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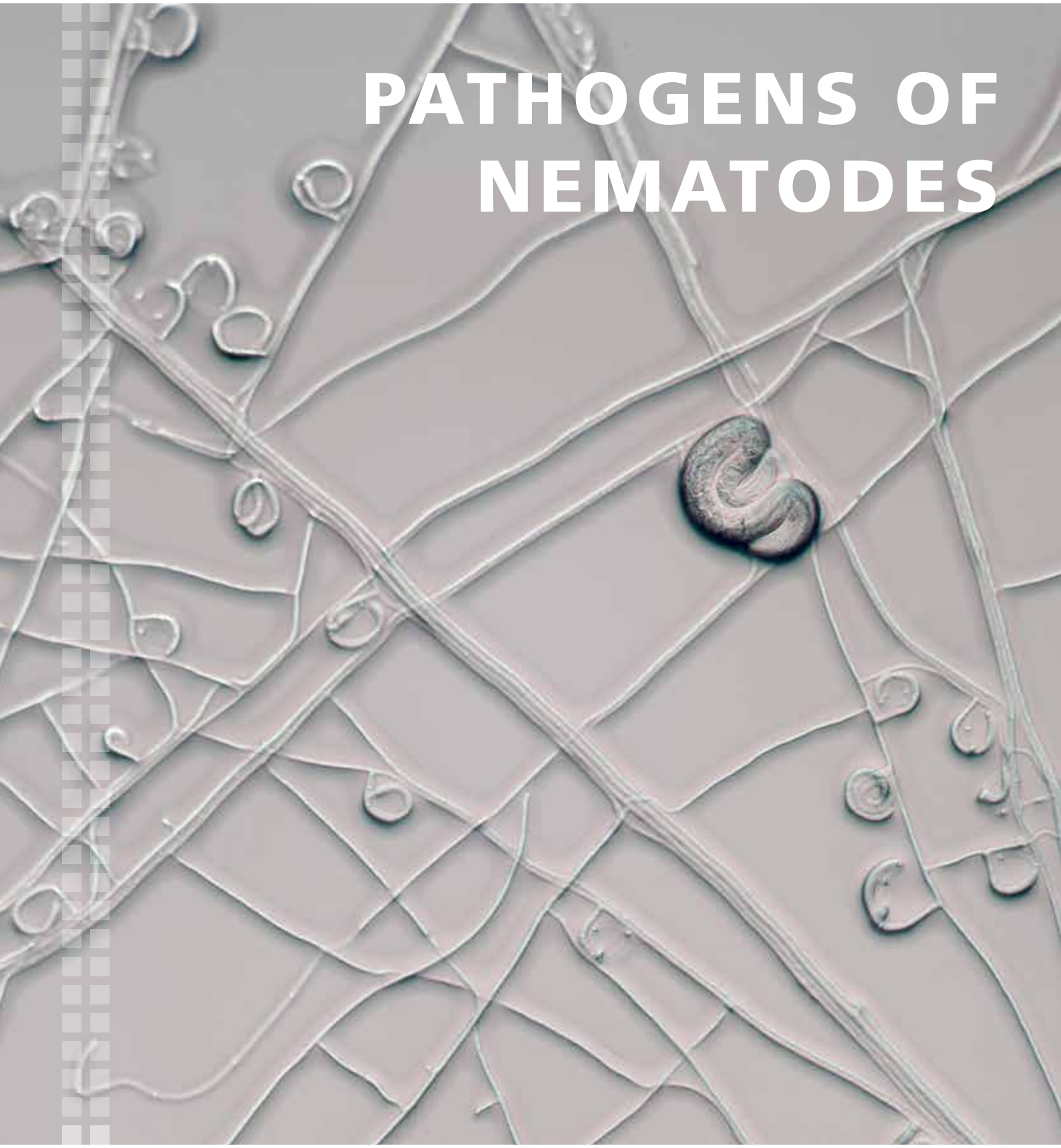
PATHOGENS OF NEMATODES



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Pathogens of nematodes

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Preface

COGEM is often asked to classify microorganisms in one of the four pathogenicity classes. The classification in one of these classes is based on the (absence of) pathogenicity for humans, animals or plants. For some of the recently assessed pathogenic microorganisms there was information available that suggested that the microorganism could also be pathogenic to nematodes (roundworms), but it was difficult to determine whether the available data were indicative of true nematode pathogenicity. To broaden its knowledge base on pathogenicity for nematodes and on methods that demonstrate true pathogenicity, COGEM commissioned a research project, which was carried out by Biotech microbials.

Biotech microbials thoroughly studied the available literature on nematode pathogens and summarized the available information in an informative report that is worth reading. The meetings of Biotech microbials and the advisory committee were pleasant and informative. Suggestions of the advisory committee were carefully weighed before being adopted. The advisory committee is pleased with the resulting report which gives a good overview of the current knowledge on nematode pathogens and on the methods that are used to study pathogenicity.

Prof. dr. J.P.M. van Putten

Chair of the advisory committee

Summary

Nematodes are the most abundant multicellular animals and play a major role in ecosystem functioning, such as the turnover of biomass and mineralization, the structuring of food webs and the population dynamics of associated species. Because of their importance in sustaining ecosystem services, it is important to understand what the effect of an accidental introduction of a nematode pathogen on the populations and associated ecosystem services would be.

In a literature survey, the available information on nematode pathogens was inventorized, in order to better understand the nature of the pathogenesis of nematode. Not for every pathogen molecular and mechanistic information was available and the experimental methods also diverged largely.

For viruses, mainly descriptive data are present, from NGS studies on the genome and transcriptome of nematodes, while for Bacteria and Fungi various virulence factors have been identified of which the infection mechanism has been experimentally dissected.

In Bacteria the primary group of reported nematode pathogens are part of the Bacillota. Nematode pathogenic representatives of this phylum make use of a repertoire of proteases that cause disintegration of the nematode cuticle and underlying tissues and in some case nematode-specific crystal (Cry) proteins, that perforate the intestinal epithelium upon specific binding.

Fungal nematode pathogens are known as nematode trap fungi (ectoparasites), obligate endoparasites and saprophytes that are able to infect eggs. Also the fungal pathogens make use of a combination of several proteases as the main virulence factor. The primary sequences of respective forms of proteases used by bacterial and fungal nematode pathogens show a strong similarity and are distinct from proteases with other functions. In some cases, also other virulence factors were reported (e.g. enterotoxins that act on the integrity of the intestine, lipases that disintegrate tissues and membranes and chitinases that allow infection of pharyngeal tissues and eggs).

The literature of nematode pathogens is largely directed to biocontrol agents for parasitic nematodes of plants and animals. From these reports and discoveries from (meta)genomic surveys it is clear that nematode pathogens are an integral part of the microbial community associated with nematodes and their environment. Several studies have failed to demonstrate a persisting disturbing effect of applying high loads of biocontrol agents on soils (and their resident off-target nematodes) in lab and field conditions. The resource limitations in soils and their partitioning in the soil cause a high competition that excludes introduced, unnatural levels of lab-reared microorganisms to sustain a persistent population. Nematode communities are also resilient in the recovery from dramatic perturbations and can recolonize an eradicated soil or sediment in months. They possess also various mechanisms, like a strong innate immune system and behavioral and developmental plasticity mechanisms to cope with the challenges of pathogens. Also, their complex metapopulation structure, something that is commonly observed in soil invertebrates, may sustain local populations thereby preventing extinction. The local and temporal reduction in ecosystem services can therefore be neglected. From this report we may conclude that it is very unlikely that (accidental) introduction of nematode pathogens will cause any persistent effects on ecosystem services.

Given the precautionary principle, it is recommended to provide at least data on the pathogenesis at the level of monolayer exposure with the model organism *C. elegans* - given the standardized assays and the molecular toolbox that are available -, the mechanisms of infection and the genome sequence of the particular microorganism, in order to make a proper assessment of its potential of nematode pathogenesis. I*n vitro* assays are elegant methods to demonstrate pathogenesis

and dissect virulence mechanisms, but they are not suited to predict the ecological outcomes of the introduction of a nematode pathogen on the populations of nematodes in the ecosystem and their roles in the ecosystem they fulfill.

Ideally, microcosm experiments with standardized nematode communities of aquatic sediments or terrestrial soils are executed. By monitoring the persistence of the introduced microorganism, the species composition of the free-living nematode communities and the nitrogen mineralization rates (the best measurable ecosystem service) the risk of undesired outcome by (accidental) introduction of the pathogen can be assessed.

Samenvatting

Nematoden zijn de meest voorkomende meercellige dieren en spelen een belangrijke rol in een aantal ecosysteemdiensten, zoals de omzet van biomassa en in de mineralisatie, de structurering van voedselwebben en de populatiedynamica van de daarin voorkomende soorten. Vanwege het belang bij het in stand houden van ecosysteemdiensten, is het belangrijk om te begrijpen wat het effect zou zijn van een accidentele introductie van een nematodenpathogeen op de populaties en bijbehorende ecosysteemdiensten. In een literatuuronderzoek werd de beschikbare informatie over nematodenpathogenen geïnventariseerd, om de aard van de pathogenese van micro-organismen op nematoden beter te begrijpen. Niet voor elke ziekteverwekker was er moleculaire en mechanistische informatie beschikbaar en ook de experimentele methoden liepen sterk uiteen.

Voor virussen zijner voornamelijk beschrijvende gegevens aanwezig, vooral uit NGS-studies op het genoom en transcriptoom van nematoden, terwijl voor bacteriën en schimmels verschillende virulentiefactoren zijn geïdentificeerd waarvan het infectiemechanisme experimenteel is ontleed.

In bacteriën maakt de primaire groep van gerapporteerde nematodepathogenen deel uit van de Bacillota. Nematode pathogene vertegenwoordigers van dit fylum maken gebruik van repertoire van proteasen die desintegratie van de cuticula van nematoden en onderliggende weefsels veroorzaken en in sommige soorten werden nematode-specifieke kristal (Cry) eiwitten beschreven, die het darmepitheel perforeren na specifieke binding.

Pathogene schimmels van nematoden staan bekend als nematodenvalschimmels (ectoparasieten), obligate endoparasieten en saprofyten die eieren kunnen infecteren. Ook de schimmelpathogenen maken gebruik van een combinatie van verschillende proteasen als de belangrijkste virulentiefactor. De primaire sequenties van respectievelijke vormen van proteasen die worden gebruikt door bacteriële en schimmelnematodepathogenen vertonen een sterke gelijkenis en onderscheiden zich van proteasen met andere functies. In sommige gevallen werden ook andere virulentiefactoren gemeld (bijv. enterotoxinen die werken op de integriteit van de darm, lipasen die vetlagen en membranen oplossen en chitinasen die de infectie van faryngeale weefsel en eieren faciliteren).

De literatuur over nematodenpathogenen is grotendeels gericht op biocontrolemiddelen voor parasitaire nematoden van planten en dieren. Uit deze rapporten en ontdekkingen door middel van (meta)genomics is duidelijk dat nematode pathogenen een integraal onderdeel zijn van de microbiële gemeenschap geassocieerd met nematoden en hun omgeving. Verschillende studies hebben geen aanhoudend storend effect aangetoond van het toepassen van hoge belastingen van biocontrolemiddelen op bodems (en de aanwezige off-target nematoden) in laboratorium- en veldomstandigheden. De beperkte aanwezigheid van middelen (zoals bijvoorbeeld nutriënten, water en niches) en de verdeling ervan in de bodem veroorzaken een hoge concurrentie die uitsluit dat een introductie van een in het laboratorium gekweekt micro-organisme, bij onnatuurlijke hoge dichtheidsniveaus een persistente populatie in stand kan houden. Nematodegemeenschappen zijn ook veerkrachtig in het herstel van dramatische verstoringen en kunnen een uitgeroeide gemeenschap in bodem of sediment in maanden opnieuw koloniseren. Ze beschikken bovendien over verschillende mechanismen, zoals een sterk aangeboren immuunsysteem en gedrags- en ontwikkelingsplasticiteit om de uitdagingen van blootstelling aan pathogenen het hoofd te bieden. Ook kan hun complexe structuur van metapopulaties, iets dat vaak wordt waargenomen bij bodeminvertebraten, lokale populaties ondersteunen en lokale extincties voorkomen. De plaatselijke en tijdelijke vermindering van ecosysteemdiensten kan daarom worden verwaarloosd. Uit dit rapport kunnen we concluderen dat

het zeer onwaarschijnlijk is dat (accidentele) introductie van pathogenen van nematoden blijvende effecten zal hebben op ecosysteemdiensten.

Gezien het voorzorgsbeginsel wordt aanbevolen om ten minste gegevens te verstrekken over de pathogenese op het niveau van blootstelling aan monolagen met het modelorganisme *C. elegans* - gezien de gestandaardiseerde testen en de moleculaire toolbox die beschikbaar zijn -, de infectiemechanismen en de genoomsequentie van het specifieke micro-organisme, om een goede beoordeling te maken van het potentieel van de pathogenese van nematoden. *In vitro* assays zijn elegante methoden om pathogenese aan te tonen en virulentiemechanismen te ontleden, maar ze zijn ongeschikt om de ecologische uitkomsten van de introductie van een nematodepathogeen op de populaties van nematoden in het ecosysteem en hun rol in het ecosysteem die ze vervullen te voorspellen. Idealiter worden microcosmos experimenten uitgevoerd met gestandaardiseerde nematodengemeenschappen van aquatische sedimenten of terrestrische bodems. Door het monitoren van de persistentie van het geïntroduceerde micro-organisme, de soortensamenstelling van de vrijlevende aaltjesgemeenschappen en de stikstofmineralisatiesnelheden (de best meetbare ecosysteemdienst) kan het risico op ongewenste uitkomst door (toevallige) introductie van de ziekteverwekker worden beoordeeld.

Abbreviations

ADP: adenosine diphosphate

CUB: complement C1r/C1s, Uegf, Bmp1 (CUB-like domain)

dsDNA: double stranded deoxyribonucleic acid

EFSA: European Food Safety Authority

GMO: genetically modified organisms

LC_{50}: exposure concentration at 50% lethality

MAMP: Microbe-Associated Molecular Pattern

MAPK: Mitogen-Activate Protein Kinase

NGS: Next Gen Sequencing

PAMP: Pathogen-Associated Molecular Pattern

PEG: polyethylene glycol

RKN: root knot nematode

rRNA: ribosomal ribonucleic acid

SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis

ssRNA: single strand ribonucleic acid

1. Introduction

1.1 Aim of this study

Genetically modified micro-organisms are widely used in experimental research. The level of containment for such experiments is partially determined by the impact of (accidental) escape of the micro-organism into the environment. Nematode populations provide essential ecosystem services. Therefore, it is important to be able to evaluate if a micro-organism is pathogenic for nematodes. This report provides an overview of the different types and characteristics of nematode specific pathogens and the potential impact of these organisms on nematode populations in the environment.

