National Institute for Public Health
and the Environment
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Colophon

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Abstract

Assessment of a GM-crop impact on soil systems using the DNA barcode-based tool for nematode community analysis

The RIVM (Dutch abbreviation for the National Institute for Public Health and the Environment) has developed with the Wageningen University (WUR) a new technique by which the soil quality can be determined accurately, the so-called nematode DNA barcode tool. This molecular method provides faster and more detailed information about disturbances in soil quality and the possible causes. This can be done because this novel information is combined with data on the overall processes by which crops are grown. Examples are the use of pesticides and effects on the soil systems of agricultural techniques such as ploughing and fertilizing. In this way a better understanding of the influences on soil quality of agricultural practices, such as genetically modified (GM) crops, can be achieved.

With the new method, the nematode DNA is determined with a special technique (quantitative PCR), by which both species (occurrence) as numbers (densities) can be derived in the soil. The nematode population reveals the important processes ongoing in the soil that support soil quality. Examples thereof are the fertility and the extent to which organic material is broken down. The DNA barcode tool is an addition to the traditional time-consuming technique, where the nematode population is determined using a microscopic examination.

The method was developed on behalf of the Netherlands Organization for Scientific Research (NWO) and the Secretary of Infrastructure and the Environment (IenM). Due to the increasing human population higher food production is needed globally, which implies more agricultural land for more crops. Not every management technique to support such a productivity increase, such as GM-crops, might be realized because they have to be safe for the environment. Hence, the fertility of the soil appears to become affected. It is therefore important to avoid possible negative effects by new forms of agriculture with a careful evaluation. Even in a broader European context, there is more emphasis on the importance of vital ecosystems belowground and on the quality of soils. One example is the 'Common Agricultural Policy' which the European Union has been promoting.

Key words: sustainable management, soil systems, nematode DNA barcode tool, ecological processes, General Surveillance GMOs

Rapport in het kort

Richtlijn om effecten van GM-gewassen te bepalen met DNA van bodemaaltjes

Het RIVM heeft met de Universiteit Wageningen (WUR) een nieuwe techniek ontwikkeld waarmee de kwaliteit van de bodem nauwkeuriger kan worden vastgesteld, de zogeheten nematode DNA-barcode tool. Deze moleculaire methode levert sneller gedetailleerdere informatie over verstoringen van een goede bodemkwaliteit en wat daarvan de oorzaak kan zijn. Dit is mogelijk doordat deze nieuwe informatie vervolgens wordt gecombineerd met gegevens over het totale proces waarmee gewassen worden verbouwd. Voorbeelden zijn het gebruik van gewasbeschermingsmiddelen en effecten op de bodem van landbouwtechnieken als ploegen en bemesten. Op deze wijze ontstaat een beter beeld van de invloeden op de bodemkwaliteit van landbouwpraktijken, zoals genetisch gemodificeerde (GM) gewassen.

Met de nieuwe methode wordt het DNA van aaltjes met een speciale techniek vastgesteld (kwantitatieve PCR), waarmee zowel de soorten als de aantallen in de bodem worden bepaald. De aaltjespopulatie weerspiegelt namelijk belangrijke processen in de bodem waaraan de kwaliteit kan worden ontleend. Voorbeelden daarvan zijn de vruchtbaarheid en de mate waarin organisch materiaal wordt afgebroken. De DNA-barcode tool is een aanvulling op de tijdrovende klassieke techniek, waarmee de aaltjespopulatie met behulp van microscopisch onderzoek in kaart wordt gebracht.

De methode is ontwikkeld in opdracht van de Nederlandse Organisatie voor Wetenschappelijk Onderzoek (NWO) en het ministerie van Infrastructuur en Milieu (IenM). Door de bevolkingsgroei is wereldwijd een hogere voedselproductie nodig waarvoor meer landbouwoppervlakten nodig zijn die meer gewassen opbrengen. Niet iedere techniek om een dergelijke toename te realiseren, zoals GM-gewassen, lijkt veilig voor het milieu. Zo kan de vruchtbaarheid van de bodem worden aangetast. Het is daarom van belang om mogelijke negatieve effecten van nieuwe landbouwvormen te evalueren. Ook in een breder, Europees kader is er meer aandacht voor het belang van bodembeheer en vitale ecosystemen in de bodem, oftewel de kwaliteit van de bodem. Een voorbeeld daarvan is de ‘Common Agricultural Policy’ die de Europese Unie uitdraagt.

Trefwoorden: duurzaam bodemgebruik, bodemecosysteem, nematoden DNA barcode tool, ecologische processen, General Surveillance GMOs

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Summary

Over the last decades, large monitoring datasets have been compiled for a wide range of taxa and ecosystems. The aim of these efforts was to identify ecological processes, including problems induced by environmental pressure. Since the early days of stress ecology (in which the focus was on single, independent predictors), many different stressors were analyzed to identify their relative importance in altering ecosystem services and hence our well-being.

