control. A gas-permeable seal was manually placed to prevent evaporation and contamination between wells, and the plates were incubated at 20°C and 150 rpm for six days. After the incubation

period, each plate was analysed for 30 minutes on the motility tracker and imaged, as described below.

Assessing impact of extracts on C. elegans

The WMicrotracker One (NemaMetrix), which was developed to measure thrashing activity of *C. elegans* in liquid media, was used in this screen as a method of identifying wells within a 96-well plate with no *C. elegans* movement. Low activity scores (less than 10) were indicative of dead or paralyzed worms.

Individual wells in screening plates were also imaged using a Nikon AZ100M microscope equipped with the AZ Fluor 2X objective lens. Image acquisition was automated and programmed to autofocus on the center of each well and capture pictures in a serpentine manner across the plate. Worms in images were scored for death/paralysis, delayed development, asynchronous development, and sterility.

Natural product purification

Extract preparation and purification for the strains in the PFL has been previously described and hit validation efforts that required more bacterial extract followed these protocols (Cook et al., 2023). An in-depth protocol for fungal strain culture, extract preparation, and purification is described in the Supplementary information for WAC10994 in this chapter.

Endoplasmic Reticulum Stress assay

Chemically-induced ER stress was evaluated using the stress-responsive reporter *hsp-4p::GFP* (SJ4005 strain) (CGC, n.d.). Worms were age-synchronized to the L1 stage and incubated in S-basal in a 96-well plate with *E. coli* OP50 and 0.6% v/v of either DMSO, 1 mg/mL tunicamycin, or WAC1490 at 20°C, 150 rpm until they reached the fourth developmental stage (L4). These L4 worms were paralyzed using levamisole, mounted onto 2% agarose pads on microscope slides, and DIC and GFP images were taken (Nikon Ni-U microscope equipped with a Nikon DS-Qi2 camera). Corrected total worm fluorescence for each image was calculated by outlining each group of worms and subtracting the background fluorescence reading from the total integrated density of the selected region.

Embryonic lethality assay

An embryonic lethality assay was conducted as previously described, serving as an indirect measure of DNA damage (Kim & Colaiácovo, 2015a). Briefly, N2 worms were age-synchronized and grown until L4 on solid NGM. They were then transferred to 96-well plates in S-basal with *E. coli* OP50 and either 0.6% of either DMSO, 1.5 mg/mL actinomycin D, or WAC466. Plates were incubated at 20 °C and 150 rpm for 24 hours until worms reached early adulthood. Worms were then transferred onto individual unseeded 35 mm NGM plates and the adult worms were picked off using a platinum wire after 24 hours. All eggs on the plate were counted

and recorded as the total number of offspring. Plates were then left to incubate at 20 °C for another 24 hours and the number of L1 worms was counted. Embryonic lethality was calculated as the number of unhatched eggs divided by the total number of offspring.

RESULTS

To identify natural compounds capable of killing or paralyzing *C. elegans*, we developed a high-throughput, liquid-based assay that accommodates the limited availability of natural product extracts (Figure 1A). We first used an activity-level readout to assess worm motility, allowing us to quickly identify extracts from the PFL that could cause death or paralysis. Movement was measured using the WMicrotracker. Plates were screened in duplicate, and motility values across screening plates could reach upwards of 200; however, there was considerable output value variability between two replicates (Figure 1B). This was expected due to the many factors that can give rise to behavioural variability (Flavell et al., 2025). For our work, only wells with activity readouts of < 10 were classified as hits and marked for further follow-up (Figure 1C). In total, we tested extracts from 384 WAC strains and identified hits from 13 strains which were selected for additional analysis. Since a brightfield image of each well was also captured, motility results could be verified

through image analysis and wells with low activity scores had an obvious lack of viable worms (Figure 1D).

While comparing images to motility scores, we noticed that inconsistent motility scores were sometimes reflective of impaired worm development. As such, to increase the sensitivity of this assay, we began manually scoring images to identify extracts that caused developmental defects in the worms. For example, asynchronous development could signify the presence of a toxic compound that is not uniformly affecting the worms. Aside from asynchronous development, delayed development, and reproductive impairments could also be indicative of exposure to a toxin (Lagido et al., 2009; Lewis et al., 2013; Lu et al., 2020). Delayed development or embryonic lethality are also important effects to consider when looking for potential nematocidal compounds. Taken together, these phenotypes could reveal compounds with unique modes of action or the presence of a lethal compound that is not present in a high enough abundance to cause death or paralysis of all exposed worms. These phenotypes were still of interest to us and guided our hit follow-up strategy. Consequently, we engaged with the Moradi Lab to develop a system to automate the image scoring (Wang et al., 2025).