Micro-organisms and other replicating biological entities are widely used as biocatalytic agents, biological control agents in agriculture and (veterinary) health, plant stimulation formulation, and as genetic vectors in biotechnology. By applying them, the risk exists that the organism escapes to the environment, infects non-target species, and creates downstream effects on the ecology of the environment. Containment measures need to be stricter for pathogens. This report provides an overview of the current knowledge on pathogens for nematodes. We will focus on pathogens that target free-living nematodes rather than higher animals or plants and describe common virulence mechanisms. Microbes that release secondary metabolites for competition in the microbial community fall outside the scope of this report.

In this report the results of a literature survey are presented in order to inventorize the knowledge about pathogens of nematodes for the assessment of the pathogenicity of micro-organisms for nematodes. We will focus on pathogens that target with a direct interaction with and subsequent infection of nematodes, with a special emphasis on free-living nematodes rather than higher animals or plants and describe common pathogenesis mechanisms in nematodes. As direct interaction we understand the infectious mechanism of a gene product (i.e., protein) on the nematode structure but also the activity of specific metabolites on the homeostasis of nematodes (narrow spectrum), but only when the authors of the respective study have proven that it is likely a specific adaptation to the infection of nematodes. Microbes that induce plant defense pathways or release secondary metabolites for competition in the microbial community fall outside the scope of this report.

1.2 Ecological roles of nematodes in the environment

Nematodes are the most abundant animal phylum in the biosphere and can be found in nearly all environments where water is present (sediments, soils and biofilms) (Yeates *et* al, 2009; van den Hoogen *et al.*, 2020). Many representatives exhibit a parasitic lifestyle in animals and plants, while the majority of free-living forms can be found in the meiofauna from freshwater to marine sediments, from arid soils to sea ice cavities, and from anthropogenic environments to the deep-surface lithosphere. Given their ubiquity of $>10^6$ free-living nematodes/m^2 decreasing from tundra to the equator and highly dependent on water (van den Hoogen *et al*,. 2020), and their divergence in several ecological niches and trophic groups, nematodes have a significant role in the functioning of ecosystems (Yeates *et al.*, 2009; Schratzberger *et al.*, 2019). More than 27,000 nematode species have been described, of which about 60% are parasitic (15% plant parasites, 45% animal parasites, both invertebrate and vertebrate-associated) (Hugot *et al.* 2001). Free-living nematodes are being described

at a high pace and increase the share of known free-living nematode species in these figures. A global meta-analysis showed that the most common trophic terrestrial groups of free-living nematodes are the bacterivores, herbivores and fungivores, followed by the omnivores and predators (van den Hoogen *et al*, 2019). With an estimated number of 40,000 up to 10,000,000 species, nematodes make an important contribution to the biodiversity on Earth (Yeates *et al.*, 2009).

Parasitic nematodes

Nematodes are best known for their nuisance as parasites in most forms of multicellular life. Parasitic nematodes of vertebrates are generally larger and produce more eggs than the parasites of invertebrates and plants and free-living forms (Morand and Sorci, 1998). Parasitism adds an additional trophic level to primary production, herbivory, predation and decomposition and as such parasites act indirectly on these other trophic levels through the productivity and behavior of their host (Dobson Hudson, 1992). Despite the fact that parasitism by nematodes creates a burden on human society (~160 billion US dollar in reduced crop yield by plant-parasitic nematodes (Singh, *et al.*, 2015), infected cattle and pets, and debilitating diseases by human parasites), parasitism has a fundamental ecological role. Parasitic nematodes that feed on the resources from the host have an impact on the population dynamics of their hosts species, hence they have a significant impact on the functioning of the ecosystem. Some parasitic nematodes (such as ascarids) that live in the gut lumen of animals are reported to have merely an indirect effect on the host, since they only feed on the microbes present, while other parasites feed on the tissues of the host. By modifying the fitness and vigor of individuals in populations their ecological interactions are maximized and energy flows are increased (Hudson *et al*, 2006). Besides this, parasitic nematodes can deliver ecosystem services to agriculture by reducing for example the abundance snails or insect pests, or by the introduction of entomopathogenic bacteria. This has been demonstrated e.g., in the *Steinernema-Xenorhabdus* and *Heterorhabditis-Photorhabdus* symbioses (Sicard *et al*, 2004)

Free-living nematodes

Free-living nematodes exhibit various trophic groups: bacterivorous, fungivorous, substrate/deposit feeders, protist feeders, plant feeders, animal predators, omnivores, as well as dispersive stages of parasites (Holterman *et al.,* 2006). Moreover, indirect heterotrophy by the direct uptake of dissolved organic compounds, or ecto- and endosymbiosis with micro-organisms for supporting the metabolism in e.g., deep sea and anoxic environments has been described. Their overall roles in ecosystem functioning are directly related to their feeding activities and digestive metabolism, whilst their specific effects are related to functional adaptation to certain niches or biotic interactions. They are the most important part of the meiofauna, the microscopic animal communities that are strongly connected to the microbiomes of decomposition in soils and sediments and that thrive on the biofilms on surfaces. In these communities, nematodes increase the turnover of biomass in both primary production as decomposition, *i.e*., nutrient cycling, by top-down control at various trophic levels, whilst increased metabolic activity in grazed bacterial populations has been observed. The share of soil mineral nitrogen, NH_4^+ and NO_3^-, set free upon decomposition induced by grazing of nematodes on the microbial populations can amount to 50% (Ferris *et al*, 1998). In mesocosm experiments, bacterial feeding nematodes can increase the nitrogen mineralization with 50-100%, as compared to a control. This has also been observed in the case of fungal grazing nematodes, but this was highly dependent on the combination of species involved (Ferris *et al.*, 1998; Chen and Ferris,1999). Cranking up the nitrogen cycle by the grazing of nematodes is essential for the development of vegetations in general and fertility in particular.

In marine sediments, an experimental reduction of meiofauna (mainly consisting of nematodes) results in a steep decline in the mineralization of organic matter. Based on the shifts in the composition of stable isotopes (*i.e.* a difference in the number of trophic levels) and community composition of bacteria the altered mineralization was explained by nematode grazing on the decomposing microbial community (Nascimento *et al.*, 2012).

Nematodes excrete up to 8% of nitrogen as ammonia upon metabolizing proteins and subsequently enhance the bioavailability of mineral nitrogen in the environment. Since they are grazed upon by larger animals (such as e.g., mites and springtails in terrestrial systems and for example polychaetes and crustaceans in marine environments), they are a major link for redistributing nitrogen to higher trophic levels.

Because of their motility, nematodes are also responsible for a significant part of bioturbation of sediments and soils and redistribution of microorganisms. Absorption of dissolved organic molecules through the cuticle is an additional trophic strategy that has been described in various groups of nematodes (including free-living marine, terrestrial and parasitic representatives). All herbivorous nematodes directly feed on higher plants by parasitizing on the sap flow by directly foraging on vascular or foliar tissues by means of a stylus or by creating a sedentary gall tissue on a nutrient rich storage tissue. In these ways they shape vegetations accordingly, by interfering with the fitness of the host plants. Nematodes can also structurally shape the community in *e.g.*, aquatic sediments and mudflats, where certain nematodes produce sheets of mucus to agglutinate detritus and micro-organisms (Riemann & Hemkes, 2002). This also contributes to sediment stability and the creation of a succession series. Given the cumulative ecological effects of the different trophic guilds and interaction with the abiotic environment, and its abundance nematodes are drivers of ecological succession in some environments, such as estuarine mud flats, grasslands and the colonization of hosts by parasites and pathogens.

Impact of changes in nematode populations

As mentioned, nematodes are the most abundant phylum of multicellular animals and involved in many essential ecosystem services, of which some of major economic importance (Yeates *et al.*, 2009). They have a major role in the trophic control of decomposition of decaying biomass and returning the nutrients back to higher trophic levels. The ecological consequences of declining nematode populations would be the most significant in the case of free-living nematodes. This would cause a reduction in mineralization rates of organic matter in soils and sediments, due to reduced grazing and bioturbation (redistribution of organic matter with micro-organisms) and a stabilization of certain aquatic sediments. These ecosystem services are essential in the food production by agriculture, the bioremediation of waste and sludge, and the management of marine resources, such as spawning areas for fish and other seafood.

Because parasitic nematodes create an extra trophic level in the food web, they strengthen the interactions in the food webs. They can modulate population levels of herbivores and predators and create variability in growth rates of plants in vegetation. They can also have direct economic benefit by suppressing populations of insects and molluscan pests. Many of these interactions are still part of the hidden biodiversity, such as those referred to as soil suppressiveness (Schlatter *et al.*, 2017), that could be attributed to undescribed interactions between parasitic nematodes, their hosts, and associated microbiomes.

Considering the important function of nematodes in ecosystem health and biodiversity, it is important to better understand how pathogens can infect and kill nematodes. This knowledge is also

essential to evaluate the risk associated with the introduction and application of various biological agents that besides their desired beneficial effects may cause serious off-target nematode population decline or extinction, resulting in loss of the abovementioned ecosystem services.

1.3 Virulence, pathogenicity and toxicity

Definitions
Commonly used terms in infectiology are pathogen, biological agent, virulence and toxicity. However, their definition is not always clear. The following chapter will elaborate on this.

Pathogens are defined in this report as organisms that *can* cause disease in another organism (Pallen & Wren, 2007). They are subdivided in a number of pathogenicity classes, according to their risk for causing disease in humans, animals or plants (https://wetten.overheid.nl/BWBR0035072/2022-07-01).

> Cass 1 microorganisms are known non-pathogenic or are safely used as based on historical evidence, or they belong to the same species as known pathogens but the particular strain is lacking the virulence factors for infection or the absence of pathogenesis can be proven by adequate test. This class also encompasses opportunists.
>
> A class 2 microorganism is a pathogen that can cause disease in humans or animals, but unlikely spreads through the population and an appropriate control, treatment or prophylaxis is at hand. Also plant pathogens are classified as Class 2 micro-organisms.
>
> Class 3 microorganisms are severe human and animal pathogens that can putatively spread throughout a population, but an appropriate control, treatment or prophylaxis is available.
>
> Class 4 microorganisms cause very severe diseases, are probably spread through populations and lack an appropriate treatment, control or prophylaxis. Also for GMO-activities the micro-organisms are graded in the same classes.

Virulence is a quantitative parameter of infection and defined as the manifestation of detrimental effects on the health, reproductive output, or survival of already infected individuals, since pathogenicity is mainly determined by the ability of transmission of the pathogen to the host (Thomas & Elkington, 2004). Virulence can vary dependent on the strain and local environmental conditions as well as on the properties of the host (Pallen and Wren, 2007). A pathogen can show commensal behavior when the host is not susceptible or resistant to it (Casadevall & Pirofski, 2001). The fact that virulence is determined by the balance between pathogenicity factors and the specific resistance or immunity of the host implies that strains that are avirulent in healthy hosts may cause disease in compromised individuals. This type of interaction is defined as opportunistic infection. Opportunistic interactions are not taken into account in this report on pathogens of nematodes.

Toxins are biological products that are harmful to other organisms. Often micro-organisms produce toxic metabolic end-product and secondary metabolites, of which the biosynthesis is induced by specific abiotic and biotic conditions. The primary function of these secondary metabolites is in competing with surrounding communities for resources, or by direct inhibition of competitors. This type of products, which have no direct effect on the host, will not be further discussed in the report. However, pathogens may produce (additional) toxins that directly target host cells or metabolism and

thus contribute to the establishment of infection. These factors as well as other determinants of virulence (Casedevall & Pirofski, 2001) will be discussed in Chapter 2.

The determinants of virulence applied to pathogens of nematodes are replication and transmission, invasiveness, required inoculum size, adherence and attachment and innate defense responses by the nematodes and the repertoire of virulence factors involved. These aspects will be further dealt with in the systematic literature overview and the subsequent discussion.

1.4 Critical biological systems for infection control in nematodes

An important step in infection of nematodes is the colonization of the host. Pathogen entry of the host occurs mainly via the external cuticle, the mucosal epithelia of the digestive or genital tract. In some cases, the egg-shell is the main barrier.

External cuticle as attachment site

The nematode cuticle represents the exoskeleton of the nematode. It is replaced four times upon molting in the four life stages of the nematode. Its protein content consists primarily of collagen. This collagen is mainly found in the basal and medial layers of the cuticle (Fetterer & Rhoads, 1993). The cortical layer consists of other structural proteins. The collagen monomers are about 35 kDa in mass and have a Gly-X-Y (X and Y, proline and hydroxyproline) motif that is interrupted regularly with other sequences (Page *et al.*, 2014). In *Caenorhabditis elegans,* 167 collagen types are present. These monomers are cross-linked by cuticlin via sulfide bridges that can be disrupted by reducing agents. Collagen is absent from the outer layer (epicuticle) which consists of lipids; the surface coat consists of non-structural glycoproteins. In some animal parasitic nematodes, an alternative crosslinking mechanism of collagen monomers by tyrosine-derived residues has been described (Fetterer & Rhoads, 1993).

The surface coat of the nematode cuticle can directly interact with pathogens and consists of glycosylated proteins structured in microvilli with mucins (Davies & Curtis, 2011). The analysis of mutants has revealed a role of the glycosylation of the surface coat protein in the adhesion of various pathogens. Besides as attachment site, the cuticle can be subjectto degradation. The sensitivity of the nematode cuticle to degradation by (microbial) hydrolytic enzymes differs between species. Furthermore, earlier life-stages of parasitic nematodes can be more resistant to enzymatic degradation as compared to the adult infecting stages (Page *et al*, 2014) likely due to the different surface coat secretions (Davies & Curtis, 2011).