A long-term monitoring network can generate data that are useful not only for its original purpose, because many novel questions continuously arise from stakeholders and policy-decision makers, questions for which the original monitoring effort was not designed. Meanwhile, many datasets are publicly available, albeit largely unexplored, and there is a huge potential to (re)analyze data although they might have been collected for other purposes. General Surveillance (GS) of genetically-modified crops, for instance, is a typical example of applied crop protection with data from rejuvenated monitoring networks. Such networks can benefit from lumping and data mining with existing trait banks and developing molecular banks.

Due to human population growth, food production needs to increase worldwide, requiring larger areas and higher yields. Any attempt to solve the food quantity problem, does not necessarily imply that all agricultural techniques are environmentally safe. Tools to evaluate potentially adverse effects of novel agricultural practices are necessary, especially in Europe, since novel techniques might affect soil fertility itself. In a broader scope, the developing Common Agricultural Policy of the European Union highlights the profitable aspects of soil management and promotes the importance of vital soil ecosystems. Currently a wide range of statistical methods and mathematical models is used to detect unexpected effects from stressors and data generated by the nematode DNA-barcode tool may serve in this respect. Diagnostics was developed to detect deviations from good ecological status and to identify to which stressors these deviations may be ascribed.

Principles and draft guidance are described for the use of nematodes as ecological indicators, keeping in mind the progress and rapid development of a DNA barcode-based tool. This tool was developed to enable assessment of the environmental safety of novel agricultural practices regarding soil quality and soil fertility.

Samenvatting

Ecologische processen bepalen in belangrijke mate het functioneren van ecosysteemdiensten en daarmee het welzijn van de mens. Om deze processen goed te kunnen identificeren zijn de afgelopen decennia monitoringsgegevens verzameld van allerlei groepen organismen en soorten ecosystemen. Onderzoek naar de effecten van omgevingsstressoren was toen gefocust op enkelvoudige, onafhankelijke stressoren.

Data uit een langlopend monitoringprogramma hoeven niet alleen bruikbaar te zijn om vragen te beantwoorden waarvoor het netwerk ooit was opgezet, maar kunnen antwoord geven op nieuwe vragen van beleidsmedewerkers en belanghebbenden. Inmiddels zijn veel datasets beschikbaar, wat de mogelijkheid biedt deze gegevens te heranalyseren in het licht van huidige vragen. In de voorliggende rapportage zijn deze in onderlinge samenhang onderzocht. 'General Surveillance' (GS) voor genetisch gemodificeerde gewassen is een typisch voorbeeld waarbij gewasbeschermingsmaatregelen kunnen worden getoetst met gegevens uit bestaande monitoringsnetwerken. Technieken zoals 'data mining', waarbij datasets verrijkt worden met gegevens uit andere databanken (zoals traits en moleculaire gegevens), kunnen aan bestaande netwerken meer waarde geven.

Wereldwijd vraagt de bevolkingsgroei een toename van voedselproductie waarbij naast een duurzame benadering ook grotere arealen en hogere opbrengsten nodig zijn. Niet alle oplossingen voor het wereldvoedselprobleem lijken echter veilig voor het milieu. Technieken die effecten van landbouwpraktijken kunnen evalueren zijn noodzakelijk, vooral in Europa, omdat nieuwe landbouwpraktijken mogelijk de bodemvruchtbaarheid aantasten. In een breder kader, de 'Common Agricultural Policy' in de Europese Unie wijst op de winstgevende aspecten van het bodembeheer en het belang van vitale bodemecosystemen. Momenteel wordt er een scala van statistische methoden en wiskundige modellen gebruikt om onverwachte effecten van stressoren op te sporen.

De principes en een ontwerprichtlijn voor het gebruik van bodemnematoden als ecologische indicatoren worden beschreven met inachtneming van de vooruitgang en ontwikkeling van een 'nematode DNA barcode tool'. Met de nieuwe methode wordt het DNA van nematoden (aaltjes) geanalyseerd op een wijze die niet alleen hun voorkomen in de bodem toont (kwalitatieve aanpak), maar ook hun dichtheden bepaalt (kwantitatieve aanpak). De aaltjespopulatie weerspiegelt namelijk belangrijke processen in de bodem waaraan de kwaliteit kan worden ontleend. Deze techniek werd ontwikkeld om een uitspraak betreffende de milieuveiligheid voor vruchtbaarheid en bodemkwaliteit mogelijk te maken van nieuwe landbouwpraktijken.

1 Introduction

1.1 Societal problem definition

Mankind influences the environment, with consequences locally, regionally and globally. Major problem areas, requiring major solutions, relate to energy, food, water, climate and limited resources (www.oecd.org). Within this context, society needs to consider food production for the 9 billion people estimated in 2050 according to the medium variant model (Cohen 2003) and the policy question must be whether the biosphere can support this human population (Running 2012). Forced strategies are the increase of agricultural land use, higher yields and efficient resource management. To gain higher yields per area, novel techniques are not only different crop management practices, but also the development (and introduction) of novel crops, mostly by conventional techniques but nowadays also by genetic modification (GM), as is happening since historical times (Ammann 2007). Despite viewpoints that "genetic modification" *sensu stricto* is regarded as societally unacceptable (cf. Ammann 2007), another viewpoint is that GM-crops should pose no (or negligible) risks or impacts to man and environment – whereby "negligible" has to be defined during a global, regional or national science-policy process.

