

Benefits and uses of nematodes in grassland soils

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Abstract. To most grassland farmers nematodes mean trouble: they are important parasites of both pasture plants and livestock. While there is no doubting the considerable losses caused by nematodes, crop and livestock pests represent a tiny minority of the approximately 26,000 described nematode species. Here I examine the beneficial effects of nematodes within grassland systems and their beneficial uses. Nematodes in grassland soils increase plant available nutrients, move beneficial microbes through the rhizosphere and control insect and mollusc herbivores. We can use nematodes as biological control agents, and also as indicators of soil health/quality. While no single group of organisms can give a comprehensive overview of soil health, nematodes offer many advantages. In field soils, analysis of nematode abundance and diversity allows us to infer much about the soils health and function. Furthermore, the short lifespan and numerous biological techniques developed for the model nematode *Caenorhabditis elegans* makes this animal an excellent species for use in ecotoxicity testing. We can measure the worm's response at the molecular, behavioural and reproductive level. Nematodes thus have much potential for assessing risks and benefits associated with novel agricultural practices, agrochemicals and transgenic crops.

Keywords: Nematodes, ecosystem services, diversity, bioindicators, risk assessment.

Introduction

Most members of the general public, if asked about bacteria, would probably know that these organisms cause disease in humans. Most would have no idea about the global importance of bacteria in driving biogeochemical cycles and providing vital ecosystem services in the oceans, freshwater and on land. Few members of the public would have even heard of nematodes, but most agricultural and environmental scientists have. However, as with the public's perception of bacteria, these scientists often have a negative perception of nematodes. This is not surprising bearing in mind the huge losses and health costs caused by nematode parasites of crops (Manzanilla-Lopez *et al.* 2004), livestock (Geary *et al.* 2012) and humans (Humphries *et al.* 2012). But, animal and plant parasites only represent a small fraction of the known nematode fauna (Hodda *et al.* 2009), and nematodes too provide many benefits.

Nematodes are the most numerous animals on earth (Poinar 1983) and are particularly abundant in soils. Soil populations range between 1–100/g dry weight (Young *et al.* 1998) and a typical soil population of 20/g equates to 60 billion/ha. Because of this abundance, it has been estimated that 80–90% of all animals on earth are nematodes (Platt 1994; Jairajpuri and Ahmad 1992).

In addition to their abundance, nematodes are both taxonomically and functionally diverse. In a recent review, Hodda *et al.* (2009) estimated that there are about 26,000 described and accepted species with a similar number of published species names that are regarded as synonyms. But, it is widely acknowledged that the described species will only represent a small fraction of

total nematode diversity with described species representing in the region of 3 – 10% of total species (Hodda *et al.* 2009). Functional diversity of nematodes is also high with most soils containing bacteriovores, fungivores, plant parasites, omnivores, predators and invertebrate parasites (Yeates *et al.* 1993).

In grassland soils, there are typically 3–4 million nematodes/m² (Coulson and Whittaker 1978; Yeates *et al.* 1997) with up to 150 species present in temperate grasslands (Hodda and Wanless 1994a;b) and up to 228 species in Kansas prairie grassland (Yeates 1998). Some soil ecologists are reluctant to work with nematodes because they are perceived to be difficult to identify. But for most nematode community analyses, nematodes only have to be identified to family level. Furthermore, a variety of molecular techniques, primarily developed for use in soil microbiology, have been adapted for use with nematodes. Techniques include denaturing gradient electrophoresis (Foucher and Wilson 2002; Foucher *et al.* 2004), terminal restriction fragment length polymorphisms (Donn *et al.* 2012) and use of next generation sequencing methods on DNA extracted from soil samples (Porazinska *et al.* 2009; 2012). While little used at present, with research and development, these techniques have potential to increase the use of nematodes.

In this paper, I review some of the beneficial functions performed by nematodes, with particular reference to grassland soils, and also describe ways we can use nematodes for pest control, soil health monitoring and assessing risks of new agricultural practices.