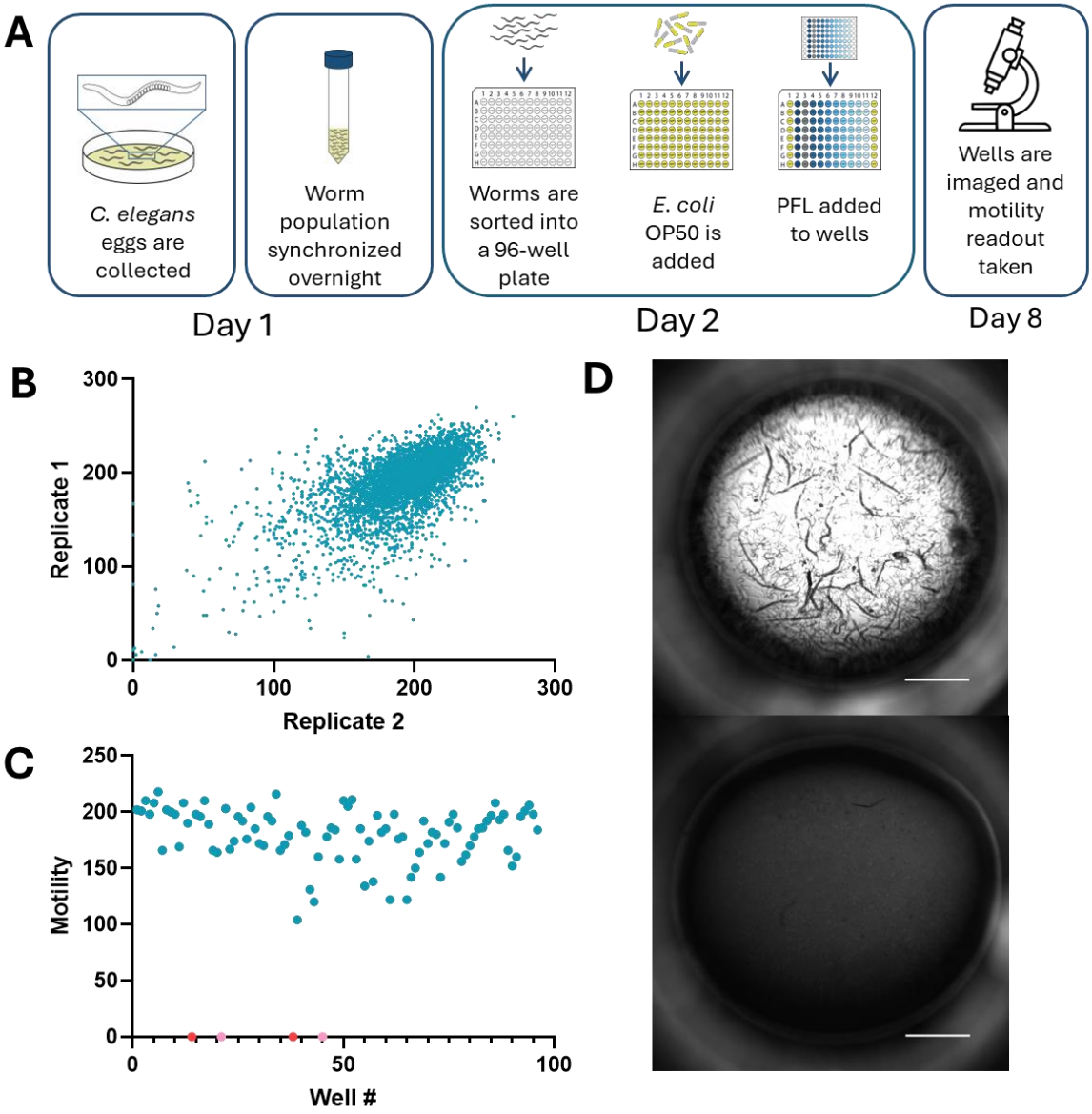


Figure 1: *Assay details and representative summary of results.* A) Workflow of the liquid assay screen set-up. Worms are age-synchronized and incubated with their food source, *E. coli* OP50, and test extract for 6 days prior to imaging and motility assessments. B) Replicate plot of PFL extracts screened. X and Y axis values indicate the activity-level readout generated by the Wmicrotracker. C) Representative motility plot for a screening plate with hit extracts. Blue dots indicate wells where activity levels were greater than 10 (no effect on worms), and red and pink dots highlight where activity levels were below 10 (hit). Red dots indicate wells treated with a crude extract, and pink dots are well treated with fractionated

extracts. D) Representative images comparing a DMSO negative control (top) versus a "hit" (bottom) well. Scale bar = 1 mm

From our combined motility and image scoring tool, we identified extracts from *Streptomyces* strains WAC1490 and WAC466, as being potent inhibitors of worm development. Previous work in the Wright Lab found that tunicamycin and actinomycin were present in WAC1490 and WAC466, respectively (Cook et al., 2023). Both of these compounds impact *C. elegans* development (Ghenea et al., 2022; Koirala et al., 2023, p. 12; Travers et al., 2000). The identification of these known nematocidal compounds demonstrate the ability of our screen to identify potent natural compounds.

To validate that these compounds are responsible for the activity in our assay, we verified their presence in the extract and compared the extracts to pure compounds to confirm that similar activities could be observed. For WAC1490, we identified three homologs of tunicamycin: tunicamycin B1 (IV), B, and D (Figure 2A). This strain was sequenced and the tunicamycin biosynthetic gene cluster (BGC) was identified through antiSMASH with 85% sequence similarity (Figure 2B). To further confirm, we analysed fractionated extract using high-resolution mass spectrometry and identified masses corresponding to ions consistent with the three homologs (Figure 2C). Tunicamycin is an inhibitor of N-linked glycosylation, which ultimately triggers an ER stress response. To determine whether the fractionated extracts

induced ER stress in the worm, we used the *hsp-4p::GFP* reporter strain, SJ4005. Increased ER stress is indicated through greater levels of GFP fluorescence, which can be observed as a result of exposure to the WAC1490 extract (Figure 2D-E).

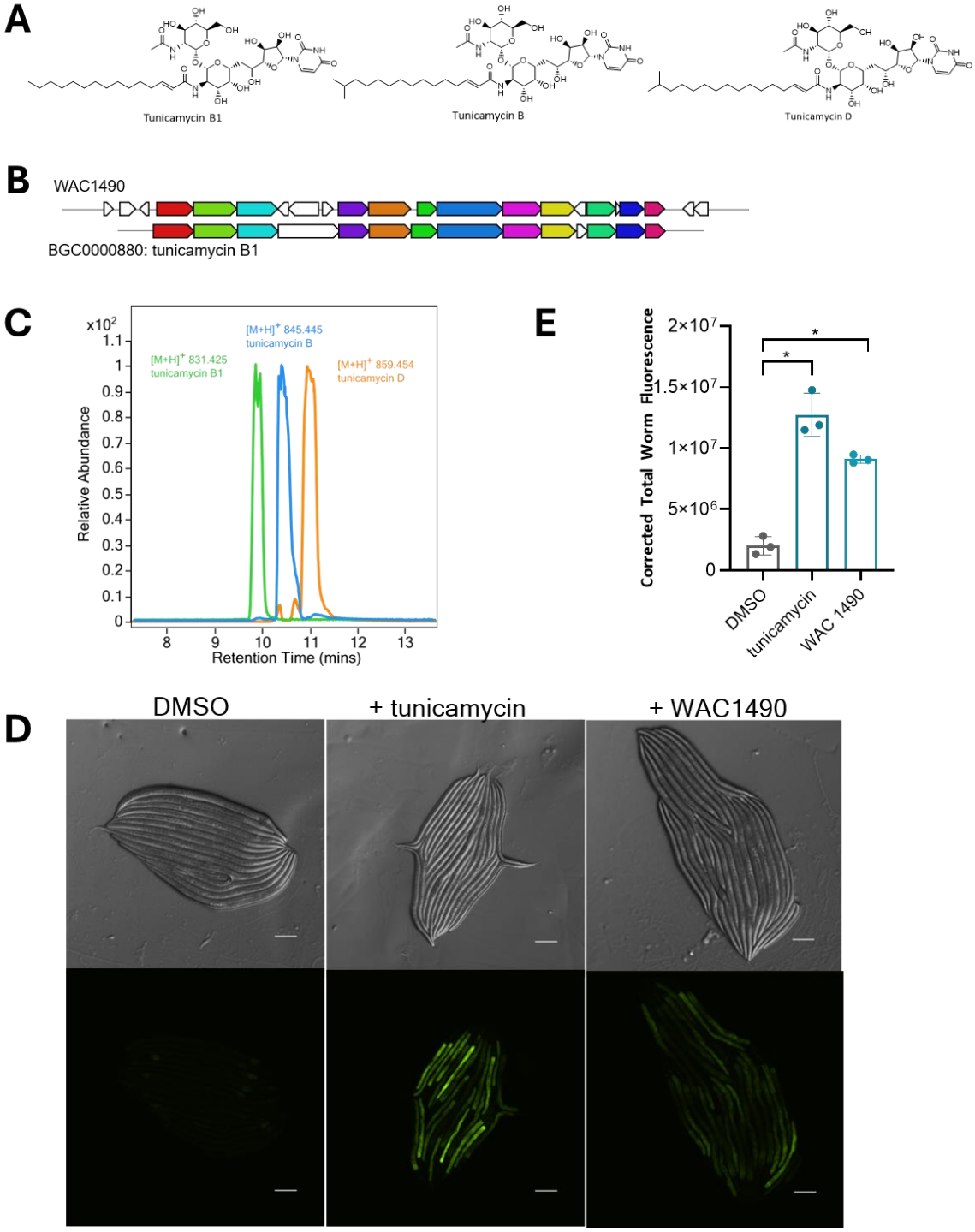


Figure 2: *Validation of tunicamycin as the active compound in WAC1490.* A) Structure of tunicamycin B1 (tunicamycin IV), tunicamycin B, and tunicamycin D. B)

WAC1490 contains the tunicamycin gene cluster as seen by sequence similarity of the WAC1490 BGC and the tunicamycin B1 cluster (MIBiG BGC0000880). C) Overlayed extracted ion chromatograms from HR ESI-QTOF-MS of a WAC1490 extract showing ions consistent with tunicamycin homologs. D) Representative DIC (top panels) and GFP (bottom panels) images of *hsp-4p::GFP* worms treated with DMSO (negative control), tunicamycin (positive control), and WAC1490. Scale bar = 100 µm. E) Fluorescence quantification for worms treated with DMSO, tunicamycin, and WAC1490. Each dot represents a group of worms. * $p < 0.05$.

Similarly, we confirmed that WAC466 extracts contained actinomycins D and V (Figure 3A). AntiSMASH analysis of the WAC466 genome identified the biosynthetic gene cluster for actinomycin D with 89% sequence similarity. HRMS revealed masses corresponding to actinomycins D and V (Figure 3B-C). For further validation in *C. elegans*, we conducted an embryonic lethality assay. This is a common test to assess for DNA damage in worms, as genotoxic stress often results in embryonic lethality, and actinomycin is a DNA intercalator (Kim & Colaiácovo, 2015b; Stergiou & Hengartner, 2004). Our results confirm that exposure to our semi-pure WAC466 extract increased embryonic lethality in worms (Figure 3D).