1.2 Report motives and focus

This report is concerned with the potential for use in ecological risk assessment of GM-crops, of a newly developed method which addresses potential crop-induced changes in nematode assemblages in agricultural soils, based on current "omics" techniques. Nematodes constitute a diverse group within the soil fauna, feeding on microbes, invertebrates (including other nematodes) and plant roots. This intimate feeding relationship with all major players in agroecosystems implies that GM-crop effects will be reflected in the nematode community. Technique is the so-called "DNA-barcode approach" to evaluate nematode assemblage compositions, which would be helpful to extrapolate "impacts" on soil systems to higher operative levels like ecosystem functioning. The development of this method was undertaken in the research program ERGO (Ecology Regarding Genetically-modified Organisms, 2007-2012) funded by the Netherlands Society for Scientific Research (NWO, The Hague). Our study is part of the research line about the effects of GM-crops on ecosystem functioning.

The start of developing this tool was triggered by scientific progress made in the field of DNA barcode-based evaluation of nematode assemblage structure (Holterman *et al.* 2006; Van Megen *et al.* 2009), in combination with practice-oriented motives. That is, the barcode approach can potentially be used as high-throughput technique in soil quality evaluation and – amongst others – GM-crop risk assessment, while it is potentially feasible in terms of assessment in comparison to ongoing methods. In developing the tool, emphasis was put on scientific and technical development, aspects of sensitivity, structure-function studies and perspectives for application in the context of Ecological Risk Assessment of GM-crops. All three potential risk assessment applications of the proposed tool (pre-market testing, case specific monitoring, and General Surveillance) were considered.

Within our project on nematode DNA barcodes, emphasis was on (1) the development of the technique, (2) determining the relative effects of disturbances using the tool, and (3) assessing the association between signals obtained by this method and soil function parameters. The focus of this report is to address briefly ERA issues in relation to the DNA-barcode tool for nematode assemblages and the implications for vital soil functions. In order to address ERA issues, the report introduces and summarizes major aspects of both the tool and of ERA-principles. The context is not only the ERGO research in the scientific sense, but also the Dutch and European legislative arena. This does not imply that the results of our research would not be valid outside Europe, it only says that the regulatory risk assessment context remains that of The Netherlands and Europe. It means that the scientific findings of this work may lead to different conclusions in the context of practical risk assessments, in short due to different (chosen policy) definitions on absence of- and negligibility of impacts. This is a common situation, as occurs for regulatory frameworks on toxic compounds.

1.3 Aims and readers guide

The aims of the DNA-barcode project in the context of ERGO were to develop and validate a high-throughput method to quantify and characterize disturbance in nematode assemblages when exposed to different forms of treatment or kinds of stress. As the problem formulation is essential in such kind of projects (Wolt *et al.* 2010), special emphasis has been given to the link between perturbations in the soil nematode community structure and soil fertility, since nematodes per se might not represent a 'valued characteristic' in an ecological risk assessment (ERA) context – they serve here as "proxy" indicator for that. Soil fertility more specifically included organic matter decomposition and nitrogen mineralization in this project. Quantifying the link between the high-throughput structural proxy and soil fertility is needed to enable final evaluations to assess if a (new) GM-crop causes effects on the soil ecosystem that are valued (by society) as adverse and are relevant for risk management.

In the project, we aimed to set out a general framework concerning types of effects, baselines, biological levels, and a versatile and (when needed) tiered approach for ecological risk characterisation of GM-crops, with a focus on effects on non-target organisms or communities in agroecosystems. Figure 1 presents an overview and the linkage of the relevant aspects of the development of the tool, till eventual use in ecological risk assessment. We only provide an overview of effects; whether or not these effects are considered harmful is not part of this report, but need to be predefined by policy.

Aims of this report are:

1. To introduce and discuss relevant elements of ecological risk assessment (ERA), both in general and specifically in the context of GM-regulatory frameworks (chapter 2)
2. To summarize and illustrate key characteristics of the DNA-barcode tool (chapter 3) and key observational findings as obtained within the context of the ERGO-program (chapter 4)
3. To identify statistical methods to distinguish between natural variability and unexpected deviations of nematode assemblages in agroecosystems (chapter 5) and to determine the relation between nematodes and biological soil fertility (chapter 6)

4. To combine the results of (1), (2) and (3) in order to provide proposed guidance for risk and impact assessment of GM-crops using nematode DNA barcode-based assessments (chapter 7) and to provide guidance for General Surveillance (chapter 8)
5. To draw conclusions on progress made and on remaining future scientific and practical issues (chapter 9)