Ecosystem services provided by nematodes

Three key ecosystem services are provided by nematodes: increasing plant available nutrients, transport of beneficial bacteria and control of pest herbivores (Fig. 1).

Enhancing nutrient availability

The majority of animals including nematodes tend to consume more nitrogen than they need. The reason for this is two-fold: nematodes often have a higher C:N ratio than their diet, *e.g.* for bacterial feeding nematodes bacteria are typically 4:1 whereas nematodes are 5.9:1 (Chen and Ferris 1999). Furthermore, ingested C is required by the consumer for both building biological molecules, and for respiratory generation of energy, whereas N is typically only used for building molecules. After respiratory loss of assimilated carbon is taken into account, nematodes typically will have consumed 18% more nitrogen than required. In the case of nematodes, excess N is excreted as ammonium that can be readily taken up by plants, or microbes.

There is also recent evidence suggesting that nematode grazing on bacteria may be important in making phosphorous available to plants. Many soil bacteria have the ability to solubilise forms of phosphorous that are unavailable for uptake by plants. The P only becomes available to plants once the P solubilising bacteria have been consumed by bacterial grazers such as protozoa and nematodes. Irshad *et al.* (2011) showed that presence of bacterial-feeding nematodes significantly enhanced both N and P availability to *Pinus pinaster* seedlings. Furthermore, Irshad *et al.* (2012) showed that the phytase-producing bacteria *Bacillus subtilis* alone did not enhance plant available P, but when bacterial feeding nematodes were present, P uptake by seedlings increased.

Most studies investigating how nematodes make nutrients available to plants have been done in microcosm or pot trials but there is evidence that

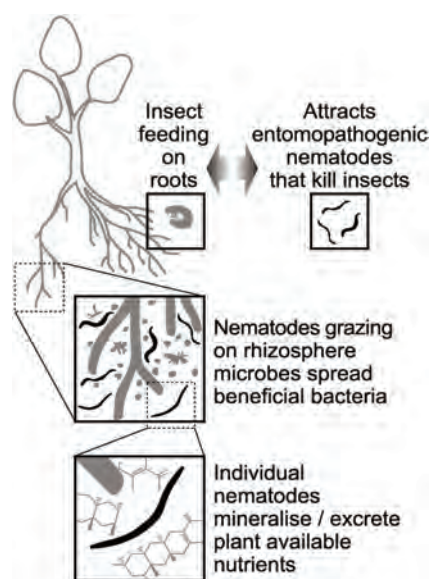


Figure 1. Ecosystem services provided by soil nematodes: control of herbivores, spread of beneficial rhizobacteria and enhancing availability of nutrients.

nematodes play an important part in plant nutrition in the field. Ekschmitt *et al.* (1999) investigated the effects of nematodes on the microbiology and soil nitrogen status in six major European grassland types ranging from Northern tundra in Sweden to Mediterranean garigue in Greece. They found profound effects of nematodes on the soil nitrogen status, with up to 27% of plant available nitrogen being attributed to nematode excretion.

Bacterial Transport

It is well known that the presence of certain bacteria in the rhizosphere and rhizoplane is beneficial to plants. The beneficial effects of bacteria include growth promotion by enhancing nutrient uptake by roots, and antagonistic effects against plant pathogens. These beneficial effects have led many researchers to develop rhizosphere bacteria as biological fertilisers or biological control agents. These are often seed applied as this allows much reduced application rate of beneficial organisms (O'Callaghan *et al.* 2012; Wilson and Jackson 2013). However, to be effective, the seed coated bacteria must colonise the rhizosphere, and the varying success of many field applications may be explained by differences in the extent of rhizosphere colonisation (Weller 1988). Bacterial feeding nematodes tend to be abundant in the rhizosphere, where they graze the numerous rhizobacteria. As they move through the rhizosphere they transport bacteria on their cuticles. The actions of nematodes is particularly beneficial in enhancing rhizosphere colonisation by beneficial seed-applied bacteria (Knox *et al.* 2003; 2004).