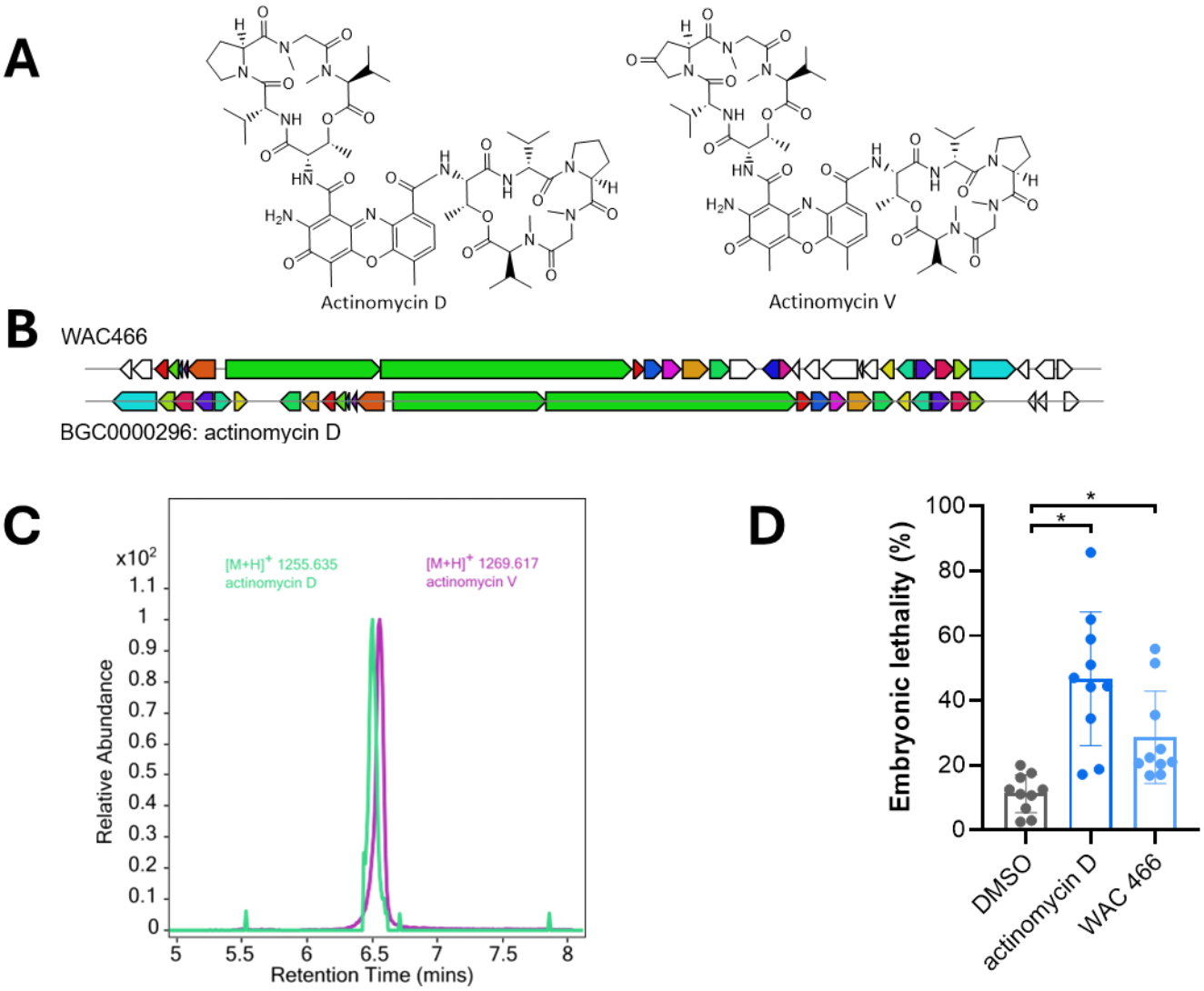


Figure 3: *Validation of actinomycin D as the active compound in WAC466.* A) Structure of actinomycin D and actinomycin V. B) WAC466 contains the actinomycin D gene cluster as seen by sequence similarity of the WAC466 BGC and actinomycin D cluster (MIBiG BGC0000296). C) Overlayed extracted ion chromatograms from HR ESI-QTOF-MS of WAC1490 extract showing ions consistent with actinomycin homologs. D) Embryonic lethality assay results. Each dot represents the hatched offspring from ~ 20 worms. $P < 0.05$

We were also able to identify an extract from WAC strain 10994 that caused asynchronous development in *C. elegans*. WAC10994 is a fungal strain from our

collection that had not been thoroughly investigated, so we sought to characterize the active component(s) from this strain that was impeding regular worm growth. After purification, we determined the bioactive components to be xanthocillin X and xanthocillin Y1 using mass spectrometry and nuclear magnetic resonance (NMR; Figure 4A-B, Supplementary Figures 1-5). These compounds exhibit both antibacterial and anti-cancer activity *in vitro* (Hübner et al., 2021; Zhao et al., 2012). The antibiotic activity of xanthocillin is said to be related to heme sequestration through direct binding of the compound to heme iron (Hübner et al., 2021). This is a likely mechanism in worms as well, since *C. elegans* is a heme auxotroph and relies on its environment as a source of this essential cofactor (Chen et al., 2012). To test the heme sequestration hypothesis in worms, we supplemented our media with hemin and found that this rescues *C. elegans*. This suggests that xanthocillin is working through heme depletion, as the addition of excess hemin mitigates the negative growth impacts on worms. Furthermore, supplementation with an iron-free form of hemin, protoporphyrin IX (PPIX), was unable to rescue worms treated with xanthocillin (Supplementary Figure 6). This result supports the existing literature that direct iron binding is required for xanthocillin activity (Hübner et al., 2021).