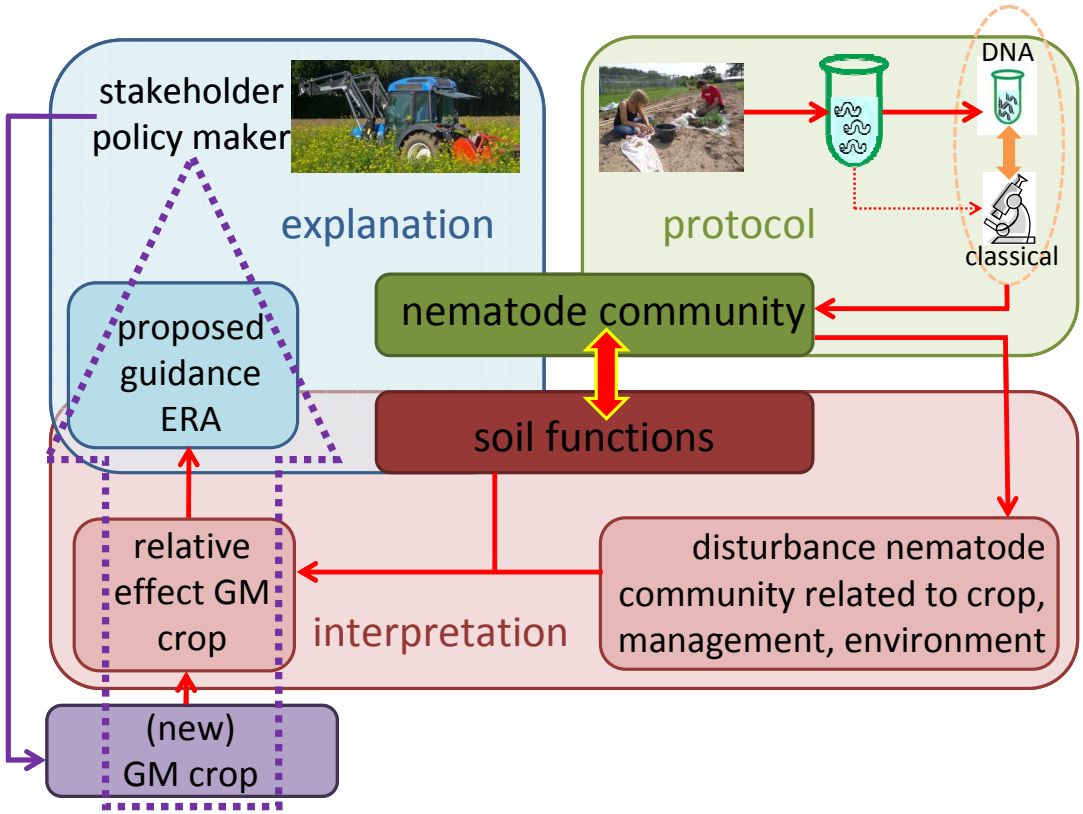


Figure 1: Overview of activities related to a DNA barcode tool to address impacts of stressors on nematode assemblages, to serve as potential early-warning signal for evaluating impacts on soil functions (like fertility), consisting of activities to (i) design a protocol for barcode assessment (green field), (ii) interpretation, especially in the context of evaluating possible impacts of GM-crops (brown field), and (iii) explanation, involving a combination of technical and societal aspects (blue field).

2 General GM-crop risk and impact assessment issues

2.1 Frameworks and options for risk and impact assessment

European regulations for GM-crops require testing for potential adverse effects at different stages and scales of pre- and post-market introduction of novel GM-crops (EEC 2001; EU 2002). The regulations describe three formats: pre-market development, and post-market environmental monitoring (PMEM) comprises of case-specific monitoring (CSM) and General Surveillance (GS). These formats are the basis to explore the risk assessment issues regarding our nematode DNA barcode tool.

2.1.1 Pre- and post-market assessments

The general purpose of pre-market ecological risk assessment of GM-crops is to address possible side-effects on ecological integrity (structure, function) of soils before introducing the crop in the environment. Market introduction is prohibited or limited when side-effects are considered too large. Due to their capacity to often assimilate compounds which are not naturally produced by the crops, ERA of GM-crops is in that sense largely comparable to the release of chemical compounds into the environment. As a general comparison, test approaches with similar pre-market investigations are very common for *e.g.,* novel plant protection products. The Mode of Action of the genetic modification (respectively the plant protection product) is taken into account in selecting the most appropriate test systems.

After an optional introduction of GM-crops on the market, post-market environmental monitoring (PMEM) is defined in the regulation as a key feature of the European legislative framework (Sanvido *et al.* 2009). In this monitoring, the focus of case-specific monitoring (CSM) being defined by considerations on the (targeted) Mode of Action (see Vonk *et al.* 2009 for use of Mode of Action) of the added trait of the GM-crop, *e.g.,* reducing damage to crop yield by pest organisms. CSM is performed in fields with GM-cropping history or in the direct surrounding environment and aims to detect direct links between effects on the soil system and GM-crops. The post-market stage also includes General Surveillance (GS). This part of PMEM is performed as a broad monitoring programme and may focus on mechanism-based expected effects or on impacts not expected from mechanisms introduced by GM-crops. In contrast to CSM, GS implies the collection and interpretation of monitoring data from a wide range of agroecosystems and/or other systems. The data are analyzed in order to detect unexpected changes which may be induced by GM-crops. Though major technical progress has recently been made on the issue of detecting impacts of stressors on biota given (bio)monitoring data sets (more details in chapters 5 and 8), the issue of GS requires demystification. As a matter of fact, GS needs a definition before it can be used operationally. It concerns, among others, working hypotheses, suitable datasets, appropriate techniques and (case-specific) criteria to define the impact of GM as compared to natural variability.