Another example of the benefits of nematodes is to rhizobacteria, of particular relevance to grassland systems related to *Rhizobium* spp. It has been shown that *Rhizobium* spp. can be vectored by the nematodes *Pristionchus iheritieri* (Jatala *et al.* 1974), *Cepholobus parvus* and *Macrolaimus crucis* (Cayrol *et al.* 1977). These studies found that the nematodes distributed rhizobial cells more evenly over the root surface, increased nodulation and thus benefited the plants.

Predation/parasitism of herbivorous invertebrates

Most animals are hosts to a broad range of parasites and numerous nematodes parasitise insects; approx 15% of all described nematode species are entomophilic (Hodda *et al.* 2009). While many nematode parasites have a highly co-evolved relationship with their hosts, and cause them little harm, some species behave more like parasitoids and kill their hosts. The best studied examples of such nematodes are the entomopathogenic nematodes of the families Steinernematidae and Heterorhabditidae. These nematodes are not closely related phylogenetically, but share similar life-cycles through convergent evolution. The nematodes have formed obligate, mutualistic associations with entomopathogenic bacteria which they carry in their intestines. The infective stage of the parasite locates a suitable host insect and penetrates, typically through natural openings, such as mouth, anus or spiracles. Once inside the insect, the nematodes release their symbiotic bacteria into the host's haemolymph. The bacteria

proliferate and produce a wide range of toxins/enzymes that kill the host. The nematodes then multiply and reproduce by feeding on tissue of the dead insect, and the developing bacteria. Once these food resources are depleted, the nematodes again form infective stages that leave the insect cadaver in search of new prey (for review see Gaugler 2002).

Entomopathogenic nematodes are widespread and common in both cultivated and natural soils throughout the world (Hominick 2002). It is difficult to estimate what level of control natural populations are having on pest insect populations but the few studies available suggest they are significant regulators of herbivorous insect populations (Strong *et al.* 1996). Furthermore, it has been shown recently that certain plants that are subjected to insect root herbivory produce signalling molecules that attract entomopathogenic nematodes to kill the insects (Rasmann *et al.* 2005) – a phenomenon that has been known in above ground systems for many years.

Another nematode that kills its host and thus is likely to exert a degree of control over pest herbivores is the slug-parasitic nematode *Phasmarhabditis hermaphrodita* (Rae *et al.* 2007). Slugs are little studied pests that cause much damage in numerous countries throughout the world. One European species of slug, *Deroceras reticulatum* (the grey field slugs) is the most widely distributed pest species and is established in North and South America, Australia and New Zealand, and many parts of Africa and Asia. In grassland soils slugs are usually considered to be a problem at establishment, and cultivation prior to sowing kills slugs both by causing mechanical damage and exposure to predatory birds on the soil surface. However when crops are direct drilled without cultivation many growers use molluscicidal bait pellets prior to sowing to reduce slug populations (Wilson and Barker 2011). However, slugs are often also present in established grasslands soils, and where clover is grown alongside grasses, as is common in New Zealand, slugs can cause considerable damage to clover. Growers tend not to treat for slugs in established pasture, but Barker and Addison (1992) showed that treating established pasture with molluscicidal baits reduced slug numbers and increased the yield of clover by 12–40%. Much less is known about this nematode than the entomopathogenic nematodes. While *P. hermaphrodita*'s lifecycle is in some ways similar to entomopathogenic nematodes, there does not appear to be any specific bacterium associated with *P. hermaphrodita* and its mechanism of killing slugs remains unknown (Rae *et al.* 2010). The nematode is widely distributed in Europe (Mengert 1953; Morand *et al.* 2004; Ross *et al.* 2010) and is present in New Zealand (Wilson *et al.* 2012) but has not yet been found in the USA despite a reasonably large number of slugs having been examined (Ross *et al.* 2010).