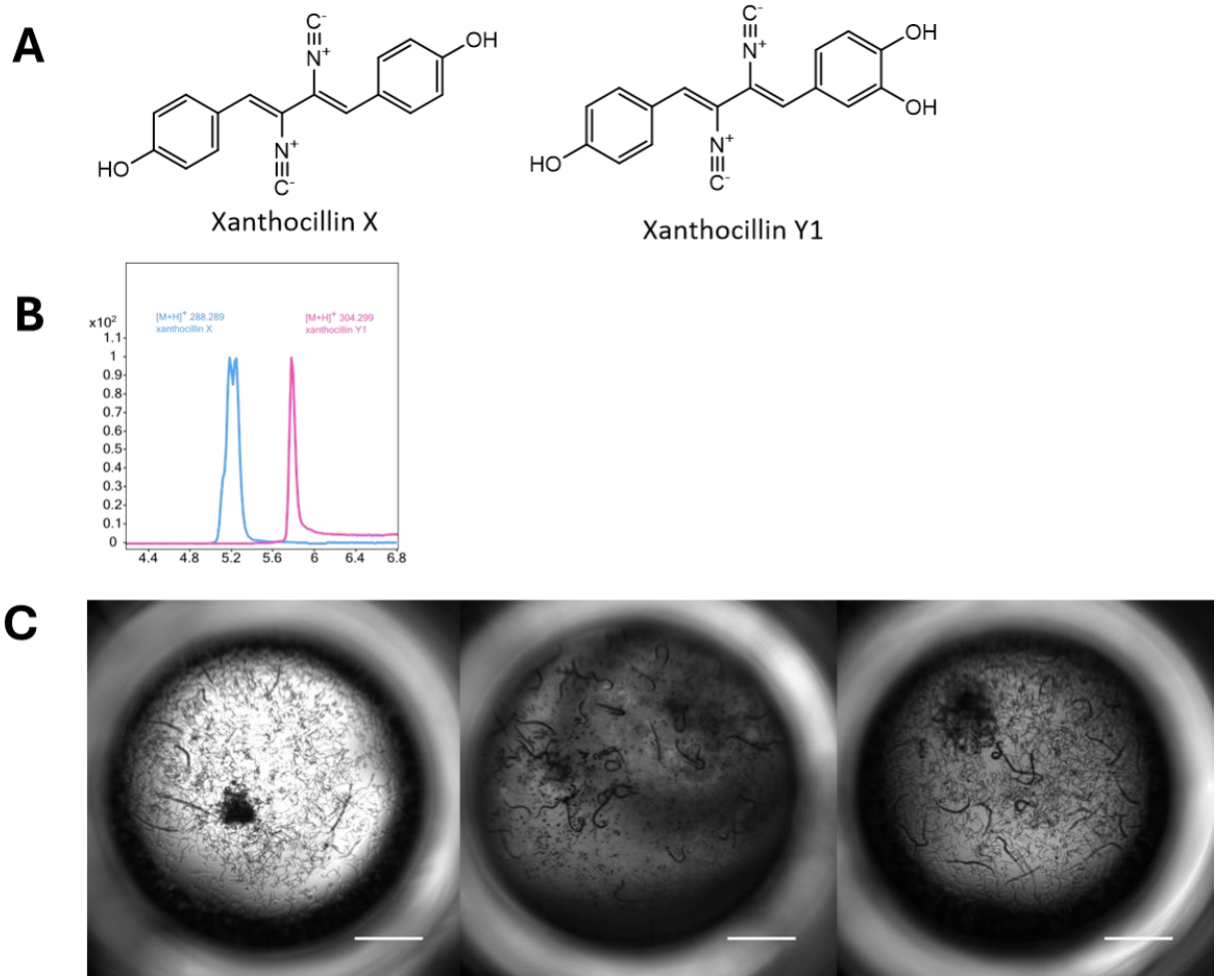


Figure 4: *Xanthocillin identified from WAC10994 is active against C. elegans*. A) Structure of xanthocillin X and Y. B) Overlayed extracted ion chromatograms from HR ESI-QTOF-MS of WAC1490 extract showing ions consistent with xanthocillin homologs. C) Representative images of worms treated with DMSO (negative control) (left), xanthocillin X (middle), and xanthocillin X + hemin (right).

DISCUSSION

Here, we describe the utility of a whole-organism high-throughput assay optimized for the discovery of natural product anthelmintics. From an experimental

set-up perspective, there are several key advantages to this screen. The first is that our assay allows for natural product extract screening by minimizing the amount of test material required per well since it is a liquid-based assay. This method allows for testing libraries that may have been previously infeasible due to the amount of reagent required for solid agar-based assays. We also use L1 worms which are more sensitive to xenobiotic exposure (Hartman et al., 2021; Vairoletti et al., 2021). This approach enables us to detect compounds with lower toxicity levels that might otherwise go undetected. Additionally, since worms in our assay are allowed to develop over a full generation, we can detect molecules that cause sterility that would not be identified in screens with shorter incubation times. Finally, there are three levels of automation to increase throughput and efficiency: sorting worms into individual wells instead of manually pipetting, using liquid handlers to add bacteria and compounds, and assessing viability using a motility tracker or an image scoring program.

Our unique in-house natural product extract library has not previously been evaluated for nematocidal compounds, and there is still much to explore in this area. One advantage of screening natural product extracts is that each sample may contain multiple potentially useful molecules, and further purification could uncover compounds whose activities were previously masked by other molecules. Therefore, screening prefractionated libraries, such as the one we have, provides the potential for uncovering greater chemical diversity (Wagenaar, 2008). The

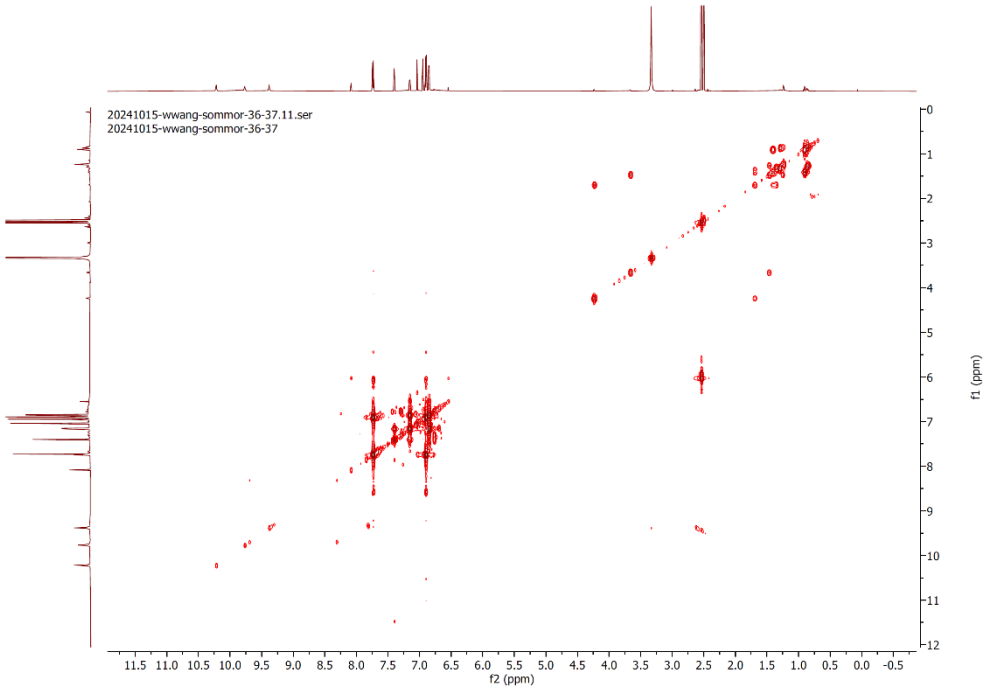
anthelmintic potential for our library is especially high, as these extracts were derived from fungal and bacterial strains isolated from soil samples. Given the shared environment of soil microbes and nematodes, it is reasonable to predict that there would be inter-species competition involving the production of toxic chemicals will occur, leaving much to be explored.

We have demonstrated the utility of our assay through the identification of known molecules, tunicamycin and actinomycin D, and a third compound (xanthocillin) that has not yet been reported to have anthelmintic activity. The high toxicity levels of tunicamycin and actinomycin in mammalian cells make them unsuitable for further development as anthelmintic in their current forms, but further investigation into the utility of xanthocillin as an anthelmintic can be explored. Our work here supports previous evidence that xanthocillin may owe its activity to heme sequestration, which can be promising as heme-targeting drugs are already common in other fields. For example, artemisinins bind to heme in the malaria parasite, and some research has suggested that these drugs could be repurposed for the treatment of helminth infections (Lam et al., 2018; Meshnick, 2002). This example suggests that compounds with similar modes of action, such as xanthocillin, may prove clinically relevant in the future.