Egg-shell as pathogen target

The outer layer of the nematode egg is important in preserving the reproductive output since nematodes do not have broodcare (except some viviparous representatives) and eggs need to protect the offspring in potentially harsh conditions. The egg-shells of the potato cyst nematode *H. rostochiensis* consist of more than 80% of protein. The chitin content amounts to 9%. (Clarke *et al.*, 1967). The egg is the most chitin-rich part of the nematode. The chitin layer is an internal part of the egg-shell and is surrounded by a protein rich vitelline layer. It gives rigidity to the egg-shell. Below the chitin layer lays a proteoglycan-rich and a lipid-rich layer, which make the egg-shell impermeable for

many compounds (Chen Peng, 2019; Stein & Golden, Wormbook). During infection, pathogens need chitinases in order to efficiently penetrate the egg-shell.

Besides the structural defenses of the nematode body, other factors, such as innate immunity and signaling, induced behavior, longevity mechanisms and the microbiome, are determining the susceptibility of nematodes for infection by pathogens.

Mucosal epithelia as infection route

The digestive system of nematodes can be divided into the foregut, midgut and hindgut. The foregut and hindgut are also covered with a cuticle (Basyoni & Rizk, 2016) under which a fixed number of intestinal epithelial cells (20 in the case of *C. elegans*) that do not differ largely from their mammalian counterparts in structure (such as microvilli enforced with actin filaments) are present. Based on the tissue-specific expression of two chitinase genes in *C. elegans* (Zhang *et al.*,2005a), chitinase was observed in the pharyngeal region. The presence of chitin was confirmed with histochemistry. Also in the animal parasite *Oesophagostomum dentatum* chitin was shown in the pharynx (Neuhaus *et al.*, 1997). It is likely that the hard structures in the buccal cavity and pharyngeal lining (including spears and teeth) consist of chitin (Zhang *et al.*,2005a; Neuhaus *et al.*, 1997). There is, however very little evidence documented in the literature on this topic.

Especially for nematodes feeding on (substrates that contain) microorganisms, the intestinal epithelia in the midgut are paramount as a defense line against infection (Cohen and Troemel, 2014). Nematodes possess just an innate immune system that triggers the expression of a battery of defense factors such as C-type lectins, ShK (Stichodactyla)-like toxins and CUB-like domain containing proteins. The main immune regulator is the p38 MAPK signaling pathway which is specifically induced through a set of G-coupled proteins and a single Toll-like receptor. In parallel additional signaling pathways for the induction of innate immune responses by specific pathogens have been described. Since no evidence exists that molecular patterns are recognized (such as MAMPS or PAMPS, like e.g., in plants), and since some of the pathways are related to stress and longevity mechanisms, it is likely that the indirect but specific effects of the pathogen on the nematode play role in the expression of the effectors (Pukkila-Worley & Ausubel, 2012).

Microbiome

The resistance of the host to pathogens not only involves physical barriers but also natural competition by other organisms, collectively named as microbiome. While the identification of a distinct gut microbiome in animal parasitic nematodes is less clear (Midha *et al.*, 2017), because it diverges between free-living and parasitic stages, and between healthy and diseased hosts, the gut microbiome of the free-living nematode *C. elegans* is better characterized. Its composition differs from the consumed bacteria. The overrepresented and persisting taxa (and isolates thereof) are e.g., *Ochrobactrum, Stenotrophomonas, Sphingomonas* and *Pseudomonas* (Dirksen *et al*, 2016). The selected taxa aid in combating environmental stressors, support fecundity, and protect against fungal pathogens. In a recent study, Han *et al.* (2021) showed that a *Stenotrophomon*as isolate can assist in the host resistance against a nematode-specific pathogen *Bacillus nematicida*. Also, in another soil-dwelling bacteriovorous nematode *Acrobeloides maximus* a significant part of the resident microbiome consisted of *Ochrobactrum* and the sphingobacterium *Pedobacter* (Bacquiran *et al.,* 2013), which suggest that these taxa may play a shared role in colonization resistance in various nematode species. A microbiome survey on 281 free-living marine nematodes isolated from various marine sediments, revealed no microbiome shaping factors (Schuelke *et al.*¸2017), such as the suspected feeding type of

the host. However, the co-occurrence of various known invertebrate pathogens indicates the potential of interactions within the microbiome. In another study on marine free-living nematodes a strong association with selective diet was found in the microbiomes of two cryptic bacteriovorous species (*Litoditis marina* complex), which can be explained by the resource partitioning within the same suspected feeding type (Derycke *et al*, 2006). Bellec *et al.* (2019) have shown a species-specific microbiome, different from the sediment with seasonal patterns, gamma-proteobacterial ectosymbionts of *Metoncholaimus albidus* that are involved in chemosynthetic sulphate reduction.

Overall, these reports suggest that the nematode-associated microbiome is partially influenced by the diet and the environment but that core microbiome members are selected upon. Their presence allows the commensal presence of pathogens and can reduce the susceptibility of the nematode for pathogens, like observed in experimental conditions with *Bacillus nematicida*. The role of the resident microbiome cannot be neglected, also in laboratory studies.

Longevity, developmental and behavioral plasticity mechanisms

Apart from the activation of the innate immune system by signaling pathways induced by pathogens, nematodes exert some developmental and behavioral plasticity mechanisms that allows them to cope better with pathogens. During periods of adverse conditions, innate immunity is induced by a longevity mechanism, which is insulin-regulated (Kurz & Tan, 2004) and was also shown to be induced by exposure to pathogens (Leroy *et al*, 2012). This pathway is also involved in innate defense pathways and can lead to the formation of a Dauer stage in Rhabditidae (a resistant propagule to withstand periods of stress). Several studies have shown evidence that the age of nematodes is inversely related with survival after pathogen colonization (Laws *et al.*, 2004) and that longevity during colonization is determined by the interaction between host and pathogen genotypes (Gravato-Nobre *et al*, 2020). Dietary restriction in the environment can induce the longevity mechanisms and hence the innate defense pathways.

Recent research has revealed that also the nervous system plays an important role in inducing the innate immune response towards infection by microbial pathogens, because not all defense reactions could be explained by the MAPK-pathways (Wani *et al*, 2020). This has been demonstrated by the stimulation of the development of CO_2-sensing neurons in *C. elegans*, as induced by Toll-like receptor signaling, and the subsequent negative chemotaxis towards pathogens (Brandt and Ringstad, 2015).

This induced negative chemotaxis by nematodes towards toxic and pathogenic bacteria has been observed in multiple cases. Beale *et al.* (2006), Zhang *et al.* (2005b) and Pradel *et al.* (2007) have shown that *C. elegans* exhibits avoidance behavior of respectively quorum-sensing molecules (*i.e.* communication signals) of *Pseudomonas aeruginosa* and the surfactant serrawettin of *Serratia marcescens* upon prior exposure to the pathogens. Akduman *et al.* (2018) observed a weak correlation trend of avoidance behavior and the survival of *Pristionchus pacificus* on the same strains. Also this nematode avoided the pathogens *S. marcescens* and *B. thuringiensis* (Rae *et al.*, 2008).

Exposure of hermaphroditic *C. elegans* to the bacterial pathogen *P. aeruginosa* induced the formation of ascaroside-receptors in sensory neurons, an increased their mating rate with males. It is believed that this mechanism of induced social behavioral plasticity facilitates the adaptation by increasing genetic diversity in exposed populations (Wu *et al.*, 2023).

2. Infection of nematodes – overview

In soils, pathogens of nematodes are part of the microbial communities and cause mortality when the conditions for their proliferation are met. As such, nematode-suppressive arable soils have been described and linked to the abundances of taxa like *Bacillus*, *Arthrobacter, Lysobacter* and several previously known fungal pathogens of nematodes (Castillo *et al.*, 2017). The suppressive and conducive microbiome effects were transferable by exchanging soil and micro-organisms between crops and could be attributed to certain cultivable isolates (Zhou *et al.*, 2019).

The concept of suppressive soils for nematodes is used in so-called conservation biological control. The strategy can be applied to facilitate an outbreak from organisms that infect nematodes (with broad or narrow specificity) with the aim to lower the burden on *e.g.,* crops by plant-parasitic nematodes (Timper, 2014; Silva *et al*, 2022). Putative pathogenic micro-organisms have also been detected in microbiomes of free-living marine nematodes (Schuelke *et al*, 2017). How these micro-organisms regulate these populations and communities is unknown.

In the following sections, an overview of the current knowledge on nematode-specific pathogens for Bacteria, Fungi and viruses is presented. Publications were collected after searching Google Scholar and via references from primary research papers and reviews.

2.1 Bacteria

Non-nematode specific bacterial pathogens

The bacterivorous nematode *C. elegans* is frequently used as an infection model to study the behavior of human pathogens (Darby, 2005). This has led to interesting discoveries that allowed extrapolation towards human innate immunity, longevity and molecular signaling (Kurz and Ewbank, 2000; Siffri *et* al, 2003; Laws *et al.*, 2004). Garsin *et al.* (2001) showed that a series of human bacterial pathogens is capable of colonizing the *C. elegans gut*, sometimes without causing an increase in mortality (e.g., *Enterococcus faecium*). Other human pathogens including *S. aureus*, *S. pneumoniae* and *Enterococcus faecalis,* did cause increased nematode mortality. The effect of *Enterococcus faecalis* was due to extracellular proteases and cytolysins which have a very broad spectrum of activity (Siffri *et* al, 2002; Coburn and Gilmore, 2003). *Pseudomonas aeruginosa* is another common pathogen that can infect a wide taxonomic range of hosts. In *C. elegans* it can manifest in a slow infection mode (colonization of the intestine) and a quick killing mode, caused by toxins such as phenazines and HCN. These effects are not nematode-specific. The pathogens *Burkholderia pseudomallei* and *B. thailandensis* exhibit variable pathogenesis in *C. elegans* which can be attributed to neuromuscular interference of presumably endotoxins (O'Quinn *et al*, 2001).

Since most of the pathogens studied in the *C. elegans* host are not nematode-specific, not competitive to obtain a niche in the environment of nematodes, tested in ecologically non-relevant conditions, and already classified in class 2 and 3 microorganisms as animal or plant pathogen, they are not further taken into account in this overview which focuses on nematode-specific pathogenic effects.

Obligate nematode pathogens

The *Pasteuria* life cycle

Probably the most characteristic and specific pathogen of nematodes belong to the genus ***Pasteuria***, closely related to genus *Bacillus.* Different *Pasteuria* species have a narrow range of hosts, on which they entirely rely to complete their life cycle and vegetative growth before the encapsulation as a spore., Currently, 323 species from 116 genera of nematodes are known to be infected by *Pasteuria* (Tian *et al.*, 2007a). *P. penetrans* is specific for a root-knot nematode of the genus *Meliodogyne*, *P. thornei* is specific for *Pratylenchys brachyurus* and some *Meloidogyne.* Cyst nematodes can be infected by *P. nishizawae* and *Belonolaimus longicaudatus* by *P. usgae* (Davies *et al.*, 2010). Still novel species of the genus are discovered infecting other species of nematodes, e.g., the plant parasitic *Rotylenchus reniformis* (Schmidt *et al.*, 2010*)*, the ash pathogen *M. ardenensis* (*Pasteuria hartismeri*) (Bishop *et al*, 2007). There are also numerous reports of *Pasteuria* infections in free-living nematodes (which are probably under-represented in the studies). From a detailed metabarcoding study in Scottish soils a strong association of *Pasteuria* species with soil parameters and a negative association with the abundances of their host nematodes was observed (Orr *et al.*, 2020).

Because of the obligatory parasitic lifestyle of *Pasteuria*, its life cycle *is* the infection cycle, that consists of Phase I: attachment and germination; Phase II rhizoid production and exponential growth, and Phase III sporogenesis (Davies *et al.*, 2011). The bacteria are present as endospores in the environment (soil) and attach to their host by means of a specific structural interaction when a second stage juvenile (J2) is encountered. This structural interaction is conferred by a collagen-like protrusion from the endospore that specifically clings to the surface coat of the cuticle of the nematode host, like in a Velcro material. Experimental evidence indicates that the nature of the interaction is between carbohydrate moieties of glycosylated proteins (Davies, 2009). This interaction determines the specificity. Populations of *P. penetrans* show detectable micro-evolution of attachment rates when confronted with alternative populations of their host (Liu *et al*, 2018). In a subsequent comparative genomics study, it was shown that a diversifying selection takes place in the evolution of *Pasteuria* collagen-like adhesive proteins. Not only in the limited match in tree topology as compared to the phylogeny based on 16S rRNA genes, but also the homology to a.o. viral collagen-like sequences points to a role of horizontal gene transfer (Srivastava *et al.*, 2019).

Germination of the endospore of *Pasteuria* species only happens when the nematode is able to enter the root of the host plant (*Meloidogyne*), but in *Heterodera* this can happen instantly. The formation of rhizoids allows *Pasteuria* to migrate through the pseudo-coelom to the reproductive tissue of the host. The molecular triggers and the nature of the disintegration at the cuticle, i.e., the enzymes involved, are still not understood (Ciancio, 2018), given the difficulties of performing experimental work with a hyper-parasitic bacterium. Both enzymatic and mechanical penetration are suggested (Tian *et al.*, 2007a). A certain isolate of *P. penetrans* is only show adherence to a nematode host, but not germination and hence arrested development, possibly due to presumed different mechanisms of specificity. This can lead to a loss of suppressiveness when a new species of e.g., plant-parasitic nematode colonizes a soil (Cetintas & Dickinson, 2004). Attachment to the nematode cuticle and simultaneous antagonism on plant-parasitic nematodes has been observed on *Meloidogyne hapla* by *Microbacterium, Sphingopyxis, Brevundimonas, Acinetobacter*, and *Micrococcus*, and caused adverse effects on *M. hapla* (mortality, reduced mobility). The attachment was not restricted to this species; the isolates also attached to the lesion nematode *Pratylenchus penetrans* (Topalović *et al.* 2019). Possibly, the attachment to the surface coat of nematodes is a more general phenomenon as compared to the interaction described in *Pasteuria.*

The exponential vegetative growth of *Pasteuria* further continues in internal microcolonies in the ovaries of the developing female, which further keep on feeding of the host plant and eventually dies. The mature endospores, up to millions per female, are released to the soil upon decomposition of the cadaver and plant roots.

The suppression of nematodes by *Pasteuria* takes place at two levels: (1) Inhibition of root invasion by mechanically disturbing the motility, and (2) Destruction of the reproductive system of the nematode host. *Pasteuria* is not directly killing the hosts but rather hijacking the life cycle of the host for its own purpose. Therefore, it is an internal pathogen that infects the reproductive system as a hyper-parasite.