Beneficial uses of nematodes

Nematodes as biological control agents

Natural populations of entomopathogenic nematodes likely play a significant role in regulating pest insects in

pasture. Many companies now mass produce these nematodes in very large scale fermenters, formulate them onto carriers and sell the nematodes as biological insecticides, or biological molluscicides in the case of *P. hermaphrodita* (see Grewal *et al.* 2005a for review). The nematodes are fairly expensive to produce and tend to be sold for use in higher value horticultural crops. It may be that through economy of scale and decreased production costs, nematodes may be applied widely to grasslands in the future. Biologically, there is every reason to believe they could work; the nematodes are sold widely to control insect pests of turf grass including scarab larvae, curculionid and lepidopteran pests (Grewal *et al.* 2005b). The only example to date where nematodes have been applied widely to grassland soils is in Florida USA. Mole crickets (*Scapteriscus* spp.) are among the most important insect pests of turf and pastures in Florida where their tunnelling and feeding causes substantial pasture loss. Mole crickets are invasive in the United States, and it is thought they were introduced accidentally from South America early in the twentieth century. As with many invasive pests, the mole crickets did much more damage in their invasive range than in their home range, and it was hypothesised this may result from enemy absence. A search for natural enemies of mole crickets in Uruguay found a new species of entomopathogenic nematode, *Steinernema scapterisci* that was highly specific for mole crickets. The nematode was introduced into Florida in 1985 and subsequently further inoculative applications since 1993 in golf courses and pastures (Parkman *et al.* 1993; 1994) have led to fairly widespread establishment. Because nematodes have somewhat limited dispersal capacity and natural spread through Florida was slow, the nematode was produced marketed commercially for several years for farmers to buy. The nematodes were applied using slit injectors in strips covering one seventh of the area of the field to reduce application costs. To date there have been no documented uses of the slug-parasitic nematode *Phasmarhabditis hermaphrodita* in grassland soils.

Nematodes as biological indicators

All agricultural practices have impacts on the soil biota, typically by replacing natural mixed plant-root communities, with simple monocultures or bi-cultures of roots. Beyond just altering the plant community, agriculture alters the soil micro and macro-biota through alterations in carbon flow, litter input and changes in soil physical properties caused by tillage. This in turn can influence many important soil functions such as mineralisation, nitrification and denitrification. In terms of functions we see as detrimental *e.g.* denitrification, we want to adopt agricultural practices which minimise these functions. Also, with increasing environmental awareness, we need to be able to measure the impacts of differing agricultural practices on soil biota. This is true of very large scale changes in practice (*e.g.* organic vs. conventional agriculture) or may relate to very specific changes, *e.g.* measuring non-target impacts of a single agrochemical, or non-target impacts of growing a genetically modified crops. Nematodes can help answer