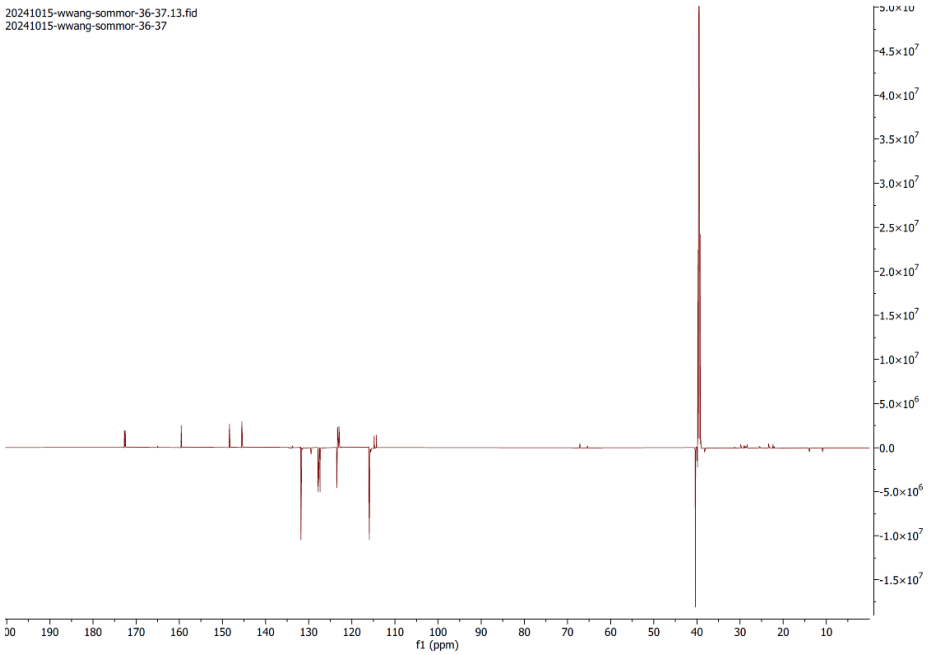
In addition to what was described here, we still have additional hits queued for follow-up from our initial screen. Most of these actives were derived from fungal

extracts due to the composition of the earlier PFL screening plates; however, the PFL has also been expanded to include extracts from additional bacterial strains, providing further diversity for screening and hit elucidation.

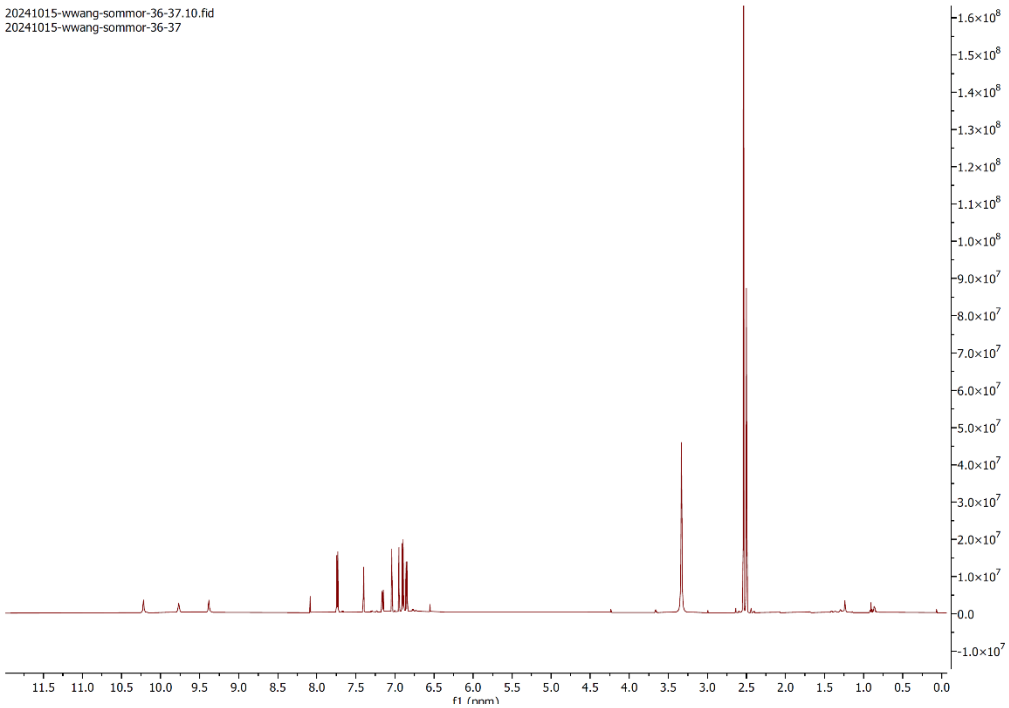
SUPPLEMENTARY MATERIAL



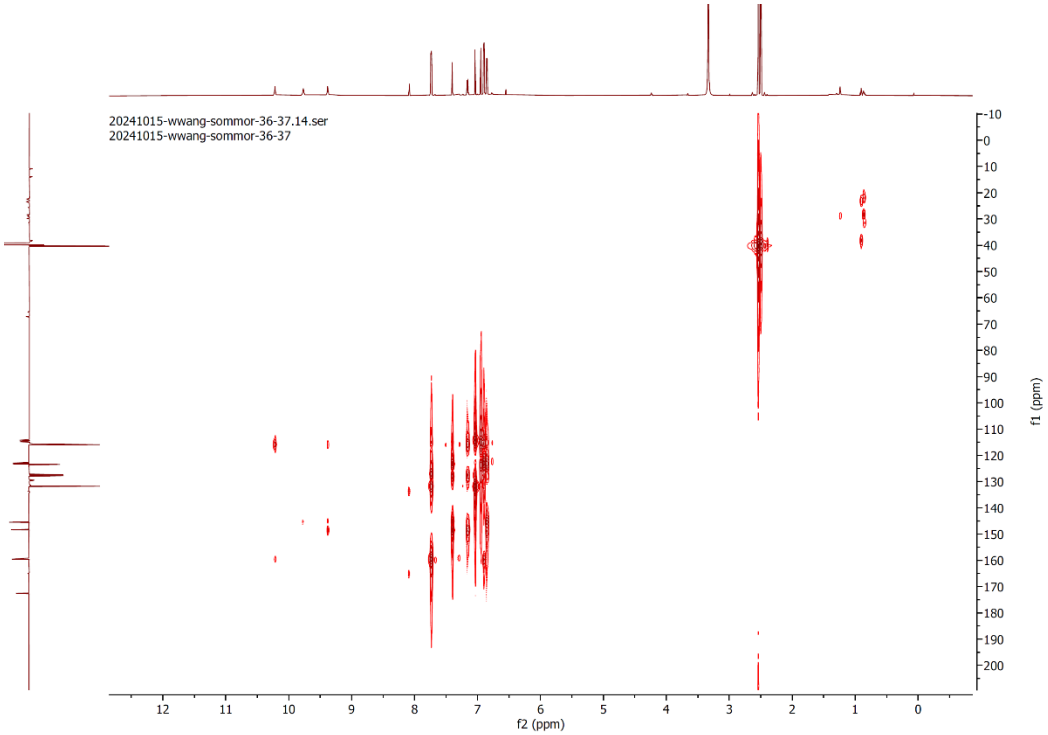
Supplementary Figure 1: ^{1}H-^{1}H COSY (Correlation Spectroscopy) NMR spectrum of xanthocillin.



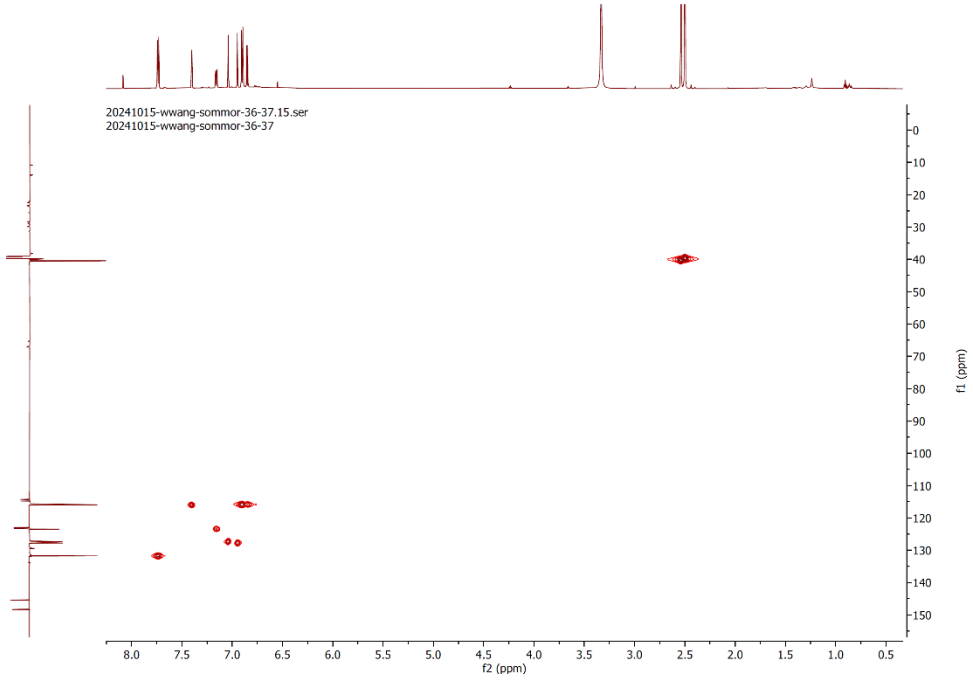
Supplementary Figure 2: DEPTQ (distortionless enhancement by polarization transfer including the detection of quaternary nuclei) NMR spectrum of xanthocillin.