Pasteuria penetrans has been shown to significantly reduce the number of nematodes in the roots and root-knot infested roots in a variety of crops when administered to the soil (Stirling 1984). The inhibitory effect on the invasion was reported by Talavera *et al.* (2002), who observed that besides an increased tomato yield the abundances of *M. incognita* in the surrounding soil were significantly higher upon *Pasteuria* treatment. These were juvenile nematodes that were unable to complete their life cycle due to restricted motility.

As discussed earlier, *Pasteuria* is present in natural soils (Orr *et al*, 2020). Their presence is often negatively associated with certain nematode taxa, because of suppression effects. Their endospores are persistent for decades in the soil. Prevalence of adhered spores to individual nematodes in field samples has been commonly observed microscopically in several studies (Orr *et* al, 2020; reviewed in Ciancio, 2018). Naturally *Pasteuria*-related suppressive soils are hypothesized to contain sufficient endospores to maintain the host densities under damaging levels. Endospores disappear from soil by irreversible attachment to host individuals, percolation into deeper layers, and possibly as diet of other organisms.

Nematode-specific *Bacillus* species

Bacillus nematicida is a nematode-specific pathogen isolated from soil (Niu *et al*, 2006a). Infection with the pathogen caused 85% mortality (over 84 h) in the free-living nematode *Panagrellus redivivus*. The identified virulence factor was a serine protease Bace16 with an alkaline optimum, however the overall mortality was higher than that of an equivalent number of units the serine protease. In a following study Niu *et al.* (2006b) identified a second protease Bae16 that was able to kill *P. redivivus* and the plant parasitic nematode *Bursaphelenchus xylophilus.* The combined action of both proteases had a lower LC_{50} (for 24 h exposure) in the two species as compared to the separate enzymes. No mortality exerted by *B. nematocida* on *B. xylophlius* was found, however. *Bacillus nematicida* has been reported to attract their prey by volatile organic compounds. When ingested by the bacterivorous nematode *C. elegans*, it is the intestine that gets infected and lysed by the combined proteolytic activity of the two identified proteases that hydrolyzed a specific set of intestine proteins (Niu *et al*, 2010). In this study the pathogenesis with nematicidal outcome was also demonstrated in microcosms with natural soil, which emphasizes the ecological relevance of this pathogen. Despite this interesting result, no follow-up studies have appeared that have evaluated the use of this isolate in biological control of parasitic nematodes.

A related bacterium ***Bacillus firmus*** also produces a strong serine protease named Sep1. This enzyme hydrolyses proteins from the intestine of nematodes. The pathogen causes high mortalities (resp. 98% and 70% over 36 h) in the root knot nematode *Meloidogyne incognita* and the soybean cyst nematode *Heterodera glycines* (Geng *et al*, 2016). The Sep1 protease was discovered after a functional screen of recombinantly produced potential nematocidal protein. The lethal activity of Sep1 was

confirmed against *M. incognita* and *C. elegans* and resulted at lower dosages in a smaller body size of the nematodes. Specific proteins were targeted as a substrate of the Sep1 in the intestine, as revealed by SDS-PAGE. *Bacillus firmus* including the strains with a direct mode of action on the integrity of nematodes and eggs has been shown effective in the field (Terefe *et al.*, 200) and is part of a commercial product (Ghahremani *et al.*, 2020), which is also registered in the Netherlands. *B. firmus* I-1582 has been shown to infect and inactivate *Meloidogyne* eggs.

A study on nematode pathogens from soil, dung and dung beetle revealed 20 out of 768 *Bacillus* isolates that caused 70-100% mortality within 5 days (Rae *et al.*, 2010) upon exposure *in vitro*. Three of these isolates, all three being a ***B. cereus***-related strains isolated from a dung beetle, caused complete mortality in *C. elegans* and to a lesser extent in *P. pacificus*. The fecundity of both species was affected as well. *C. elegans* mutants with a Cry-protein resistance for Bt-toxin or a mutant with an increased longevity were also susceptible to infection with these three strains. The cause of the lethal effect is still unknown. The three isolates were PCR-positive for a series of hemolysins, ceretoxins, enterotoxins and phospholipases. Earlier work by Fincher (1975) showed that an abundance of dung beetles reduces the incidence of parasitic nematodes (with a free-living juvenile stage) in the pastures and the cattle grazing on it.

Bacillus thuringiensis is a well-known insect pathogen, a hallmark in biological control of herbivorous insect pests with applications of *Bt* as a microbial agent and in GMO-technology of crops. More recent work showed that this bacterium also infects nematodes (Bel *et* al, 2022). In several field trials, Zuckerman *et al.* (1993) have shown that application of nematicidal *B. thuringiensis* as a drench or a seed coat can reduce the pressure of *M. incognita*, *R. reniformis* and *P. penetrans* on the yield of crops. This is caused by the paralyzing activity of a specific crystal protein (Cry) that is present in the parasporal coat. Cry binds specific receptors in the intestinal epithelium of the host. Disintegration of the epithelial barrier gives access to the internal tissues for further digestion and colonization. *Bacillus thuringiensis* was originally described from an infection in *Bombyx* mori, but since subsequent research mainly focused on its application in combatting insect pest, the host range of the bacterium has been overlooked, also because numerous isolates were not infectious for insects. But *Bacillus thuringiensis* has been found in regions with scarce insects, e.g., Antarctic soils, has been detected on and isolated from nematode carcasses, and has evolved both commensal and pathogen lifestyles in nematodes (Ruan *et al*, 2015). Given these observations, it was suggested that nematodes likely play a prominent role in the natural history of *B. thuringiensis*, as a vector towards other hosts and a reservoir of vegetative cells associated with living or decaying nematodes and endospores in the soil. Some of the types of Cry-protein are specifically targeting nematodes. Cry6A, Cry14A, Cry21A, and Cry5B, Cry13 caused elevated mortality of *C. elegans* and on most representatives in a series of free-living nematodes (*Acrobeloides* sp, *Panagrellus revidivus, Distolabrellus veechi* and *P. pacificus*) and the animal pathogen *Nippostrongylus brasiliensis*. The effect was also visible as reduced growth and reproductive output (Wei *et al.*, 2003). Later studies have extended this list of nematicidal Cry proteins, such as Cry31Aa in *Aphelenchoides besseyi* (Liang *et al*, 2022) and Cry5C and Cry5D in *Meloidogyne incognita* (Geng *et al.*, 2009). A comparative genomic approach by Zheng *et al.* (2017) has provided evidence of clustered Cry-genes co-located in pathogenicity islands and mobile elements, facilitating horizontal gene transfer. Unfortunately, only four nematode-infecting *Bacillus thuringiensis* genomes were included in this study, and these were distributed over different clades in a phylogenetic analysis. However, the earlier identified nematode-specific Cry-genes were associated with each other in a network analysis, indicating a degree of physical linkage in the analyzed genomes.

Griffitts *et al.* (2001) have reported the development of resistance against Cry5 and Cry14A by loss-of-function of a post-translational modification gene. This type of resistance does not prevent infection

caused by other *Bacillus*-specific virulence mechanisms (Rae *et al.*, 2010). For example, in addition to the parasporal crystal toxins a metalloprotease ColB (collagenase B) has been described, which synergistically interacts with the activities of nematode-specific Cry proteins by doubling the mortality exerted by them (Peng *et al.*, 2015). In this study, *Bacillus thuringiensis* strain 010 was found to have significantly detrimental activity against *C. elegans*. To further characterize this activity, the toxicological mechanism was elucidated at molecular level. Genes encoding for crystal protein and chitinase were isolated, cloned, and sequenced. However, the toxicity was detected only for the chitinase (Zhang *et al*, 2014).

Brevibacillus laterosporus G4 produces an extracellular protease that creates a hole in the nematode cuticle and allows it to enter the body of the host. The pathogen caused a mortality of 90 and 82% in *Panagrellus redivivus* and *Bursaphelenchus xylophilus* after 48 h of controlled *in vitro* exposure (Huang *et al.*, 2005). In analogy with *Bacillus nematicida*, *Brevibacillus laterosporus* secretes a combination of two extracellular protease, an alkaline and a neutral (Tian *et al.*, 2006; Tian *et al.*, 2007b). The neutral protease does not seem to have a lethal effect or a lytic effect on the cuticle. However, it fortifies the activity of the alkaline protease. In experiments in natural soils, the use of the sterile supernatant of a sporulating *B. laterosporus* culture caused 80% mortality of *M. incognita* (Hamze & Ruiu, 2020).

The importance of ***Bacillus* species** as pathogen for nematodes was further confirmed in a large-scale screen for nematicidal activities using 120 strains of spore-forming *Bacillus* (30 species) (Zheng *et al*, 2016). The pathogens causing the most severe effects belonged to the following nine species: *Bacillus thuringiensis*, *B. cereus*, *B. subtilis*, *B. pumilus*, *B. firmus*, *B. toyonensis*, *Lysinibacillus sphaericus*, *Brevibacillus laterosporus* and *B. brevis*. *Bacillus thuringiensis* was by far the strongest nematicidal species. The Bacilli share a number of virulence factors, *i.e.*, the nematode-specific Cry-proteins, some types of alkaline and neutral proteases for cuticle and intestine degradation, beta-metalloproteases and collagenases, Trojan horse mechanism for the attraction of nematodes, and in a lesser extent the activity of chitinases. However, not in all the strains the harmful effect on nematodes could be explained by this arsenal of virulence factors, indicating that also other virulence factors (like *e.g.*, the exotoxin thuringiensin) may be contributing to the mortality.

Other nematode-specific bacterial pathogens

Actinomycetes are known for their extensive biosynthetic capacity to produce secondary metabolites that affect the growth and survival of nematodes. An overview on the toxicity of the bacteria to a series of isolates from *Pristionchus* pacificus-associated environments showed strong mortality and negative chemotaxis to *P. pacificus* (Akduman *et al.*, 2018). Actinomycetes were one of the three groups with strongest toxicity to nematodes and caused negative chemotaxis in this study.

From several independently contaminated *C. elegans* cultures, a contaminant was isolated and described as ***Microbacterium nematophilum***. This bacterial pathogen causes colonization and infection of the cuticle in the rectal and anal region and causes swelling of the tissue as a result of the evoked innate defense response upon ingestion by bacterial grazing nematodes, such as *Caenorhabditis* spp. The bacterium can infect several representatives of this genus, but the mortality varies widely from 0-100% between and within *Caenorhabditis* species, (Akimkina *et* al, 2006; Parsons & Cipollo, 2014). These mechanisms lay at the interplay of immunity and longevity and have been used as a model in *C. elegans* for chronic gastrointestinal infections (Gravato-Nobre *et al.*, 2020). Whether this pathogen is affecting nematodes outside the genus *Caenorhabditis* is unknown.

A related member of the Microbacteriaceae, ***Leucobacter chromiireducens*** subsp. *Solipictus,* was discovered as another contaminant in a *C. elegans* lab stock (Muir & Tan, 2008). It infected the uterus of *C. elegans* via a non-oral infection route resulting in a moderate increased lethality (50% in 5 days). The growth of the nematode was retarded with a less developed internal fat storage and exposed adults became infected in the uterus. According to the chemotaxonomic and biochemical properties this species is a saprotrophic bacterium of damp environments and forms strong biofilms that contribute to the lethality by congesting the nematodes. It is presumably a mild opportunistic pathogen that has been described only from *C. elegans*. It lives as a commensal on *C. elegans* and becomes lethal under certain conditions.

From two independent rotting fruit samples, from France and India, the Gram-negative strain ***Chryseobacterium nematophagum*** (Flavobacteriacea) was isolated. It exhibits 100% mortality in *C. elegans* and *C. briggsae* (7 h) and in several free-living bacterivorous stage of animal parasites (24 h, *Haemonchus contortus, Trichostrongylus vitrinus, Teladorsagia circumcincta, Cyathostomin sp., Ostertagia ostertagi, Parastrongyloides trichosura, Cooperia curtecei, Cooperia oncophera, Nippostrongylus brasiliensis* and *Ancylostoma caninum*) (Page *et al.*, 2019). The potato parasite *Globodera pallida* did not show mortality, presumably due to the non-bacterial diet. In this study, the specificity towards nematodes was also shown, because the larval stage of *Aedes aegypti* did not show adverse effects upon exposure to *C. nematophagum*. The bacterium degraded the pharyngeal tissue of the nematode as confirmed by fluorescent gene fusions with pharyngeal genes and monitoring of breakdown products in the body lumen. The primary factor causing infection is a collagenase. Collagen was degraded by *C. nematophagum* but not by another species from the same genus. The gene for this enzyme is shared between the genomes of the two *C. nematophagum* genomes. Additionally, a chitinase, a secreted astacin (type of endo-metallopeptidase), and multiple pertussin-toxin-like and cytolysin-like genes were discovered in the pathogen. The authors suggest that the first three virulence factors contribute most to the pathogenicity of this bacterium. After discovery of this pathogen, no attempts have been reported to apply it in biological control.

Pseudomonas protegens is a well-known plant growth promoting bacterium with a diverse arsenal of secondary metabolites and extracellular enzymes. In a specific isolate (15G2) a strong inhibitory effect on the growth (>75%) and development of *C. elegans* larvae (82%) was observed (Wei *et al.*, 2017). The supernatant of the bacterial culture contained a protein with limited similarity to ADP-ribosyl transferase (cholera-toxin-like and heat-labile toxin-like). This enterotoxin consists of two subunits encoded by two distinct ORFs and is also detected in the genomes of two other *P. protegens* genomes. The bacterium also had a strong negative effect on the growth of other species of free-living nematodes. No adverse activity was found upon exposure of a series of fungi and insects, which indicates a nematode-specific mechanism, which is not fully understood yet. It is not known if any of the applied commercial biocontrol strains also contains the abovementioned enterotoxin or other nematode-specific virulence factor.

Among a series of isolates from grapevine roots Canchignia *et al.* (2017) have identified ***Pseudomoas veronii*** R4, a severe pathogen of *Xiphinema index*. A mortality of 100% is attained in just 3 days. In comparison to the reference strain used in this study, *P. protegens* CHAO, the lethal activity is caused without the production of 2,4-diacetylphloroglucinol, a broad-spectrum secondary metabolite. A Zn-dependent metalloprotease, a lipase and a phospholipase were identified as virulence agents, which exhibited degradation of the cuticle and body wall sections.