2.1.2 *Ecological risk assessment (ERA) of GM-crops*

The ERA of GM-crops on non-target organisms, populations or communities can be performed in different ways. First, there is the classical type of assessment that can be used if the GM-crop releases new type(s) of chemical compound(s) into the environment induced by transgenesis (*e.g.,* release of specific proteins by *Bt* corn; Mulder *et al*. 2006; Icoz and Stotzky 2008; Coll *et al*. 2009). In this context, it is common to compare predicted environmental concentrations (PEC) of new compounds due to commercial growth of the GM-crop are compared to the predicted no-effect concentration (PNEC) of the compound for specific (groups of) organisms. This results in risk characterization and the assessment of potential risks of the GM-crop. Risk characterisation values larger than unity indicate an impact beyond policy-accepted effects. The PEC is based on modelling of the GM-crop area and chemical properties of the compound to determine release and distribution of the compound in the environment. The PNEC can be derived from laboratory toxicity tests to establish dose-response relationships using a specified endpoint or from models based on quantitative structure-activity relationships (QSARs) to calculate the toxicological effects of the compounds. This way of ERA is only suitable for GM-crops that release specific chemical compounds which are also available to perform laboratory toxicity studies.

For other GM-crops, the environmental risks are determined based on their effects on specific (test) organisms or on communities or functional groups occurring in the field. Recently, a large scale British national project, whose experimental design included more than 250 fields with spring crops of beet, maize and both spring and winter oilseed rape, was monitored from sowing to harvest aiming to quantify the effects of GM herbicide-tolerant crops (Bohan *et al.* 2007; Hawes *et al.* 2009; Squire *et al*. 2005, 2009; but see also Squire and Gibson 1997). Despite these authors discovered only very minor ecological effects, there was an unusual clamour to disband this experiment (Squire 2004). This paradox is very interesting: on one hand, field tests are an accepted test option in the evaluation of risks of plant protection products, and this option is triggered usually by lower-tier laboratory test outcomes. On the other hand, as soon a set of field tests is established, many people ask to stop the experiment. The field test has still to be seen as the most realistic exposure condition. In this second way to perform ERA, the effects of GM-crops on organisms, communities, or functional groups are compared to a control treatment, and/or to the effects induced by traditional crop(s). This assessment should be based on the most suitable endpoint. For laboratory test organisms, this endpoint is often related to mortality or reproductive activity, for communities from the field the most appropriate level of biological aggregation can be related to diversity, abundance, or function of the studied biota. Intensity of disturbance from GM-crops should be related to relative changes in the chosen endpoint.

The variation of effects induced by non-GM crops can be determined either using normal operating range (NOR) or from selected reference locations. This variation can be induced by differences in environment (*e.g.,* soil type, seasonality), agricultural management (*e.g.,* tillage, fertilizer, plant protection products), or by crops themselves (type, cultivar). Since the environment already has large effects on organisms and communities, variations need to be assessed within environmental boundaries, such as climate and soil type, before the possible additional effects of management or crops can be assessed.

2.2 Contextual issue: impact of agriculture *per se*

Agricultural practices primarily influence agroecosystems and hence these systems can be characterized as relatively adjusted to disturbance as compared to natural systems. Comparisons between the effects related to 'traditional' crops farming and those related to GM-farming could provide information on the potential secondary disturbance from GM-farming on agroecosystems. Three routes of risk assessment were defined above based on 1) pre-market testing, 2) post-market CSM and 3) General Surveillance. However, there is another major contextual issue when considering developing application of a novel tool for ERA: additional GM-crop effects on soil productivity need be evaluated in the context of variability related to current, conventional management.

Whilst this aspect may be different for introgression, the possible side effects of GM-crops on soil biota (and hence soil functions) need to be characterised in comparable response variables as side effects of conventional cropping. For example, soil microbial respiration might be affected by plant exudates and this is not seen for conventional crops as 'beyond a negligible effect', in contrast to effects induced by GM-crops. (Side effects are neither quantified nor evaluated for non-GM crops.) In this research line on soil functions within ERGO, the absolute value of any response variable will not be *per se* relevant for risk assessment, but the relative value needs to be analysed whether or not this is an excessive (quantitatively) or a particular (qualitatively) impact in comparison to natural variability.