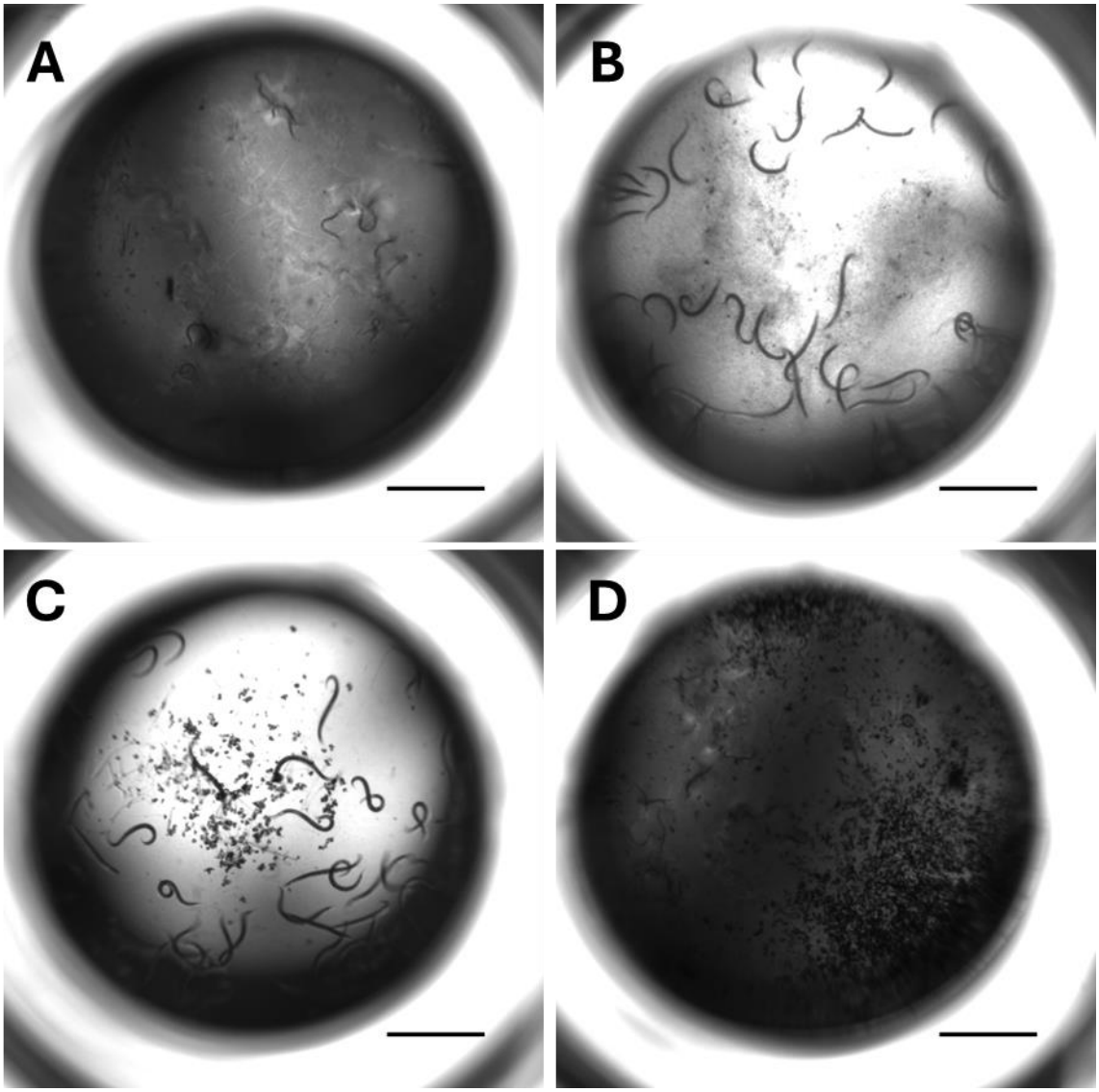
Supplementary Figure 3: ^{1}H NMR spectrum of xanthocillin



Supplementary Figure 4: HMBC (heteronuclear multiple bond correlation) NMR spectrum of xanthocillin.



Supplementary Figure 5: HSQC (heteronuclear single quantum coherence) NMR spectrum of xanthocillin. Results compared to previously published xanthocillin NMR spectra for identification (张长生 et al., 2020).



Supplementary Figure 6: Representative images of C. elegans treated with A) xanthocillin X, B) DMSO, C) xanthocillin X + hemin, and D) xanthocillin X + PPIX. Hemin supplementation can rescue worm development in the presence of xanthocillin, but PPIX cannot. Scale bar = 1 mm.

SUPPLEMENTARY PROTOCOLS: Fungal growth optimization and purification of xanthocillin from WAC10994

Included here is a step-by-step protocol for the growth optimization and culture of WAC fungal strains. We also outline the purification steps taken for the isolation of xanthocillin. A fungi-specific protocol was not previously established and was developed during the project in collaboration with Dr. Wenliang Wang of the Wright Lab.

Supplementary Protocol 1*: Fungal growth optimization*

1. Generate a fungal seed culture by inoculating 5 mL of seed medium (2 g glucose, 5 g malt extract, 2 g yeast extract, ddH_2O to 500 mL, pH 6.5) with a fungal strain and incubating at 30 °C, 170 rpm for 5 days.
2. In 250 mL flasks, prepare small-scale cultures of the same fungal strain in three different media types to determine optimal fungal growth conditions for bioactivity. Media components are included in the table below. Each recipe can be scaled up to down to accommodate the number of samples tested. Every 250 mL flask should have no more than 50 mL of media and all flasks with media should be sterilized prior to adding fungi.

Potato Dextrose Media		Fermentation Media		Rice	
12 g	Potato dextrose broth (Difco)	10 g	Mannitol	80 g	White rice
500 mL	ddH_2O	10 g	Maltose	45 mL	ddH_2O
		5 g	Glucose		
		5 g	Monosodium glutamate		
		0.25 g	Potassium phosphate monobasic		
		0.15 g	Magnesium sulphate heptahydrate		
		1.5 g	Yeast extract		
		0.5 g	Corn steep liquor		
		500 mL	ddH_2O		
		pH 6.5			

3. Inoculate each media type with the indicated amount of seed culture and incubate under the following conditions:

Media type	Inoculum volume	Incubation conditions
Potato dextrose media	1 mL	30 °C, 200 RPM shaking, 7 days
Fermentation media	1 mL	30 °C, static (no shaking), 7 days
Rice	2.5 mL	30 °C, static (no shaking), minimum 7 days

4. Make crude extracts from each of the media types:
    a. Potato dextrose media and fermentation media
        i. Transfer culture to a 50 mL falcon tube
        ii. Centrifuge at 5000 rpm for 15 minutes (or longer) to collect fungal cell pellet

iii. Separate the supernatant from the pellet and transfer 1 mL supernatant to a glass tube for evaporation (this will serve as the supernatant/cell-free extract)

iv. To the cell pellet, add a 70:15:15 ratio of methanol:ethyl acetate:water and sonicate for 1 hour

v. Centrifuge the solvated pellet to separate any large particles and transfer 1 mL to a glass tube for evaporation (this is the pellet extract)

b. Rice

i. Add a solution of 70:15:15 methanol:ethyl acetate:water to the fungi grown on rice, enough to cover the rice

ii. Cover flask with aluminum foil and incubate at 20 °C with shaking at 50 rpm overnight

iii. Sonicate flasks for 10 minutes

iv. Transfer extracts into 50 mL falcon tubes and centrifuge to pellet large particles

v. Transfer 1 mL supernatant to a glass tube for evaporation

NOTE: By now you should have 5 extract samples of 1 mL each: one supernatant and pellet extract from each of the potato dextrose media and fermentation media, and one pellet extract from the rice.