At this time, novel bacterial pathogens specific for (certain taxa of) nematodes are still being discovered. Recently, a novel Gram-negative bacterial pathogen from the rhizosphere, merely

infecting the tissues of the J2 stage of the RKN *Meloidogyne* sp., was described, but not taxonomically positioned by molecular methods (Ciancio, 2021).

2.2 Fungi

Ectoparasites - the nematode-trapping fungi

Nematode-trapping fungi are asexual stage of representatives of the Orbiliaceae (Ascomycota) and are the most iconic ectoparasites of nematodes. They can produce hyphal structures in several forms, like nets, adhesive knobs or lasso-like (constrictive or not-constrictive) structures that catch nematodes that pass by. They are indiscriminate against the nematode preys they catch (Niu & Zhang, 2011). These structures are inducible and within a species a variety of morphologies can emerge (Nordbring-Hertz, 2004).

Arthrobotrys oligospora is the best described nematode-trapping fungus and has been detected in and isolated from a variety of soils, fecal and surface water samples. It is a saprotrophic species that under nitrogen deficient conditions can turn into a predator (but also induction factors like nematodes (Hsueh *et al.*, 2013) and soil bacteria are reported) (Niu & Zhang, 2011). Volatile attractants are produced to recruit nematodes to the traps (Hsueh *et al.*, 2017). Upon interaction with a nematode, the animal is immobilized by adhesion to the cuticle or constriction. Subsequently an appressorium is formed, through which the perforation of the cuticle and infection takes place. Apart from the lectins (carbohydrate-specific protein) for adherence and the nematotoxic metabolites that have been identified, the proteolysis of the cuticle is the main mode of action of infection. Tunlid *et al.* (1994) have identified one of two secreted proteases as lethal to *P. redivivus*. It was characterized as a serine protease and was induced by carbon and nitrogen deprivation. The enzyme can hydrolyze nematode cuticle proteins. Field studies with this fungus have shown biocontrol of *C. oncophora* free-living stages in pastures with up to a tenfold decrease in cow pats treated with the fungus (Grønvold *et al.* 1987), but also a reduction of *M. incognita* on tomato roots (Soliman *et al.*, 2021)

Duddingtonia flagrans is also a nematode trapping fungus that can be used in the prevention of free-living stages of animal parasites in cattle. It produces chitinases and proteases that cause a strong reduction of the activity cyathostomin L3 larvae (Braga *et al.* 2015). The fungus is also able to catch *M. incognita* and kill J2 larvae and eggs with the volatiles it produces (Mei and Li., 2021). This species is already in use as a safe feed-additive for the prevention of animal parasites, but not in the EU, although the EFSA does not foresee an environmental risk for this product (EFSA, 2020). Moreover, its chlamydospores survive gastrointestinal passage, which implies it can be administered as a feed-additive.

Monacrosporium haptotylum is a nematode-trapping fungus in which the mechanism of trapping has been studied mechanistically. It deviates from the abovementioned nematode-trapping fungi, that it does not have the constricting structures to immobilize nematodes, but adhesive knobs instead. Within these structures subtilisin-like serine proteases are upregulated during the attack on nematodes, but also proteins with carbohydrate binding domain (for binding the glycosylated surface coat of the nematodes), as compared to the mycelial hyphae (Ahrén *et* al, 2005, Anderson *et al.*, 2013). No reports of successful application in the field have appeared until now.

Another mechanism of nematode-trapping is shown by the oyster mushroom ***Pleurotes ostreatus*** and many of its congeneric species (Lee *et al.*, 2020). This mushroom is grown commercially at very large

scales for human consumption but is not generally known to be a very vicious predator of nematodes. Within a few minutes upon contacting hyphae, nematodes from the genera *Caenorhabditis*, *Diploscapter*, *Oscheius*, *Rhabditis*, *Pristionchus*, *Panagrellus*, *Acrobeloides*, *Cephalobus*, *Mesorhabditis*, and *Pelodera* are killed and subsequently digested. All stages of nematode development are quickly paralyzed (eggs were not tested, however). Like the mechanism of *A. oligospora*, the infection by *Pleurotus* also takes place via the sensory cilia (verified by mutant analysis in two species), but at a second time frame as compared to the death struggle of hours in the case of *A. oligospora*. Subsequently a general necrosis spreads (via the nerve system) over the body, after which the nematode is digested by the fungus. *Pleurotes* mushrooms have been applied in a number of field studies to control parasitic nematodes, e.g., on the reduction of *H. schachtii* in sugar beet (Palizi *et al.*, 2009). However, they can be preyed upon by nematodes themselves (and by bacteriovores that switch to fungal hyphae) (Marlin *et al.*, 2019).

Egg-directed fungal ectoparasites

Besides nematode-trapping fungi, several fungal ectoparasites have a preference or restriction to eggs of nematodes, as demonstrated by the following two example species.

Paecilomyces/Purpureocilium lilacanus: is a fungal saprotrophic fungus that can switch under some conditions to the infection of nematode eggs, including the plant-parasitic nematodes *Meloidogyne* sp., *Heterodera* sp. and *Globodera* sp. (Cannayana & Sivakumar, 2001), although it also infects the mobile free-living stages. It has been proven to strongly reduce plant-parasitic nematodes on crops, e.g., *Pratylenchus* and *Meloidogyne* on chrysanthemum were strongly decreased by this fungus, the formation of root knots to a lesser extent (Sanchez & Cardona, 218). A serin protease (subtilisin-like) has been isolated and characterized and linked to the infection and inactivation of *M. hapla* eggs (Bonants *et al.*, 1995). This effect was especially noted when the fungus had been cultivated in the presence of the main protein component of the egg-shell: vitellin.

Pochonia (Verticillium) chlamydospora is another cosmopolitic fungus involved in nematode egg infection. It is a saprophytic, sometimes endophytic, fungus that can turn into a nematode pathogen under certain conditions (Manzanilla-Lopez, 2013) A secreted protease Vcp1 was isolated from this fungus. This enzyme removes the outer layer of the *M. incognita* egg (Segers *et al*., 1994; Morton *et al.*, 2003), but possibly chitinases play also a role in infection. Following the annotation of the genome, it was understood that this species is not an obligate parasite, but has rather multitrophic, including a saprophytic and endophytic lifestyle (Larriba *et al.*, 2014). The strict need for a partially decomposed and rich substrate in order to get an elevated egg infection was observed in biocontrol trials with this species (van Damme *et al*, 2005; Luambano *et al.*, 2015). This indicates that this species has a narrow niche for growth and application. Administration of this fungus in a field trial to suppress *Heterodera schachtii* demonstrated a lack of persistence (Jalali *et al.*, 1998).

Endoparasites

Endoparasitic fungi have an infectious lifestyle in which they enter through the cuticle and proliferate in the nematode body. Such fungal species can produce non-motile or motile zoospores.

A fungus that produces motile zoospores for infection of nematodes is the facultative endoparasite ***Catenaria anguillulae***, a member of the Chytridiomycota with a broad geographic distribution that has been reported to cause epidemic events among soil nematodes (Vaish, 2012). After torrential rains the prevalence among free-living nematodes can amount to 60% of the individuals, as observed by microscopic investigation of nematodes direct from field soils. The fungus

produces flagellated mobile zoospores that migrate until recognition, adhesion and encystment to body apertures of nematodes and subsequently to germinate in the body. The grown out hyphae stretch throughout the entire length of the nematode body, with intermittent sporangia developing, from which spores are released via duct-like structures. No molecular mechanisms of virulenceare known at the moment.

Drechmeria coniospora produces infectious haploid spores that attach to the cuticle (in the head and vulva region) of certain nematodes (*C. elegans*). After adhesion and the development of an appressorium structure occurs, from which the cuticle is penetrated. Hypha grow throughout the host and eventually form conidiophores that produce new spores. Legrand *et al.* (2016) performed genome analysis of *D. coniospora* and identified hundreds of potential virulence factors: a.o. saposin A containing domains (interfering with components of the nematode defense system), enterotoxins, biosynthetic gene clusters, and subtilisin (serine protease) that is possibly involved in the infection mechanism. The fungus contains less genes for carbohydrate and lignocellulosic decomposition as compared to other fungi, which indicates a dedicated nematode pathogenic lifestyle. The exact mechanism of the enterotoxin damage is still enigmatic since the fungus does not need to be ingested for infectivity. Also, the myriad of secondary metabolites produced by this fungus contributes to its toxicity to nematodes (Wan *et al.*, 2021).

In a comparative genomics study on ant-infecting fungi, De Bekker *et al.* (2017) included the nematode pathogenic fungi *Ophiocordyceps minnesotensis* and *Drechmeria coniospora,* which contain respectively 19 and 25 enterotoxin genes (protein family sPF01375) in their genomes. In a phylogenetic analysis, the nematode infectious enterotoxins failed to cluster together from the enterotoxins from insect pathogens. Courtine *et al.* (2021) reported that subculturing in lab conditions created a stronger virulence towards *C. elegans*. However, a number of regulatory pathways were mutated during lab condition. This phenomenon may imply less risks for adverse ecological effects from lab-populations of *D. coniospora.*

Hirsutella rhossiliensis is an endoparasitic fungus that was discovered from a series of isolates when infecting *H. glycine* (Wang *et al*, 2009). This fungus produces specialized conidiospores that are ingested by nematodes and internally infects the host in the intestine. The crude extract with a highly expressed alkaline protease was reported to kill al juveniles in an experiment. In addition to the latter enzyme other virulence agents are produced by this fungus.

No convincing successful application of the obligate nematode pathogens *D. coniospora* or *H. rhossiliensis* as biocontrol agent of parasitic nematodes have been published. The species grow slowly on media (Liu *et al.*, 2009). The same applies for other strict nematode fungal pathogens, which are ineffective for biocontrol because of their requirement of sufficient nematode populations for persistence in the soil.

2.3 Viruses

Several studies have also used *C. elegans* as a model organism for viral infection, in particular to study the innate immune response to viruses (Shaham, 2006) and to demonstrate the role of RNAi and active cell death as players in the innate defense against ssRNA and dsDNA viruses, respectively. In order to circumvent the lack of match between host receptor and virus, sometimes experimental treatments for artificial intracellular infection, like PEG and Vaccinia virus (Liu *et al.*, 2006) or injection of vector with the genomic information (Flock House Virus) (Lu *et al.*, 2006) were needed for viral

replication in *C. elegans*. Vesicular Stomatitis Virus (negative strand ssRNA) can infect a *C. elegans* cell line when high viral loads are used (Schott *et al.*, 2005), three to tenfold viral particles per cell. Still, this remains an artificial situation that does not relate to conditions in the field.

Nematode viruses

Current knowledge on nematode-associated viruses is very limited. Most isolated viruses appear to show commensal behavior and have thus far barely linked to pathology. The first report on nematode pathogenic viruses came from an experiment with *M. incognita* in which the population in one particular field treatment appeared sluggish and was not capable to form any root knots. A filtrable agent (different form a bacterium or a fungus) was able to transmit the symptoms to healthy stock of nematodes (Loewenberg, 1959). The characteristics of this possible virus were not further investigated. Poinar and Hess (1977) have detected virus-like particles in the mosquito parasite *Romanomermis culicivorax* by means of microscopic observation of tissues. Ibrahim (1978) described a phenomenon called "swarming behavior" (congregation in masses), which he observed in several species of plant-parasitic nematodes, to be associated with a viral infection in *Tylechorhynchus martini*. Microscopic investigation revealed structures that point to an infection related to the insect virus Cytoplasmic Polyhedral Virus. In addition to some other studies, pre-molecular research had already confirmed that field populations nematodes are also susceptible to infection with viruses. With the onset of NGS, more evidence became available upon non-targeted analysis of the genetic material in nematodes.

Felix *et al.* (2011) have described two commensal Nodaviridae (positive strand ssRNA) from *C. elegans:* Orsay virus and *C. briggsae* (Santeuil virus), respectively. These viruses induce a distinct epithelial cell morphology but are not lethal and have no or little effect on the reproductive output. Genetic variation in susceptibility between different strains of nematodes has been shown.

Bekal *et al.* (2011) discovered four novel negative strand ssRNA viruses in the transcriptome of a laboratory population of the soybean cyst nematode *H. glycine*. They were related to arthropod viruses from nyaviruses and bornaviruses, rhabdoviruses, bunyaviruses and tenuiviruses families, respectively. A vertical transmission was shown, because of their presence in eggs and larvae. No integration of viral sequences in the host genome was detected. In the same research group, a positive ssRNA virus was described later from the same lab cultures as well as in field populations. The virus was transferred throughout all life stages and was also present in males, so it was possible sexually transmitted (Bekal *et al.*, 2014). The virus was closely related to *Pestivirus* in the Flaviviridae.

The above five viruses were discovered in a monitoring survey of 25 greenhouses and fields in North-Carolina and Missouri and retrieved in the majority of locations. In general, the overall prevalence was higher in greenhouse populations (32.6-88.4%) of *H. glycine* compared to the field populations (0-80%). Interestingly, other congeneric species like *H. schachtii* and *H. trifolii* were also carrying (a subset of) these viruses, but all other plant-parasitic nematode species tested negative (Ruark *et al.*, 2017). In a later study also a Nyami-like Virus and a novel Bunya virus were described from the soybean cyst nematode and related species. In the same study Ruark *et al.* (2018) described a picorna-like virus (Secoviridae) specific for the potato cyst nematodes *Globodera rostochiensis* and *G. palida*.

A novel virus, belonging to Order Picornavirales, Family Secoviridae (positive strand ssRNA), vertically transmitted throughout eggs and juveniles was described by Lin *et al.* (2018) in the beet cyst nematode. *H schachtii.* When also a root lesion nematode *Pratylenchus penetrans* infecting virus was described by Vieira and Nemchinov (2019), also belonging to the Secoviridae family, it was understood

that the clade of nematode infecting viruses (not detected in the plants) from these family of plant viruses, were phylogenetically related.

Also in animal parasitic nematodes virus have been described, in the porcine parasite *Ascaris suum* a posavirus-like sequence was described (Hause *et al.*, 2015). Other novel viruses discovered in animal parasitic nematodes are the Fulton Virus (new clade in the Order Bunyavirales, negative strand ssRNA) and Amsterdam Virus (Lipiviridae, negative stranded ssRNA) in the liver parasite *Capillaria hepatica* in house mice and brown rats (Williams *et al.*, 2019).