2.3 Levels of comparison

GM-crops related effects on the environment can be compared at different levels. Broadly, four levels of comparison can be identified:

(1) GM cultivar and non-GM cultivar which is the most restricted comparison and takes only into account possible negative effects,
(2) GM cultivar and natural variation within agroecosystems using different crops
(3) GM cultivar and non-GM cultivar including management-related effects to account also for possible positive effects,
(4) GM cultivar and non-GM cultivar using a life-cycle impact assessment to account for the whole process from growing crops till the final product.

While in this study we will focus on the first two levels, we shortly provide here an example for the third and fourth level of comparison. As an example for issue 3, changes in crop management related to certain genetic modifications can result in a different exposure of soil biota to crop protection agents (Ammann 2005). The overall effect of a GM-crop is therefore not restricted to the direct effects induced by the plants, but the changes in overall management can induce additional (positive) effects. During a workshop on "New challenges in risk assessment of genetically modified plants" in Copenhagen (December 2011), this third issue lead to a prominent discussion. (A report of this workshop is expected.)

Regarding issue 4, life-cycle impact assessment (cf. Finnveden *et al.* 2009) is a method to compare different effects of products during cropping, processing and recycling. An example for the fourth level comparison can be the growing of GM-starch potatoes that contain only one type of starch to reduce the amount of chemicals needed to produce potato-starch. A life cycle impact assessment can compare the overall impact of potato-starch production on the environment by

taking into account effects of reduction in the use of these production-related chemicals as well as possible effects related to the GM cultivar. In life cycle impact assessment, addition or loss of traits can be of high distinctive relevance for final outcomes. However, there are no separations within current regulations for GM-crops between cis- and transgenesis or between the addition of traits (*e.g., Bt* or round-up genes) and the loss of traits (*e.g.,* amylase-potato).

2.4 How to assess effects of GM-crops?

It is not possible to assess all organisms present in an agroecosystem, therefore a representative selection of the organisms will be analysed (Mulder and Lotz 2009; Ricroch *et al*. 2010). Still, the effect of GM-crops can be compared at different levels. Comparing specific reference locations or NOR and effects of GM-crops depends on the data distributions (normality, skewness) and for the latter the measurement range taken into account (all, 99 % or 95 % confidence intervals). When data sets are smaller, various approaches can be chosen to improve data quantity, for example by grouping the data from sets of GM-crop experiments by similar types (*e.g.*, mode of action), so that mode-of-action related effects can be better assessed per modification in order to identify possible differences.

An important policy aim is to stimulate sustainable agroecosystems by protecting key ecosystem services, and thus key ecosystem functions that provide these services (*e.g.*, in formulating a European Union Common Agricultural Policy). Possible GM-crop induced effects in agroecosystems need to be related to changes in ecosystem functions. In order to link the effects observed at individual, population or community level with the effects on ecosystem level, a quantitative relationship between these two levels would in general need to be established. This requires a selection of the ecosystem functions considered to be important to maintain the ecosystem services desired by land users and policy makers within these agroecosystems. The studied organisms/communities can either have a direct influence on these ecosystem functions of interest or they can serve as a proxy for these ecosystem functions. The type of ecosystem functions under consideration strongly influences the choice for a group of organisms used to identify possible effects (Mulder *et al.* 2011).

2.5 Tiered approach

Studies focussing on possible effects at different levels within agroecosystems are necessary to provide an overview of the overall risk of GM-crops on soil systems. Different tools are needed to assess potential effects on different ecosystem services. Priority setting for the application of tools, based on their sensitivity, cost effectiveness or reproducibility, might be desirable to optimize use of resources. In this way, a tiered approach can be applied, as often used in ERA. The type of GM-crop can possibly influence the tool kit (set of tools) used to identify effects. Lower-tier tools (early-warning tools) are commonly designed such that they are conservative, that is: they are meant to over-estimate risks. Soil, sediment and water quality criteria are amongst such easy-to-apply tools, by which users can easily define a compartment as 'clean' (with no unacceptable risk) or not (below and beyond the criterion, respectively). In this context, PNEC values are often derived using Safety Factors, like 10, 100 or 1000. The lowest known impact concentration is then divided by this value to obtain the criterion.

Hence, this lower tier is considered protective, but it may be overprotective such that impacts are not directly observed when the criterion is exceeded. For plant protection products, triggered by such lower-tier risk characterizations (PEC exceeds PNEC), semi-field and field tests are the higher-tier approaches provide refined information on risks.

Any final conclusion whether the overall risk is negligible or not, largely depends on risk analysis specificity, policy choices within the risk assessment, and risk perceptions. By placing effects of GM-crops in context to effects related to current crops and variation between agroecosystems, we aim to provide additional information for the assessment of GM-crop related effects. Finally, it is important to realize that results derived from the nematode DNA barcode tool need to be suitable to be implemented within current legislation and guidance (*e.g.,* OECD, EU, EFSA, US-EPA).