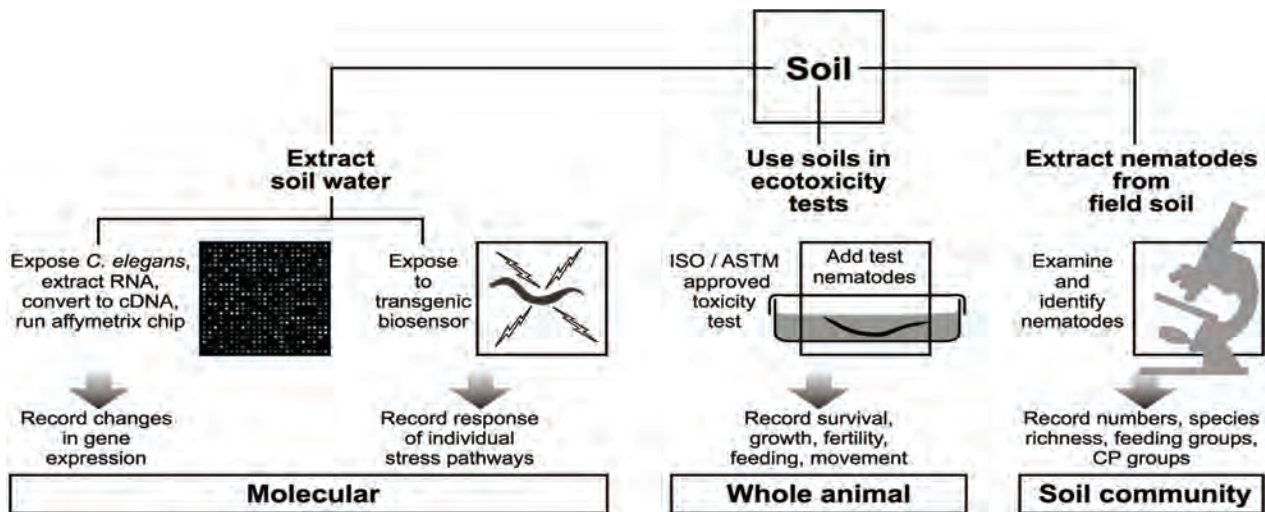


Figure 2. Using nematodes as biological indicators at the molecular, whole animal and community ecology scale.

all of these questions and can be used to indicate changes at the level of gene expression, whole animal or community ecology (Fig. 2).

Nematodes as indicators of changes in gene expression

The first animal to have its entire genome sequenced was the nematode *Caenorhabditis elegans* (The *C. elegans* Sequencing Consortium, 1998). The intense research interest in this organism, which was originally chosen as a model organism for use in developmental biology research, has generated a wealth of techniques for studying and manipulating gene expression and resulting phenotypic consequences. It is now possible and relatively straightforward to study changes in transcription of the entire *C. elegans* genome using DNA micro-arrays (glass slides spotted with thousands of test gene sequences). Affymetrix produce and market the GeneChip *C. elegans* Genome Array - a whole genome array that can record expression levels of the 22,500 different coding genes from *C. elegans*.

These chips can be used to measure specific responses in transcription to any environmental factor. This is usually done using a dual-colour DNA microarray experiment. Briefly, mtRNA is isolated from control and treated nematodes and reverse transcribed into cDNA which is labelled with different coloured probes for the two treatments. The two batches of cDNA are combined and hybridized to the array, which is then scanned using a laser scanner that records colours associated with each gene. The relative abundance of the treatment vs. control probe colours report whether the transcription of individual genes is unaffected, up-regulated or down regulated when exposed to the treatment.

This technology is still relatively new, and most examples of its use have looked at responses to individual pollutants added to soil. One study of relevance to agricultural soils is that of Menzel *et al.* (2005) who investigated the effects of humic materials in natural organic matter (known to have a variety of toxic effects) on gene expression in *C. elegans*. These authors found that the most active humic-like substance induced

up-regulation in 554 genes and down regulation in 885 genes.

Transgenic nematode biosensors

Numerous ways of studying gene function have been developed for use with *C. elegans* including transformation of nematodes by addition of DNA, gene knock-out technology and RNA interference. This has enabled the development of whole-organism, transgenic *C. elegans* biosensors. Two general approaches have been used in developing transgenic nematode biosensors: biosensors that report on the induction of stress promoters and biosensors that report on perturbation of energy balance. In the former, a reporter gene that produces an easily visualised phenotype (*e.g.* fluorescence, luminescence) is linked to a promoter of a stress induced gene. These can be promoters that respond to very specific stresses, *e.g.* presence of lead, or general stress response promoters *e.g.* those associated with heat shock proteins. Biosensors using energy balance approaches rely on measuring stress induced metabolic perturbation using reporter genes that report on levels of cellular ATP (Lagido *et al.* 2001).