To conclude, current knowledge on nematode-associated viruses indicates a high or strict taxonomic specificity for the host and suggests a high degree of commensalism. This view may be biased by the limited number of studies. More research may be needed to fully appreciate the possible virus-infection associated nematode pathology and its effect ecological processes.

2.4 Commercial applications of nematode pathogens

As mentioned above, the effects of pathogens on nematodes have in some cases resulted in biocontrol applications. Table 1 gives an overview of currently available commercial formulations that are based on or contain pathogens of nematodes.

Table 1: List of known commercial formulations that are based on or contain pathogens of nematodes.

Pathogen	*Brand name*	*Target species*
Pasteuria penetrans	Econem (Syngenta)	*Meloidogyne*
Pasteuria nishizawae	Clariva N (Syngenta)	*Heterodera glycine*
Pasteuria sp. Ph3	Naviva ST (Syngenta)	*Rotylenchus reniformis*
Pasteuria usage Bl1 + Pasteuria Ph3	NewPro (Syngenta)	*Belonolaimus longicaudatus*
Bacillus firmus	BionemWP (Agro-Green)	*Meloidogyne, Heterodera, Helicotylenchus*
Bacillus firmus I-582	Flocter (bayer) / VoTiVo (BASF) ***NL***	*Meloidogyne*
Bacillus laterosporus (and other)	Biostart (Bio-Cat)	*Meloidogyne*
Duddingtonia flagrans	BioWorma (IAHP)	*Several animal parasitic nematodes (grazers)*
Purpureocilium lilacanus	BioAct WG (Bayer)	*Meloidogyne*
Pochonia chlamydosporia	Green Nema Free (Green Heal)	*Plant-parasitic nematodes*

The formulations of biological control agents that make use of nematode pathogens included in the list mainly contain Gram-positive bacteria like *Pasteuria* and *Bacillus/Brevibacillus*, which is probably caused by the history of discovery of such pathogens. *Pasteuria* is a highly specific pathogen that is restricted to certain varieties of the host species and needs to be applied in high titers (10^8

spores per seed or 10^{10} spores per liter of soil) but it is difficult to culture outside the nematode host (Ciancio, 2018). Surveillance of endospore titers and match with the resident variety of the plant parasitic nematode remains necessary.

Representatives of the genus *Bacillus* have many applications as plant growth promoting rhizobacteria, often with plant stimulating or microbiome shaping effects. This includes one product (VoTiVo, currently BASF) that is on the Dutch market, that also makes use of the infectious nature of *B. firmus*. The two well-known nematodes egg parasites *P. lilacanum* and *P. chlamydospora* have also made it to the market after their success in field trials. The obligate endoparasitic fungi *D. coniospora* and *H. rhossiliensis* did not make it to a product, probably because they grow very slow on media (without a nematode host) and their narrow optimum of soil conditions in which they effectively operate.

In a combined greenhouse and field study with cotton, which was monitored up to 90 days after treatment, the application of *P. liliacanus* (up to 0.3% vol/vol in water) and *B. firmus* (up $1.4.10^{7}$CFU/seed) a significant decrease of the plant parasite *R. reniformis* was observed, however the densities of free-living nematodes were not reduced (and increased in some cases) (Castillo *et al*, 2013).

The capability of *Duddingtonia flagrans* to keep its highly effective activity against animal parasitic nematodes after transit though the gastrointestinal system in cattle, has contributed to it commercialization. It is registered outside the EU. Saumell *et al.* (2015) have conducted an ecological impact study on the use of *D. flagrans* as a biocontrol, administered as a feed-additive, against parasitic nematodes in sheep. They did not find an effect on the densities of free-living soil nematodes or the biodiversity of local fungal pathogens in the soil. A strong decline was visible in the persistence of the biocontrol strain in the topsoil layer (Saumell *et al.*, 2015), indicating that resource partitioning and competition prevented attaining dominance, despite the relative high dose (250.000 chlamydospores/kg body weight for 90 consecutive days). In an earlier study it was also described that *D. flagrans* is unable to survive outside the faecal patches of sheep dung (Faedo *et al.*, 2002). Treatment of carnation beds with *P. liliacanus* did not have an effect on the abundance of the resident free-living nematodes or shifts in the composition of the community (Langat *et al.*, 2008). The dependence on the resource is for some biocontrol agents, like *Pochonia chlamydospora*, so specific for their application and persistence in the soil (Jalali *et al.*, 1998; van Damme *et al.*, 2005; Luambano *et al.*, 2015).

In general, the short and long-term effects of the application of biocontrol of nematodes on the surrounding local ecosystem in the field are not (well) studied, but initial reports indicated no measurable effects on the fate of free-living nematodes, while effective but temporal reduction of nuisance nematodes for crop and cattle are mostly attained.

3. Experimental techniques for study and predicting the nematode pathogen interaction

In order to determine whether micro-organisms are pathogenic for nematodes and disturbing for their role in the ecosystem, solid validation procedures would be of great help. These can vary from relevant experimental infections in the laboratory, omics-analysis for virulence-associated genes in

putative pathogens, and standardized field experiments for the assessment of risks for non-target species and ecosystem services.

The interactions between pathogens and nematodes have been investigated experimentally primarily using bacteria and fungi and has gained insights in the virulence factors that are involved in the infection process.

The viral work (on genuine nematode-associated viruses in field populations), until today, has remained at the level of description after discovery in histological analysis, but more recently also after data analysis of nematode NGS data (mainly transcriptome). From these observations it was seldomly clear if the viruses behaved as commensals, symbionts, or pathogens.

3.1 Bioassays to assess nematode-pathogen interaction

The most often used method for the primary discovery of pathogenic isolates in nematodes involves the exposure of nematodes to lawns of monocultures of test pathogens on a solid agar medium exposed to air, in which nematodes contact or (in the case of bacterivorous nematodes) graze on the pathogens. Mixed lawns have also been applied in order to moderate lethality or to create a dose response effect. Subsequently, the mortality, reproductive output or growth are often determined by counting motile individuals, eggs, or larvae, and by image analysis of the populations on the agar medium.

Besides using intact putative pathogens, purified proteins derived from extracts or recombinant expression (in e.g., *E. coli*) have been used to separately investigate the effect of distinct gene products on nematode survival and reproduction. Especially the work on *Bt* Cry-proteins has been made possible by performing experiments with recombinantly expressed gene products, sometimes offered as a *E. coli* prey to the tested bacterivorous nematodes. Occasionally, the test may yield invalid results as discussed by Wei *et al.*, (2003), e.g., because of the inability to crush the *E. coli* by *P. pacificus* or experimental correction after feeding purified crystal proteins in an additional exposure. Also, in the case of the cuticle degrading proteases the effect of the different forms could be dissected independently and in interaction. This shows that this approach allows to investigate any interactions between virulence factors. Despite the limitations of *in vitro* exposure of nematodes to recombinant virulence factors, these tests remain a useful approach to assess the overall effect of the pathogen on the nematode and to dissect the infection mechanism.

The primary pitfall in the myriad of combinations of used approaches of exposing nematodes to pathogens or to separated virulence factors is that the dose or duration that is applied is variable. This remains an issue even when the dose of a purified virulence factor is expressed as an LC_{50}-value for that particular experimental approach. The used values are difficult to translate to the exposure of a varying densities of nematodes to pathogens in a lawn on a solid agar medium and even impossible to compare with the situation in the field. A comparison in a meta-analysis is therefore cumbersome. The mechanistic investigations on *Pasteuria* are all conducted in a combination of descriptive *in vitro* and *in planta* experiments, because of the obligate hyper-parasitism of this pathogen.

The conditions of growth of the pathogens can also have an effect on the outcome of an infection test. For example, the actinomycete isolate *Streptoverticillium albireticulli* was able to cause more than 90% of mortality in *C. elegans* after 6 h due to colonization within the nematode when grown on Fish Meal Extract Agar, but not on other media (Park *et al.*, 2002). Limited chitinase and no protease activity

could be determined in the extracellular environment. The mechanism of infection is still unknown for this pathogen and it is therefore possible that this conditional effect is due to the induced production of toxins, rather than the consequence of specific virulence factor for nematodes.

3.2 Parameters of virulence

As mentioned above, nematode mortality is often used as the primary parameter of virulence of a putative pathogen. Mortality has been mostly determined by touching the nematode during or after the bioassay and looking for a tactile response under a binocular loupe, or by looking at mobility or lysis. A physiological output like the pumping frequency of the pharynx, can also has been used a readout of nematode stress. Some studies have looked at nematode survival (Kaplan-Meier curves in *C. elegans* research groups) and growth analysis relative to a control. An entirely different bioassay is the hatching of eggs or the survival of newly hatched larvae as a virulence endpoint.

Besides mortality, physiological parameters and hatching, microscopy has been used to investigate damage to tissues and eggs, and lesions in cuticle and intestine. In the experiments fluorescently labelled strains of nematodes or pathogens have been used for observing the disintegrations of the nematode body or the colonization of the tissue by the pathogens. In combination with Nomarski microscopy this has provided clear histological evidence of infection. In some cases, the activity of proteases on nematode cuticle was visualized using subsequent SDS-PAGE (sometimes 2D) on intestinal or cuticular proteins and by the characterization of substrates for the respective forms of protease.

3.3 Ecological assays for the impact of nematode infections

The literature concerning pathogenic micro-organisms for nematodes is focused on *C. elegans* and plant-parasitic nematodes. To a lesser extent, some studies have taken along free-living representatives and occasionally some animal parasites. No experimental studies have been performed with pathogens of marine nematodes and there is a considerable bias towards terrestrial nematodes. The pathogens that have been isolated were also sampled mostly in terrestrial habitats (mainly soil, dung beetles/dung, arable land with a natural suppressiveness for plant-parasitic nematodes).

Nematode specificity of pathogens

The ecological risk of an introduced nematode pathogen is inverse related to its host specificity. The nematode specificity is highest by *Pasteuria* strains, which also show intraspecific specificity for their host species. Also, for the Cry-proteins, nematode selectivity has been observed, probably because of the interaction with receptors in the intestine. For the other bacterial pathogens, it is difficult to estimate the susceptibility of the various tested nematodes for the pathogens or their isolated virulence factors, mainly because different nematodes (and feeding types) were deployed in the various studies. The fungal pathogens were mostly tested on plant-parasitic nematodes (except *D. flagrans* (mostly animal parasites) and *P. ostreatus* (free-living))

The feeding type of the nematode can be of importance for the susceptibility to the pathogen (Page *et al.*, 2019). For example, *Globodera pallida* (with a stylus mouthpart) did not show any mortality when exposed to *C. nematophagum*. This indicates that for some pathogens the entrance into the digestive system is necessary for virulence. On the other hand, the Cry-protein can be taken up by *A. besseyi* (plant-parasitic nematode) with needle-like mouthparts (Liang *et al.*, 2022) and *P. pacificus* (Wei *et al*, 2003). Also, the presence of attracting VOC (Trojan horse principle) and chemotaxis determine the consumption by the nematodes. The least specific are the fungal pathogens of nematodes, in particular the nematode-trapping fungi. They show no specificity and are understood to catch every appropriately sized that binds their specialized hyphae .

Also, the susceptibility of the nematode determines the virulence of a pathogen. Some taxa, like *P. pacificus* (associated with cadavers and dung), are less susceptible to nematode pathogens (*e.g.*, against some of the Cry-proteins in Wei *et al*, 2003, and the dung beetle isolated *B. cereus*-like isolates in Rae *et al.*, 2010) as compared to other nematode taxa. Avoidance behavior can play a role in this, but this is not the case during exposure on bacterial lawns. It is more likely that members of the core-microbiome prevent the colonization of intestinal pathogen, like shown by Han *et al.* (2021). In this *C. elegans* study, the nematode pathogen *B. nematocida* was inhibited colonizing nematodes that pre-fed on a *Stenotrophomonas* spp. from the *C. elegans* microbiome. It was assumed that this also takes place in field conditions when the challenge due to exposure to pathogens is lower (no monoculture lawns). The colonization resistance is based on the principle of the presence of a host-associated microbiome that is selectively recruited by interaction with the host (Macke *et al*, 2017). The protecting microbiome members can produce antimicrobials compound or compete out the pathogens by means of space and resources. Also, the preparedness of the innate defense system of the host can keep defense pathways standby. This has been proven in *C. elegans* (Wang *et al.*, 2019), in which a *Phytobacter* strain prevented the colonization of *B. nematocida* due to adhesion to the intestinal cells and the competition for iron. Its dynamics has been demonstrated by artificially evolving a bacterium to the gut environment of *C. elegans* resulting in a reduced colonization by (some) pathogens (Dahan *et al.*, 2020). In order to better understand the variation in virulence in the experimental studies, a report of the microbiome composition of the tested nematodes would be desirable. This is, however, often outside the scope of the respective studies.

Studies of direct ecological impact of nematode pathogens

It should be noted that the possible short or long-term effects of infection-mediated nematode mortality or behavior on the surrounding ecosystem has rarely been studied, except for the abovementioned biocontrol studies that monitored the off-target effects of *B. firmus* (Castillo *et al*, 2013), *D. flagrans* (Faedo *et al*, 2012, Saumell *et al.*, 2015) and *P. liliacanus* (Castillo *et* al, 2013; Langat *et al.*, 2008). None of these studies has indicated a negative long-term effect of the introduction of the pathogen.

3.4 Remarks

There are only a few studies that report on the effect of the pathogen on nematodes (including chemotaxis) kept in natural soil under controlled conditions in the lab, as has been done for *B. nematicida* (Niu *et al.*, 2010). The activity of some pathogens has been studied under field conditions (such as in an application as biocontrol agent, *e.g.*, *B. firmus, B, thuringiensis, Pasteuria,* and most of the investigated pathogenic fungi (except the endo-parasitic obligate fungi) (Liu *et al.*, 2009). Other

pathogens were isolated from *C. elegans* cultures from diverse geographical locations or from samples with a strong natural history background, which provides evidence for the ecological relevance of the observed interactions and molecular mechanisms.

The experimental conditions in most consulted studies were appropriate to demonstrate the virulence of the pathogens on the nematodes and to allow a better understanding of the involved mechanisms. However, because of the large number of approaches and combinations of techniques and assessed endpoints, it is virtually impossible to make a good comparison of the virulence between all pathogens.