3 Essentials of a nematode DNA barcode-based assay

3.1 Introducing the DNA barcode tool

Identification of individuals using light microscopy is the classical method to characterize soil nematode assemblages. However, this method is both very time consuming (on average several hours for 150 individuals per sample) and the resolution depends on the level of taxonomic expertise. Various molecular methods are being / have been developed to provide an alternative method for the analysis of faunal communities and their trophic interactions with relative energy flow (Carreon-Martinez and Heath 2010). The small subunit ribosomal DNA (SSU rDNA) gene is very conserved and therefore often used for resolving phylogenetic relationships. For nematodes, SSU rDNA sequences were used for phylogenetic reconstruction (*e.g.*, Blaxter *et al.* 1998) and to develop a phylogenetic tree (Van Megen *et al.* 2009) in which many nematodes families appeared as monophyletic groups. DNA barcode-based identification of nematodes has been performed at different taxonomic levels (species, genus, and family: *e.g.* Floyd *et al.* 2002; Holterman *et al.* 2006). By using real-time PCR, not only can a specific taxon be detected in an assemblage (qualitative analysis), but also an estimation of the number of individuals can be provided (quantitative analysis). The latter will allow the determination of population density and persistence (*sensu* Johnson *et al.* 2009) in soil communities (Vervoort *et al.* 2012). The copy number and quantities of the (taxon-specific) target template (SSU rDNA) are inversely proportional to the cycle number (Ct) and the number of individuals can be calculated by direct comparison with Ct values for known standards (Brunborg *et al.* 2004; Atkins *et al.* 2005). Taxon specific primers were and are being developed to determine a wide range of nematode taxa (Table 1). (More details for primer development and testing in Vervoort *et al.* 2012.)

3.2 Nematode sampling and extraction

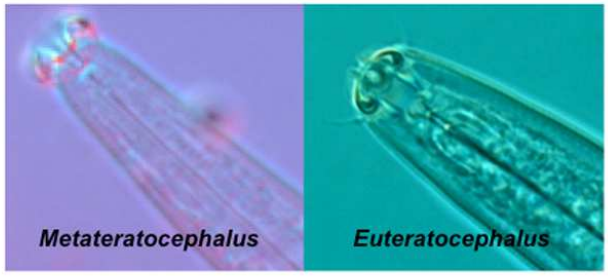
Nematode sampling in the field is best performed according to currently used protocols, since this enables comparisons between newly generated nematode data (using barcode-based detection) and existing long-term datasets on nematodes. Within the Dutch Soil Quality Network (DSQN; overview in Rutgers *et al.* 2009 and Mulder *et al.* 2011) nematodes have been collected in the field using small corers (2.3 cm diameter, 10 cm depth) for over 15 years. Here, a bulk sample consisting of 320 cores randomly collected from across the study site was mixed and 500 g soil was kept in glass containers and stored at 4 °C prior to extraction using the Oostenbrink method (Oostenbrink 1960). The protocol for extraction of DNA from (free-living) soil nematodes and consequent storage of DNA samples is provided by Vervoort *et al.* (2012).

Table 1: Overview of nematode taxa for which primers have already been developed and used (left column) and taxa for which primers are still in development or not tested yet (right column). Families as in De Ley et al. *(2006), further division into sub-clades (additional codes behind family names) according to Holterman* et al. *(2008).*

qPCR primers for nematode taxa	
Already used in field experiments	**In development or not yet field tested**
Achromadoridae	Anatonchidae M4
Alaimidae	Aphanolaimidae
Aphelenchidae	Bastianiidae
Aphelenchoididae	Choanolaimidae
Cephalobidae	Chromadoridae
Cruznema	Dorylaimida D2
Diphterophoridae (*Tylolaimophorus*)	Dorylaimida PP1
Diphterophoridae (*Diphterophora*)	Dorylaimida PP3
Diplopeltidae	Ethmolaimidae
Dolichodoridae (*Amplimerlinius*)	Heterorhabditidae
Dorylaimida D1	Ironidae
Dorylaimida D3	Mononchidae M2
Metateratocephalidae	Ohridiidae (*Domorganus*)
Monhysteridae	*Paratylenchus*
Mononchidae M3	Dolichodoridae (*Tylenchorhynchus*)
Mylonchulidae M1	Steinernematidae
Panagrolaimidae	Telotylenchidae (*Tylenchorhynchus*)
Plectidae (*Anaplectus*)	Tobrilidae
Plectidae (other genera)	Tripylidae (*Tripyla*)
Prismatolaimidae	Tripylidae (*Tripylella*)
Rhabditidae (Mesorhabditidae)	
Teratocephalidae	
Trophurus	

3.3 Sensitivity, accuracy and reproducibility of the DNA barcode method

Sensitivity
Quantification of the primers used in the DNA-barcode tool was performed using 1 to 100 individual nematodes. With a standardized protocol, all primers were sensitive enough to detect single individuals. An example of a quantification curve (here for Metateratocephalidae), in which the Ct value derived from qPCR is plotted against the number of nematodes in reaction, is provided in Figure 2.