Examples of using transgenic nematode biosensors relevant to grassland soils include the work of McLaggan *et al.* (2012) and Anbalagan *et al.* (2013). McLaggan *et al.* (2012) used a metabolic biosensor to assess toxicity of sewage sludge (biosolids), the waste product of sewage treatment. This by-product is frequently applied to agricultural land (including grasslands) as a cheap source of nutrients, and a convenient disposal method. The sludge contains numerous potentially toxic materials but mostly at levels thought not to be harmful. McLaggan *et al.* (2012) tested the combined toxicity of sewage sludge extracts using a luminescent metabolic biosensor that showed that the complex mixture of pollutants had subtle adverse effects on *C. elegans*. Anbalagan *et al.* (2013) investigated the potential toxicity of a range of agricultural pesticides to nematodes using 24 GFP-reporter *C. elegans* strains representing genes from four different stress-response pathways. Some tested pesticides *e.g.* the herbicide diuron had no

detectable effects, whereas others, including the organophosphate insecticide diruon induced multiple stress pathways.

Nematode Ecotoxicity tests

Nematode toxicity tests can be used to monitor either toxicity of test substances or the toxicity of field soils suspected to contain toxins *e.g.* agrochemicals. Typically, known numbers of nematodes are incubated in soil, extracted and survival, development and movement studied. Toxicity is tested at the whole animal level (usually *C. elegans*) and is measured using a wide range of endpoints. Nematodes were first used in ecotoxicity tests some 40 years ago (Boroditsky and Samoiloff 1973) and have numerous positive features for use as test organisms: firstly, they are animals, having reproductive and nervous systems, and thus have potential to respond to neurotoxins and endocrine disrupting compounds. Secondly, their small size, and ease of handling means that nematode-based ecotoxicity tests can be done much more rapidly, and with much smaller amounts of soil than needed for other animals *e.g.* earthworm tests. But, despite these advantages, nematodes tend to be underrepresented in toxicological assessments (Höss and Williams 2009).

A variety of different end points can be used to assess toxicity of compounds to nematodes. While survival is the easiest to assess, it is a much less sensitive measure than many sub-lethal effects. For example, Boyd and Williams (2003) measured the effects of copper on survival, reproduction and movement of *C. elegans* and found that sub lethal effects on reproduction and movement could be detected at concentrations 40 times lower than those that induced mortality. Thus, when, for example, testing a new agrochemical for non target effects, a range of parameters should be recorded including movement behaviour, development time, adult size and reproduction capacity.

In order to standardise methods both the American Society for Testing and Materials (ASTM) and the International Organisation for Standardisation (ISO) have drawn up standard procedures for using *C. elegans* in soil toxicity tests (Anon 2001; 2009). From the evidence that is available, the standards appear to be robust: Höss *et al.* (2012) compared data from eight separate laboratories testing toxicity of aqueous solutions of benzyl-cetyldimethylammonium chloride as well as native sediments and soils against *C. elegans* following ISO 10872. They found that both across and within laboratories the standard test gave a good degree of repeatability and reproducibility.

Nematode community analysis

As mentioned previously, nematodes in soils are abundant and diverse. Their relatively short lifespans and limited mobility means that the community composition changes relatively rapidly in response to perturbation. The effects of these changes are long-lived as re-colonisation by nematodes is slow (Yeates and van der Meulen 1996). Extracting the entire nematode community from soil samples and identifying them is slow and technically demanding. But, data from *in situ*