We recommend the following approach for demonstrating genuine pathogenesis of a microorganism on a nematode:

1. Significant effect on mortality, reproductive output, or growth in a matter of days of exposure.
2. Identification of the virulence factor(s) and indication of their mode(s) of action (recombinant proteins for exposure and degradation experiments)
3. Observation of altered histology and colonization by the pathogen.

The distinction of between pathogenic and opportunistic behavior, *i.e*., infection upon treatment with an additional stressor or forced feeding in monoculture lawns, cannot be made by monolayer exposure and grazing alone. Monolayer lawns lack ecological relevance, but provide a baseline for determining the susceptibility to any pathogen.

Within nematode field populations, variation in ecological and biotic stress exist that may convert the susceptibility of nematodes to non-specific, opportunistic pathogens. With aid of additional experimentation, the specificity for nematodes can be determined. In only in a small number of cases the specificity for nematodes has been tested by exposing *e.g*., insect larvae or fungi.

4. Conclusions

4.1 Literature review

Review of the literature learned that most of the bacteria that have been shown to have a pathogenic effect on nematodes belong to the phylum Bacillota/Firmicutes. Especially *Pasteuria* spp., *Bacillus nematicida*, *Bacillus thuringiensis*, *Brevibacillus laterosporus* and *Bacillus firmus* show pathogenic behavior. In addition, the Actinobacteria *Microbacterium nematophilum* and *Leucobacter chromiireducens* infect nematodes. This is in line with the phylogenetic position of the isolates that exhibited the strongest mortality on *P. pacificus* in Akduman *et al.* (2018). The high prevalence of pathogenic *Bacillus cereus*-like species (Rae *et al.,* 2010) and of the selected bacilli (Zhang *et al.,* 2016) confirms the patterns observed in the respective primary research papers. Especially the study by Zhang *et al.* (2016) demonstrates the reproducibility of the pathogenesis caused by the virulence agents of the bacilli.

With regard to fungal pathogens for which nematode pathogenesis has been described, it can be noted that all belonged to the subphylum Pezizomycotina, except the oyster mushroom *Pleurotes ostreatus* a member of the Basidiomycota.

An overview of the virulence factors that are used by bacteria and fungi to infect nematodes is given in Table 2. P**roteases** and **Cry-proteins** are the most prominent virulence agents that have been reported. Chitinases play a relatively minor role in these processes and there are some minor virulence agents like e.g., enterotoxins (see Table 2). For several pathogens, the virulence mechanisms remain enigmatic.

Cry-proteins are important virulence factors in the infection of several nematodes. *B. thuringiensis* (and *B. toyonensis*) may express up to eight classes of crystal proteins (Cry) (see Figure 1). This role in virulence has been demonstrated in recombinant assays and by exposure of nematodes to strains that harbour these genes. From the paper by Zheng *et al.* (2016) the highest mortality was observed with *Cry*-gene containing isolates.

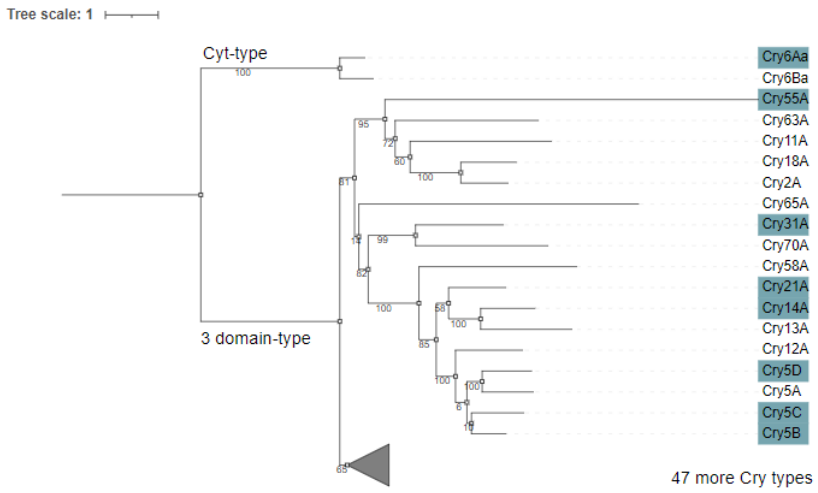

Figure 1: *ML-tree based on the representatives of the different Cry proteins (https://www.bpprc-db.org/). The Cyt-type crystal proteins (Cy6A and Cy6B) were used as an used as an outgroup. The Cry-*

proteins that can paralyze the intestine of nematodes are marked in green, and all but one belong to the same clade. The remaining clades of Cry-proteins are collapsed (47 more types).

Proteases play the second most important role in the infection mechanisms of nematode pathogens. Their combined action creates access to the body tissues by hydrolyzing the cuticle and underlying protein structures. They show specificity for substrates but no obvious nematode species specificity. The primary sequence analysis of the respective proteases of fungal and bacterial nematode pathogens (alkaline, neutral and metalloproteases) indicates that they are structurally distinct from proteases with other functions (see Figure 2). We have extended the protease phylogenetic analyses of Wang *et al.* (2009) with the other accessions collected from the literature overview and listed in Table 2. Li *et al.* (2010) corroborated this analysis, based on 189 subtilisin-like sequences from Pezizomycotina (major subphylum of the Ascomycota, to which many nematode pathogenic fungi belong). A clear distinction of proteases that are involved in the infection of the nematode body can be observed. The fungal nematode trap neutral protease and the alkaline proteases of the egg pathogens and endoparasite egg pathogen neutral proteases cluster together, just like the alkaline and neutral serine proteases of the Bacilli. The auxiliary proteases (collagenase-like and metalloproteases) are also well recognizable as a separate clade. These results suggest that nematode pathogens can be recognized by comparing the amino acid sequence of their protein virulence factors to the sequences from confirmed nematode pathogens.

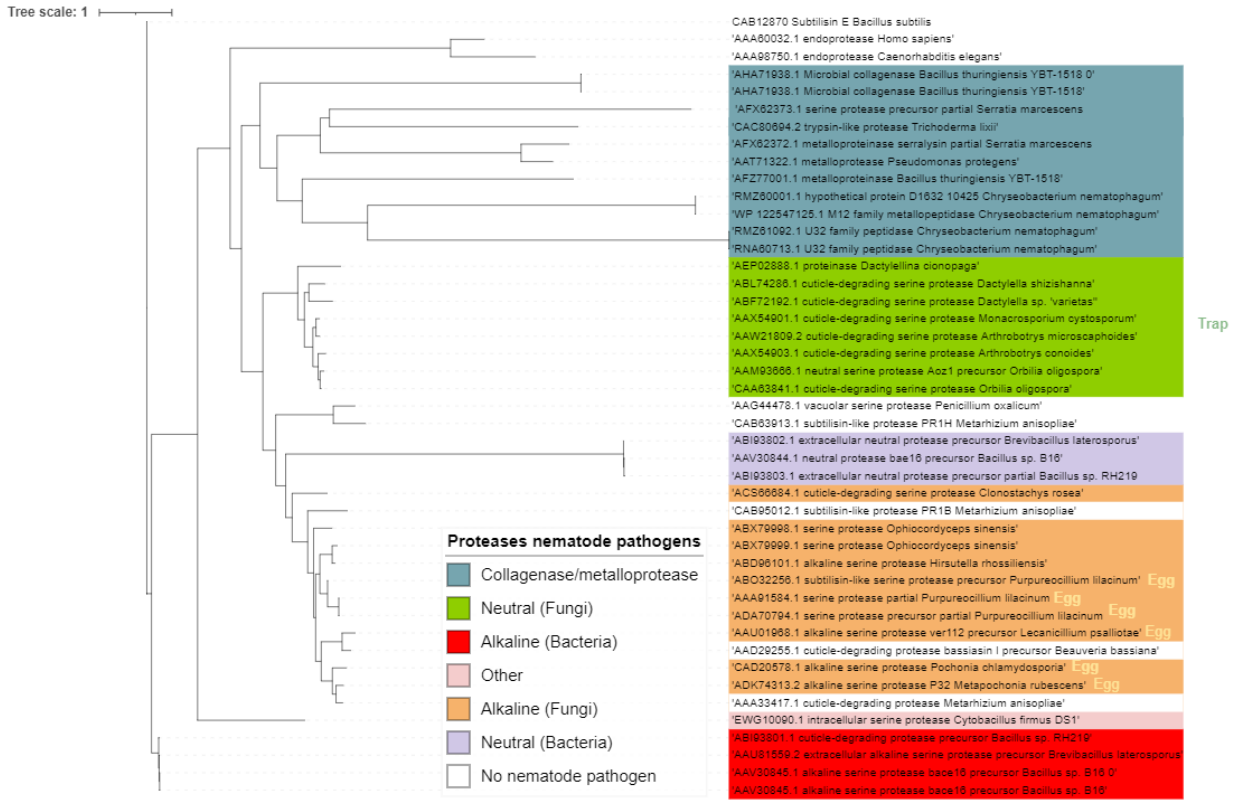

Figure 2: *ML-tree based on the protein sequences of the proteases.* Subtilisin E *of B. subtilis 168 was used as an outgroup. Some new taxonomic names relative to those used in the literature review were*

introduced (Arthrobotrys microcaphoides: Monacrosporium tactopylum; Orbilia: Arthrobotrys; Cytobacillus firmus: Bacillus firmus).

It should be noted that risk assessment of microorganisms for application in food and agriculture currently needs the provision of genome data in order to verify the presence of undesired antibiotic resistance genes, mobile elements, and biosynthetic gene clusters that can produced undesired toxins (EFSA, 2018; https://food.ec.europa.eu/plants/pesticides/micro-organisms_en). By comparing the virulence factors in the investigated strain to those identified in other nematode pathogens a first estimate of the pathogenic potential can be made. The taxonomic identification alone is not a good predictor of the pathogenic potential of a micro-organism.

4.2 Knowledge gaps and recommendations

In general, it seem fair to conclude that the knowledge of the natural pathogens of nematodes is still in its infancy and largely restricted to terrestrial and mostly crop-related systems. There are some upcoming insights about viruses in nematodes and pathogens in the microbiome of marine nematodes, but these are still of a very descriptive nature and often a "bycatch" of sequencing efforts for other reasons. This is remarkable, given the importance of nematodes in various ecosystem services. We can foresee that in the nearby future associations between quantitative metagenomics and ecological data patterns emerge in the dynamics of disease in population of nematode species and that we get a better understanding about the specificity, magnitude and any ecological effects (if measurable due to resilience or redundance).

The structural biological approaches on the adaptation of the proteases by Liang *et al.* (2009 & 2011) or chitinases (Zheng *et al.*, 2016) of certain nematode pathogens or the experimental screens executed by Wei *et al.* (2003) on the lethality or reproductive effects of the different Cry-proteins are excellent studies to demonstrate the principles and help to assess the pathogenicity. Molecular data for this assessment are not lacking anymore. Nowadays, sequencing the genome of any micro-organism in use is available to anyone and common practice and these data can be compared to the clades of the virulence agents that are associated with the infection of nematodes. However, the biological and chemical diversity of (non-target) nematodes and micro-organisms in the environment is very large and the ecological interactions too complex to ever make predictions on the ecological effects outside the feasibility of the bacterial lawn under lab conditions.

If there would be a local extinction of non-target nematodes by the escape of nematode pathogen or the application of a biocontrol agent, their high fecundity will allow them to recolonize the disturbed community in a short time (Schratzberger *et al.*, 2019). From a study on the effects of microwave or steam treatment for sustainable disinfection of greenhouse soils, in which the total nematode community was radically eradicated up to 20 cm deep, the community composition and densities of nematodes was not different from the control situation after 8 to 10 weeks (Bonnet *et al.*, 2022). The recolonization from surrounding soils and deeper layers can explain the observed resilience. Also in marine sediments the recolonization of dramatically disturbed communities. e.g., iceberg scouring, is taking place at relatively short time frames (30 days) (Lee *et al*, 2001). Repeated (re)colonization by nematodes has been described taking place from a complex structure of metapopulations (Folkertsma *et al*, 2007; Sloat *et al.*, 2002).

Given the arguments of ecological interactions in the environment, the natural history of nematode-suppressive soils and the resilience of nematode communities to various stressors, it is highly unlikely that when a nematode pathogen is released in the environment will create a permanent shift in the community composition.

The risk of the environmental introduction of nematode pathogens has not been reported for other pathogens than strains used in the biocontrol of plant and animal parasitic nematodes. This implies that we have to deal with the information provided in these publications.

Microbial biocontrol agents are generally applied at densities of several orders of magnitude larger of what they actually would reach in the soil, on the plant, or in the treated animal. It can be expected to rapidly return to the levels that the carrying capacity the ecosystem allows, and subsequently the interactions with the resident microbiome in the environment are taking place. The monoculture lawns of the *in vitro* assays are therefore not representative for the *population effects* of the pathogen.

Soils are generally resource limited, which means that the chemical warfare with the resident microbiome will make it difficult for the biocontrol agent to find an appropriate niche, if it is still available (Köhle *et al.*, 2019). A biocontrol strain, which has been grown under optimal conditions and applied with adjuvants for quick spread an efficient colonization, will have to cope with conditions like drought, UV-stress and fluctuations in temperature. Also other ecosystem control mechanisms will take place like predation or pathogenesis on the applied strain. The applied doses of microbial biocontrol agents on arable land are approximately 10^{12} cells/ha for fungi and 10^{13} cells for bacteria, which are negligible doses of the 10^{9}-1^{10} microbial cells/g soil. Also for other microbial treatments of biological production systems application of micro-organisms are taking place, e.g. the fertilization with manure (can be rich in nematode pathogens like *Bacillus* spp., as seen in the papers with isolates from dung) introduces 4000x more bacteria than an application with a microbial control agent.

By adhering to the precautionary principle, the ecological risk assessment of mass-reared potential nematode pathogens could be assessed in bioassays in which both the community composition and a key ecosystem service (e.g., nitrogen mineralization) are investigated. A standardized *C. elegans* ISO10872 is already used to perform toxicological evaluation of compounds and samples. For the risk assessment of the escape of a putative nematode pathogen, microcosms experiments consisting of the intended soil types with standardized nematode communities (Griffiths *et al*, 2018; Höss *et al.*, 2021) should be used in order to look at the resilience of the free-living nematodes towards the perturbance (if any). These can be followed by experiments on larger surfaces (arable land, pastures, greenhouse) in order to allow colonization and gene flow of the non-target nematodes.