5. Evaporate solvent from all samples using a centrifugal solvent evaporator.

6. Dissolve extracts in 100 mL of DMSO and test against worms to confirm activity. Make note of which growth condition generated the extract with the greatest activity, as this will be the preferred media for a larger scale culture.

Supplementary Protocol 2*: Bioassay-guided purification of Xanthocillin from WAC10994*

Prior to a large scale growth, it was determined that growing WAC10994 in fermentation media resulted in extracts with the strongest activity against worms. The following purification protocol is based around the pellet extract but can be modified to accommodate other growth media.

1. Prepare six flasks of seed culture with 20 mL per flask and incubate at 30 °C with shaking at 170 rpm for 5 days.
2. Autoclave 600 mL of fermentation media in six 3 L flasks.
3. Inoculate large flasks with 15 mL of seed culture each and incubate at 30 °C without agitation for approximately 6 weeks. There should be a thick mat of fungal growth at the surface of the media prior to extraction.
4. Centrifuge cultures to pellet fungal cells. If this step does not sufficiently separate cells from media, an additional vacuum filtration step can be included. Vacuum filter the culture through a stack of three milk filter discs.

Combine the cell material left on the filter with the rest of the cell pellet from centrifugation.

5. Add enough 80% methanol to cover the cell pellet and stir on a magnetic plate with a stir bar for 10 minutes, then sonicate pellet for 5 minutes.
6. Vacuum filter the extract through three new milk filter discs into a clean container.
7. Repeat steps 5 and 6 two more times and pool material together. This is the pellet extract.
8. Remove 1 mL of pellet extract and supernatant for the bioassay. This can be done by evaporating using a centrifugal solvent evaporator and dissolving the pelleted material in 100 µL of DMSO. The supernatant extract testing step here is optional. If it is already known that there is no activity from the supernatant extract, this can be skipped to just focus on the pellet extract.
9. After confirming activity, add 120 grams of HP20 resin (Diaion #13606) to the pellet extract and evaporate the solvent using a rotary evaporator.
10. When the sample is completely dry, pack the HP20 resin into a column and elute with 500 mL each of: 100% ddH_2O, 5% MeOH, 10% MeOH, 20% MeOH, 40% MeOH, 60% MeOH, 80% MeOH, and 100% MeOH, in that order. Collect each fraction separately.

11. Remove 500 µL of each fraction for testing by evaporating solvent and dissolving the remaining sediment in 100 µL DMSO. Conduct bioassay to determine active fractions.
12. Combine all active fractions and remove solvent using a rotary evaporator.

    Note: as an additional test for pH stability, remove 500 µL after combining samples and add 0.1% trifluoric acid. Remove solvent, dissolve in DMSO and test extract against worms.
13. Dissolve the remaining residue in 15 mL of 50% acetonitrile (in water) and aliquot into two 50 mL falcon tubes.
14. Pack each falcon tube with silica gel and mix well with extract.
15. Lyophilize falcon tubes until fully dry.
16. Pack a column with a 1:1 ratio of fresh silica gel:sample and elute with 500 mL each of the following solvent mixtures, collecting each as separate fractions:

Solvent	Hexanes :	Ethyl acetate :	Methanol
1	80	20	0
2	50	50	0
3	25	75	0
4	0	100	0
5	0	98	2
6	0	95	5
7	0	90	10
8	0	80	20
9	0	50	50
10	0	0	100

17. Take samples of each fraction and repeat bioassay (see step 11).
18. Repeat step 12 and resuspend sample in ~ 5 mL of DMSO.
19. For the final purification step, use medium pressure C18 reverse-phase chromatography (Teledyne NextGen CombiFlash) with the following parameters:
    - Separation column: 86 g C18 RediSep column (Teledyne)
    - Solvent A: ddH_2O
    - Solvent B: acetonitrile
    - Collect fractions across a linear gradient of 5% to 95% of solvent B
    - Flow rate: 20 mL/min
20. Evaporate solvent from all samples and dissolve in DMSO to bioassay.

    Note that there may be upwards of 70 fractions so it may be more efficient to first test every 4^{th} fraction to identify the approximate range of the active compound.
21. Analyse active fractions using mass spectrometry and NMR to determine chemical structure.

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CHAPTER 4 – CONCLUSIONS

Summary of Work

This project aimed to develop and validate a screening platform for identifying anthelmintic compounds from natural product sources. In the work presented, we established an assay that could detect toxins affecting worm growth. The set-up was optimized with several features in mind. First, we needed a simpler nematode model to avoid the complexities associated with host-reliant growth, hence the use of *C. elegans* in our assays. Next, we needed a liquid-based platform that could minimize the amount of test material required, to limit the quantity of natural product extracts needed, as these are labour-intensive and costly to make. Thirdly, we wanted to increase assay sensitivity, as active components in crude extracts can often be in low abundance and masked by other compounds. We accomplished this by using younger, first larval stage (L1) worms for screening. Lastly, to detect as many desirable phenotypes as possible, we set the screening length to accommodate two generations of worms. This incubation time allows us to determine if there are any alterations to overall growth, like asynchronous or delayed development, and reproductive capacity. It also ensures that any slower-acting compounds can be detected.

We screened an in-house natural product extract library against *C. elegans*, testing a total of over 300 crude and over 3,000 semi-pure extracts. We used both

motility and image analysis methods for characterizing resulting phenotypes, and this yielded a combined 34 hits. At the time of screening, most extracts in the library were derived from soil-isolated fungi. Therefore, 24 of these hits were traced back to extracts from fungal isolates while the remaining 10 were bacterial extracts. There have been ongoing efforts to expand this library, and it now includes more *Streptomyces* bacterial strains that should be tested against worms in future work.

From initial follow-up work, we were able to identify three compounds: tunicamycin, actinomycin D, and xanthocillin. The former two compounds are known to be toxic against various organisms including worms and bacteria, and have been identified in other screening efforts (Cook et al., 2023; Ghenea et al., 2022; Koirala et al., 2023, p. 12; Travers et al., 2000). Xanthocillin, however, does not have any previously reported nematocidal activity, but it is known to directly sequester heme in bacteria (Hübner et al., 2021). In our work, we show that it may have a similar effect in worms, as heme supplementation rescues worms from the toxic effects of xanthocillin.

To advance the field of anthelmintic drug discovery beyond traditional phenotypic screens, we also extended our work to develop a nanoluciferase-dependent target-based assay. We chose the nematode cuticle as a potential target, as its essentiality in worms expands its promise as an effective target, and the absence of a cuticle in mammals suggests that off-target effects would be limited.

There are already developments in this field showing that the nematode cuticle is of increasing interest as an anthelmintic target (Greiffer et al., 2022; Njom et al., 2021; Page et al., 2014).

Altogether, this work integrates both phenotypic and target-based approaches to improve the ease of early-stage compound discovery. We used a variety of output methods for hit identification, including motility and image scoring, with the intention of developing an assay with maximized automation and sensitivity.