assays are more ecologically relevant than molecular or single organism assays. While identifying nematodes to species level is challenging to all except a few trained individuals, nematode community analysis does not need such high taxonomic resolution. For community analysis, nematodes are principally categorised by two factors, their trophic group and their coloniser/persister status. The former can readily be ascertained by examining the mouthparts and requires little training, and the latter requires identification only to the family level. A key advance in nematode community analysis was the development of the Maturity Index (MI) by Bongers (1990). The MI was developed as a gauge of the condition of the soil ecosystem and ranked nematode families on a coloniser/persister (akin to *r* and *K* strategists) scale ranging from 1 (rapid colonisers) to 5 (long term persisters). Since 1990, nematode community indices have evolved and numerous other indices based on, but expanding on the MI concept have been developed. Further indices have also been developed based on the concept that presence of certain MI group 1 colonisers is an indication of enrichment of the system, MI group 2 nematodes indicate 'basal' fauna, and certain higher MI groups are an indication of food-web structure. Differences in the trophic groups of nematode taxa have been incorporated into the system such that nematode analysis can give an indication of habitat disturbance, enrichment, primary decomposition channels (fungal vs. bacterial) and food web structure. Detailed further description of the calculation of these indices and their uses is given in Ferris and Bongers (2009).

Examples of where these indices have been used to test impacts of agricultural practice on grassland soils include that of Wei *et al.* (2012). These authors used nematodes to study how nitrogen addition to grassland soils influenced the soil community. They found increasing nitrogen enrichment lead to significant reductions in total nematode abundance, diversity (H' and taxonomic richness), MI, and the abundance of fungivores, omnivores and predators. These authors attributed the reduced nematode diversity to ammonium suppression.

Nitrogen leaching from dairy pasture can be a considerable problem, and one way to decrease runoff is the use of nitrification inhibitors. In New Zealand, the nitrogen inhibitor DCD is frequently used. Aalders and Bell (2008) used nematodes to investigate possible non-target effect of DCD. They found no differences in any of the nematode community indices that they calculated suggesting that frequent, small applications of DCD over a single year had no major effects on soil nematodes.

Integrating assays at different levels

One of the key advantages of the battery of ways in which nematodes can be used as environmental indicators, is their potential for integration. A long term goal will be to determine whether changes at the molecular level in individual animals can predict long term ecological consequences. We are a long way from answering this question but people are starting to combine short term and long term assays. Höss *et al.*

(2011) used a combination of nematological techniques in a tiered approach to assess the risk posed to free living nematodes by genetically modified maize. Use of transgenic crops in agriculture has been hugely successful, but controversial in some geographical regions e.g. most of Europe and New Zealand. In such countries, detailed risk analysis needs to be conducted prior to general release. Höss *et al.* (2011) investigated the risk associated with maize genetically modified to express a *Bacillus thuringiensis* toxin, (CRY3Bb1) which confers resistance against the corn root worm *Diabrotica virgifera*. These authors tested acute toxicity of purified Cry3Bb1 protein; the changes in gene expression in *C. elegans* when exposed to sub-lethal toxin levels; toxicity of rhizosphere soil from transgenic crops; and also studied the composition of natural nematode community *in situ* in plots growing transgenic and control maize plants. They found that while the protein was not acutely toxic to nematodes, high doses did inhibit growth and reproduction of *C. elegans*. Furthermore, the authors showed that Cry-protein-specific defence genes were up-regulated in the presence of the toxin. However, there were no inhibitory effects when the nematode was grown in soil taken from the rhizosphere of transgenic crops. This latter finding was in keeping with the acute toxicity test data, when levels of the toxin in the rhizosphere were measured. Finally these authors showed no significant sustained changes of functional diversity or maturity of the nematode communities living beneath transgenic and conventional maize crops, and concluded that the transgenic maize posed little risk to soil nematodes.

Conclusions

Nematodes are the most abundant animals on earth, but most research on this phylum has concentrated on the few species that are parasites of crops, livestock, and humans. However, nematologists are starting to appreciate that the vast abundance and diversity of nematodes in agricultural soils provide several beneficial ecosystem services including making nutrients available to plants, spreading beneficial bacteria and predation of herbivores. This abundance and diversity means that analysing nematode communities can give much information on the biological state of soils. The numerous established molecular techniques developed for the model nematode *Caenorhabditis elegans* gives nematodes much potential for use as bioindicators of perturbation generating useful data at the molecular, whole animal and community levels.

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