Table 2: *Overview of virulence factors that target nematodes (modified from Zheng et al., 2016). The items in bold are discussed in the body of the report.*

proteins	organism	gene ID	nematode target	effects on nematodes	references
Proteases					
Fungal proteases					
Serine proteases					
alkaline serine protease Hasp	***Hirsutella rhossiliensis***	**ABD96101.1**	***Panagrellus redivivus***	**Hasp killed the juveniles of the soybean-cyst nematode (*Heterodera glycines*5 and degraded proteins of the nematode cuticle**	**Wang *et al.* (2009)**
alkaline serine protease VCP1	***Pochonia chlamydosporia***	**CAD20578.1**	***Meloidogyne incognita***	**The purified enzyme hydrolyzed proteins in situ from the outer layer of the egg shell of the host nematode *M. incognita* and exposed its chitin layer**	**Morton *et al.* (2003)** **Segers *et al.* (1994)**
alkaline serine protease P32	*Pochonia rubescens*	ADK74313.2	*Globodera pallida*	The purified protease was able to degrade certain cyst nematode proteins, involving nematode egg penetration by *Verticillium suchlasporium*	Lopez-Llor *et al.* (1990)
alkaline serine protease Ver112	*Lecanicillium psalliotae*	AAU01968.1	*P. redivivus*	The purified protease degraded nematode cuticle with 81% of a	Yang *et al.* 92005ab and b)

					nematode being degraded after treating with Ver112 for 12 h	
cuticle-degrading protease Ac1	serine	*Arthrobotrys conoides*	AAX54903.1	*P. redivivus*	Ac1 can degrade a broad range of substrates including casein, gelatin, bovine serum albumin, collagen, and nematode cuticles, can immobilize the free-living nematode *P. redivivus* and the pine wood nematode *Bursaphelenchus xylophilus*	Yang *et al.* (2005c)
cuticle-degrading protease PII	**serine**	***A. oligospora***	**CAA63841.1**	***P. redivivus***	**P Ⅱ immobilized *P. redivivus* in bioassays and hydrolyzed proteins of the purified cuticle. The enzyme hydrolyzed several protein substrates including casein, bovine serum albumin and gelatin, but not native collagen**	**Tunlid *et al.* (1994)** **Ahman *et al.*(1996)**
cuticle-degrading protease PrC	serine	*Clonostachys rosea*	ACS66684.1	*P. redivivus*	The purified protease could degrade a broad range of substrates including casein, gelatin and nematode cuticle	Liang *et al.* (2010) Li *et al.* (2006)
cuticle-degrading protease Ds1	serine	*Dactylella shizishanna*	ABL74286.1	*P. redivivus*	The purified protease could degrade purified cuticle of *Penagrellus redivivus* and a broad range of protein substrates	Wang *et al.* (2006)
cuticle-degrading protease Dv1	serine	*D. varietas*	ABF72192.1	*P. redivivus*, *Caenorhabditis elegans*	This protease could immobilize the free-living nematodes *P. redivivus* and	Yang *et al.* (2007)

				C. elegans and hydrolyze the purified cuticle of *P. redivivus*	
cuticle-degrading serine protease Mc1	*Monacrosporium cystosporum*	AAX54901.1	*P. redivivus*, *B. xylophilus*	Mc1 could degrade a broad range of substrates including casein, gelatin, BSA (bovine serum albumin), and nematode cuticle, could immobilize the free-living nematode *P. redivivus* and the pine wood nematode *B. xylophilus*	Yang *et al.* (2008)
cuticle-degrading serine protease Mlx	*M. microscaphoides*	AAW21809.2	*P. redivivus*	This protease could immobilize the nematode *Penagrellus redivivus* in vitro and degrade its purified cuticle	Wang *et al.* (2006)
neutral serine protease Aoz1	*A. oligospora*	AAM93666.1	*P. redivivus*	Degrade nematode cuticle and immobilize *P. redivivus* in vitro	(Minglian *et al.*, 2004)
Protease	*Dactylellina cionopaga*	AEP02888.1			unpublished
serine protease Csp1	*Ophiocordyceps sinensis*	ABX79998.1	*Hepialus* sp.	Degraded the cuticle proteins of larval *Hepialus* sp. in vitro	Zhang *et al.* (2008)
serine protease Csp2	*O. sinensis*	ABX79999.1	*Hepialus* sp.	Degraded the cuticle proteins of larval *Hepialus* sp. in vitro	Zhang *et al.* (2008)
serine protease precursor	*Purpureocillium lilacinum*	ADA70794.1	*M. incognita*	Overexpressed enhanced the virulence of *Paecilomyces lilacinus* against *M. incognita* eggs	Wang *et al.* (2010)
subtilisin-like serine protease precursor	*P. lilacinum*	ABO32256.1			unpublished

Bacterial proteases					
alkaline serine protease Bace16	***Bacillus nematocida* B16**	**AAV30845.1**	***P. redivivus***	**The purified protease can hydrolyze several native proteinaceous substrates, including collagen and nematode cuticle**	**Niu *et al.* (2006a)**
neutral protease Bae16	***B. nematocida* B16**	**AAV30844.1**	***P. redivivus***	**The purified protease could destroy the nematode cuticle and its hydrolytic substrates included gelatin and collagen.**	**Niu *et al.* (2006b)**
Metalloproteinase Bmp1	*B. thuringiensis* YBT-1518	AFZ77001.1	*C. elegans*	Purified Bmp1 protein showed toxicity against *C. elegans*, enhanced the toxicity of Cry5Ba and degrades intestine tissues	Luo *et al (2012)*
Metalloproteinase ColB	***B .thuringiensis***	**AHA71938.1**	***C. elegans***	**Metalloproteinase ColB destroy the intestine thereby facilitates the adaptation and colonization of *B. thuringiensis* in *C. elegans*.**	**Peng *et al.* (2016)**
cuticle-degrading protease precursor Apr219	*Bacillus* sp. RH219	ABI93801.1	*P. redivivus*	Nematicidal and cuticle-degrading activities for *Bacillus* sp. RH219 were mostly due to the extracellular serine alkaline protease Apr219	Lian *et al.* (2007)
extracellular neutral protease precursor Npr219	*Bacillus* sp. RH219	ABI93803.1	*P. redivivus*	The addition of neutral protease Npr219 increased the mortality of Apr219	Lian *et al.* (2007)

extracellular alkaline serine protease BLG4	***Brevibacillus laterosporus***	**AAU81559.2**	***P. redivivus***	**The protease hydrolyzed relatively broad substrates including collagen and the cuticle of nematodes**	**Huang *et al.* (2005)**
extracellular neutral protease precursor NPE-4	***B. laterosporus***	**ABI93802.1**	***P. redivivus***	**NPE-4 could degrade proteins from the inner layer of purified cuticles from nematode *P. redivivus* in vitro.**	**Tian *et al.* (2006)**
Metalloprotease AprA	*Pseudomonas protegens*	AAT71322.1	*M. incognita*	AprA inhibited egg hatching and induced mortality of *M. incognita* juveniles	Siddiqui *et al.* (2005)
Metalloprotease (Zn-dependent)	*Pseudomonas veronii R4*	WP_017845712	*Xiphinema index*	Cell-free supernatant, containing milk-induced AprA, caused disintegration of nematode cuticle.	Canchignia *et al.* (2017)
metalloproteinase serralysin	*Serratia marcescens*	AFX62372.1	*B. xylophilus*	Serralysin showed nematicidal activity to *B. xylophilus*	Paiva *et al.* (2013)
serine protease precursor	*Serratia marcescens*	AFX62373.1	*B. xylophilus*	Serine protease precursor (AFX62373.1) sowed nematicidal activity to *B. xylophilus*	Paiva *et al.* (2013)
collagenase	*Chryseobacterium nematophagum*	RNA60713.1 RMZ61092.1 WP_122545897.13	*Caenorhabditis elegans* *Caenorhabditis briggsae*	Infection through the pharynx. Complete digestion from the animal, starting from the intestine. Probably also attraction by a Trojan horse mechanism.	Page *et al.* (2019)
astacin	*Chryseobacterium nematophagum*	WP_122547125.1 RMZ60001.1	*Globodera pallida* *Haemonchus contortus*		

			Trichostrongylus vitrinus *Teladorsagia circumcincta* *Cyathostomin sp.* *Ostertagia ostertagi* *Parastrongyloides trichosura* *Cooperia curtecei* *Cooperia oncophera* *Nippostrongylus brasiliensis* *Ancylostoma caninum*		
Trypsin protease					
trypsin-like protease PRA1	*Trichoderma harzianum*	CAC80694.2	*M. incognita*	Pure PRA1 preparations reduced The number of hatched eggs of the root-	Suarez *et al.* (2004)

				knot nematode *M. incognita* significantly	
Chitinases					
Chitinase Lpchi1	*L. psalliotae*	ABQ57240.1	*M. incognita*	The purified chitinase LPCHI1 was found degrading chitinous components of eggs of the root-knot nematode *M. incognita* and significantly influence its development.	Gan *et al.* (2007b)
Chitinase PcChi44	*Metacordyceps chlamydosporia*	ABW96521.1	*M. incognita*	The chitinase PCCHI44 could damage eggs of both the root-knot nematode *M. incognita* and the insect *Bombyx mori*	Mi *et al* (2010)
chitinase precursor PLC	*P. lilacinum*	ABP37997.1		*P. lilacinum* is nematode egg-parasitic fungus	Dong *et al.* (2007)
extracellular chitinases Chi46	*P. variotii*	AAL78814.1(43 kDa endochitinase of *Hypocrea virens*)	*M. incognita*	*P. variotii* DG-3 is a chitinase producer and a nematode egg-parasitic fungus, the purified Chi46 showed chitinase activity bands with 0.01% glycol chitin as a substrate	Nguyen *et al.* (2008)
Chitinase	*Chryseobacterium nematophagum*	RNA61966.1; WP_122546716.9	*(see above)*	Infection through the pharynx. Complete digestion from the animal, starting from the intestine. Probably also attraction by a Trojan horse mechanism.	Page *et al.* (2019)

Lipases	*Pseudomonas veronii* R4				
Lipase (LipA) and Phospholipase (ExoU)		WP_017845717; WP_017844966.	*Xiphinema index*	Cell-free supernatant, containing milk-induced AprA, caused disintegration of nematode cuticle.	Canchignia *et al.* (2017)
Cry proteins					
	B. thuringiensis and *B. toyonensis*	Eight clades are associated with nematode pathogens		Perforation of gut epithelium	Wei *et al.*, 2003; Zheng *et al.* (2016)
Other proteins and peptides					
Nel	*B .thuringiensis*	AHZ54746.1	*C. elegans*	Nel exhibited inhibition activity to nematode, and the toxicity of Nel to nematodes targets the intestine, showing that Nel triggered heat shock pathway and necrosis pathway in nematodes.	Ruan *et al.* (2015)
nematicidal protein AidA	*Burkholderia cenocepacia* J2315	YP_002153683.1	*C. elegans*	Expression of aidA is regulated by the cep quorum sensing system. AidA is involved in killing of *C. elegans*	Holden *et al.* (2009)
Amidophosphoribosyltransferase PurL	*B. subtilis* OKB105	NP_388531.2(*B. subtilis* subsp. *subtilis* 168)	*Aphelenchoides besseyi*, *Ditylenchus destructor*, *B. xylophilus* and	The *purL* gene regulated the production of purine biosynthesis intermediates which affected the nematicidal activity of strain	Xia *et al.* (2011)

			M. javanica	OKB105	
Heat-labile enterotoxins (44)	*Drechmeria coniospora*	XP_040654756.1 KYK55404.1 XP_040657615.1 KYK58263.1 XP_040654225.1 XP_040653304.1 XP_040658204.1 XP_040658203.1 XP_040656590.1 XP_040655711.1		Genome annotation of the obligate nematode pathogen *D. coniospora*	*De Bekker et al. (2017)*

	XP_040661411.1
	XP_040661408.1
	XP_040661010.1
	XP_040660855.1
	XP_040660694.1
	XP_040660643.1
	XP_040660094.1
	XP_040659930.1
	XP_040659929.1
	XP_040659463.1
	XP_040659275.1
	XP_040658996.1

	XP_040658307.1
	KYK62059.1
	KYK62056.1
	KYK61658.1
	KYK61645.1
	KYK61503.1
	KYK61342.1
	KYK61291.1
	KYK60742.1
	KYK60578.1
	KYK60577.1
	KYK60111.1
	KYK59923.1
	KYK59644.1
	KYK58955.1
	KYK58852.1
	KYK58851.1
	KYK57238.1
	KYK57217.1

		KYK56359.1 KYK54873.1 KYK53952.1			
Pseudomonas protegens 15G2	Binary enterotoxin (two domains Pp-ANP1a and Pp-ANP2a)	ASO45453.1 ASO45454.1 ASO45455.1 ASO45456.1 ASO45457.1 ASO45458.1	*C. elegans,* *Pristionchus pacificus, Panagrellus redivivus,* and *Acrobeloides sp.*	Potent and specific activity nematodes (growth retardation and death).	Wei *et al.* (2017)
Unknown mechanism			*0*		
Microbacterium nematophilum	?	?	*Caenorhabditis* sp.	Infection of anal and rectal cuticle	Akimkina *et al.* (2006)
Pleurotes ostreatus	?	?	*Caenorhabditis nigoni* *Caenorhabditis briggsae* *Caenorhabditis remanei* *Caenorhabditis elegans*	Instant death within seconds, mechanisms via the sensory cilia upon contact.	Lee *et al.* (2020)

	Caenorhabditis angaria
	Caenorhabditis drosophila
	Diploscapter coronatus
	Oscheius myriophilus
	Rhabditis colombiana
	Oscheius tipulae
	Rhabditis rainai
	Pristionchus pacificus
	Panagrellus redivivus
	Acrobeloides apiculatus
	Cephalobus cubaensis
	Mesorhabditis sp

			Pelodera teres		
Catanaria anguillula	?	?	*P. redivivus*	Colonization of body apertures by adhesion and encystment of motile zoospores. Development of chain of sporangia along the infecting hyphae in the nematode body.	Vaish (2012) (review)

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