Overall Significance

Helminth infections continue to pose a significant threat to human health. They primarily affect lower-income regions, where populations are already vulnerable due to limited resources. Current preventative measures include handwashing when touching food, wearing shoes, and avoiding water or soil that may have come into direct contact with human feces (Branda et al., 2025). However, these actions are difficult in areas where sanitation infrastructure is poorly developed and individuals often do not have the financial means to ensure personal health and safety. Fortunately, the World Health Organization recognizes the severity and impact of helminthiases and have implemented interventions to control and eradicate them, including mass drug administration (MDA) programs (WHO, 2025a). These involve the treatment of entire populations in endemic regions with existing anthelmintics. This has proven effective for many neglective tropical diseases,

including helminthiases (Engels and Zhou, 2020; Richards et al., 2020; Turner et al., 2021). However, its application is not without roadblocks or risks. Most notably, MDA programs can introduce selective pressure that promote anthelmintic resistance (Konopka et al., 2022). Preventative chemotherapy is at the forefront of MDA efforts; however, the implementation of widespread treatment without diagnoses of infection may further increase resistance (Tinkler, 2019; WHO, 2025b). It is also important to note that interventions such as MDA were recently deprioritized as global health resources and research efforts were re-allocated to support pandemic response for the global covid outbreak (Kabore et al., 2021; Turner et al., 2021). Although exact numbers are unknown, this likely resulted in an unintended increase in infection numbers, and delayed progress towards the control and elimination of these diseases. As efforts towards NTD control ramp up again, the concern shifts back towards rising resistance levels, and a limited treatment arsenal will produce further complications.

To combat treatment difficulties associated with increased resistance, new and effective drugs need to be identified. A bottleneck in the anthelmintic discovery process is that, currently, there is no gold-standard screening assay. There are a number of groups dedicated to similar drug discovery efforts; however, their assays often differ in various aspects of experimental design such as media type, incubation time and temperature, desired readout, and the type of chemical library tested (Boyd et al., 2010; Fahs et al., 2025; Katiki et al., 2011; Martel et al., 2020;

Partridge et al., 2017; Risi et al., 2019; Smout et al., 2010). This variation across studies can cause issues in verifying reproducibility and comparing results generated by different research groups, which supports the establishment of a universal screening protocol. There has also been consistent interest in mining natural sources for anthelmintic compounds, but these efforts are largely focused towards plant natural products (Jayawardene et al., 2021; Liu et al., 2020). At the moment, one of the most widely used anthelmintics is ivermectin (Crump and Ōmura, 2011; Martin et al., 2021). The 'wonder drug' and Nobel prize-winning compound is produced by the actinomycete *Streptomyces avermitilis* (Callaway and Cyranoski, 2015; Crump and Ōmura, 2011). Despite the historic success of soil microbes producing potent anthelmintics, there has been little effort to continue searching for microbial secondary metabolites with relevant activity. The preference for plant products is likely rooted in the historical and traditional uses of medicinal plants for treating different ailments, including those of helminthiases (Liu et al., 2020).

Since we have access to an in-house library of extracts generated from edaphic bacterial and fungal strains, we sought to fill this discovery gap by developing a robust assay that is optimized for these natural product extracts. This will hopefully influence the field to expand the screening repertoire beyond bioactive libraries, as this can encourage novel discoveries rather than focusing on drug repurposing efforts.

In tandem with generalized phenotypic assays, our work in developing the target-based nanoluciferase assay is also intended to promote more selective screening strategies. Although research into compounds that disrupt the nematode cuticle is limited, existing studies demonstrate its potential as a therapeutic target. The work here can serve as a tool to facilitate both the identification and validation of cuticle disrupting compounds.

Limitations and Future Directions

While the work completed in this thesis fills a gap in the current research field, there remain some experimental limitations that could be addressed. Although *C. elegans* has been accepted as a suitable nematode model due to its physiological similarity to pathogenic species and a similar treatment response to existing anthelmintics, it is not a parasitic worm (Burns et al., 2015; Coghlan et al., 2019; Hahnel et al., 2020; Suárez et al., 2022). As such, there are gene families that are absent in the *C. elegans* genome that may play pivotal roles in helminth pharmacokinetics. For example, genes involved in host survival and invasion, and immune system evasion are absent in *C. elegans* (Hahnel et al., 2020). For our work, this means that an additional validation step will need to be conducted for all hits, to verify activity in a parasitic nematode species. Other studies have used ruminant and murine helminths such as *Haemonchus contortus* and *Trichuris muris* for screening (Klementowicz et al., 2012; Schärer et al., 2023; Wimmersberger et al., 2013). Therefore, *ex vivo* and *in vivo* testing against this species would be an

appropriate next step. While actinomycin D and tunicamycin are unsuitable for continued testing due to their toxicity against mammalian cells, xanthocillin could be further evaluated against parasitic worms in a whole animal infection model.

In terms of assay optimization and design, we have prioritized data collection and interpretation. All steps of the process are automated, from liquid transfer to imaging, which reduces the risk of human error. The incorporation of endpoint image collection can also provide more mechanistic insight based on the phenotypes observed; however, whether manual scoring or computer-vision scoring should be used can be debated. Manual scoring is labour-intensive and time-consuming, but more phenotypes may be able to be identified. We have not yet tried to train an image analysis algorithm to identify specific phenotypes. Therefore, this technology is currently limited to binary scoring: normal versus abnormal. While this may be sufficient for preliminary results, the high hit rate could be a deterrent if screening a larger library of compounds/extracts.

With respect to immediate future directions, there are still over two dozen hits that could be further investigated for bioactivity. The remaining hits are extracts derived from soil bacterial and fungal species. The order in which to prioritize these will depend on overall objectives. Bacteria are typically easier to work with due to their shorter generation time and smaller genomes. However, fungal extracts are also important to consider as fungi have larger genomes, giving rise to greater

chemical diversity that could prove more desirable for the discovery of novel compounds. Bacteria, fungi, and soil-transmitted helminths all occupy similar environmental niches. This suggests the presence of interspecific competition through chemical production, but further work will need to be conducted to determine which microbes are more prolific producers of nematocidal compounds.

We also described a luminescence-based assay here that could serve as an indicator of cuticle damage. Due to the high output variability, we propose that this method would serve better as a secondary assay for mechanistic studies or should be reserved in cases where test compound is not limited. Previous efforts to reduce variability were unsuccessful due to the challenge of lysing worms without interfering with nanoluciferase integrity. However, if a protocol could be established that eliminated the need for worm lysis, either by improving furimazine penetration into *C. elegans* tissues or improving the lysis procedure, this assay would be more broadly applicable in primary screening efforts. Nevertheless, we have still generated a nanoluciferase-expressing worm strain that can provide valuable insights into a compound's target.

Concluding Remark

Drug discovery has consistently been an important research endeavour in human health and medicine. For NTDs, typical challenges associated with innovation in the field are amplified by lack of funding due to the limited economic

power of the affected population. This generates a positive feedback loop where limited financial return discourages discovery efforts, which leads to limited advancements and a worsening treatment landscape for the individuals that are already socioeconomically vulnerable. Although the translation of screening hits into compound leads for *in vivo* infection models is beyond the scope of this thesis, there is evidence to suggest that microbial natural products are a relevant source for anthelmintics.

Beyond therapeutic development, anthelmintic discovery can also contribute to our understanding of resistance mechanisms, parasite physiology, and host-parasite interactions. These can in turn support additional prevention initiatives such helminth vaccines, which have been difficult to develop due to the complex lifecycles of parasites (Maizels, 2021; Perera and Ndao, 2021; Zawawi and Else, 2020).

Looking forward, many fields will continue to be shaped through advancements in technology. Already we demonstrate here that automation through employing microfluidic devices and high-throughput imaging can reduce the labour associated with complex assay set-up. Artificial intelligence and machine learning are also emerging as crucial approaches in the drug discovery process to help accelerate progress and prioritize compounds (Catacutan et al., 2024; Dara et al., 2022; Vamathevan et al., 2019). As these tools become more accessible in

academic settings, the potential for rapid discovery and validation in resource-limited academic settings skyrockets. This is promising for the field of anthelmintic drug discovery, as it creates opportunities for the development of new, effective, and accessible treatments that will ultimately improve global health outcomes.

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