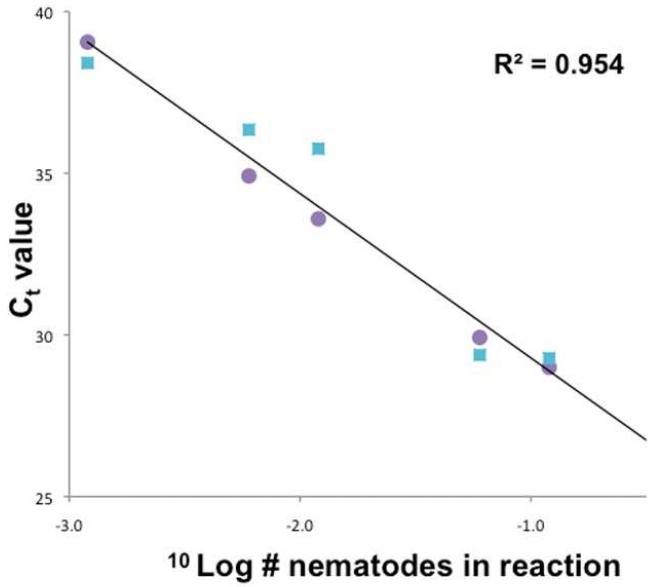


Figure 2: Example of a quantification curve for the genera Metateratocephalus *(purple circles) and* Euteratocephalus *(blue squares) of the family Metateratocephalidae. Individual nematodes were collected from field samples and in the reaction 1, 5, 10, 50 and 100 specimens were used to determine the relationship between the Ct value derived from qPCR against the number of nematodes in reaction. Figure reproduced from Vervoort* et al*. (2012).*

Accuracy and reproducibility
All primers were tested against possible false positives. We used ARB software (Ludwig *et al.* 2004) to identify potential false positives. Potential false positives were not *per se* taxonomically closely related to targets, but the available extensive database (~ 2,400 taxa; Van Megen *et al.* 2009) enables a phylum-wide screening. The smallest gap between the target and the non-target, expressed as ΔCt, was determined for all primers. Primers with ΔCt values smaller than 12 were discarded (Vervoort *et al*. 2012). Figure 3 provides an example of primer testing for Metateratocephalidae. The reproducibility of qPCR analyses is expected to be about 25 % for the taxa used in field experiments (Vervoort, unpublished data).

Targets	Clade	C_t value
Metateratocephalus sp.	6	19.52
Euteratocephalus sp. 1	6	21.21
Euteratocephalus sp. 2	6	21.74
Non-targets	**Clade**	**C_t value**
Diplogaster sp.	9A	47.72
Anomyctus sp.	10B	N/A
Wilsonema sp	6	N/A
Paraplectonema sp.	6	N/A
Chromadoridae sp.	3	N/A
Procamacolaimus sp.	6	N/A
Leptolaimus sp.	6	N/A
Tylocephalus sp.	6	N/A
Anaplectus sp.	6	N/A
Pristionchus sp.	9A	N/A
Mylonchulus sp.	2A	N/A
Negative water control		N/A

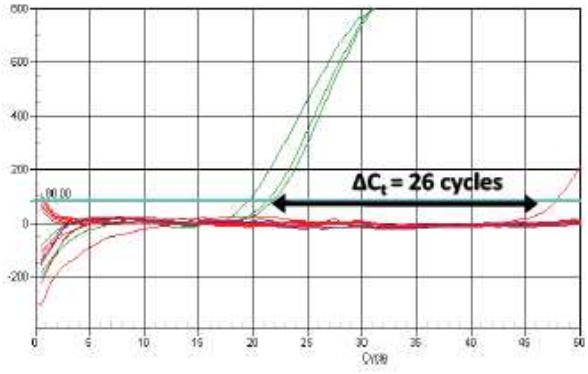


Figure 3: Specificity test of a primer for Metateratocephalidae. SSU rDNA fragments from three target species (green lines), 11 potential false positives (red lines) and a negative water control (blue line) were tested. Clade numbers are according to Van Megen et al*. (2009). The gap between the target and the first non-target signal (ΔCt) is shown. N/A: no signal detected for the non-target). (Figure reproduced from Vervoort* et al*. 2012)*

3.4 Comparison of barcode-based and classical taxa identification

The selection of taxa used to determine seasonal changes in the nematofauna (Vervoort *et al.* 2012), consisted of 13 families and 3 genera, and accounted for 29.8 % of the nematode density in arable fields on clay, 32.1 % in arable fields on sand, and 42.6 % in arable fields on Loess, as identified by microscope (data from Mulder and Vonk 2011). Rhabditidae (around 25 % of the nematofauna in these arable fields) and Tylenchidae (around 18 %) are the two main families not included in this qPRC analysis. For Rhabditidae, no single all-encompassing primer can be developed since this family is poly- and paraphyletic and genus-level primers will be developed. For the herbivorous Tylenchidae, primers are still in test.

From the study on seasonal changes in nematofauna, we can provide an overview of the accuracy of overall nematode densities derived from qualitative PCR analysis (Vervoort *et al.* 2012). This density, calculated as the sum of the densities of the quantified taxa, was compared to density counts using light microscopy (Figure 4).

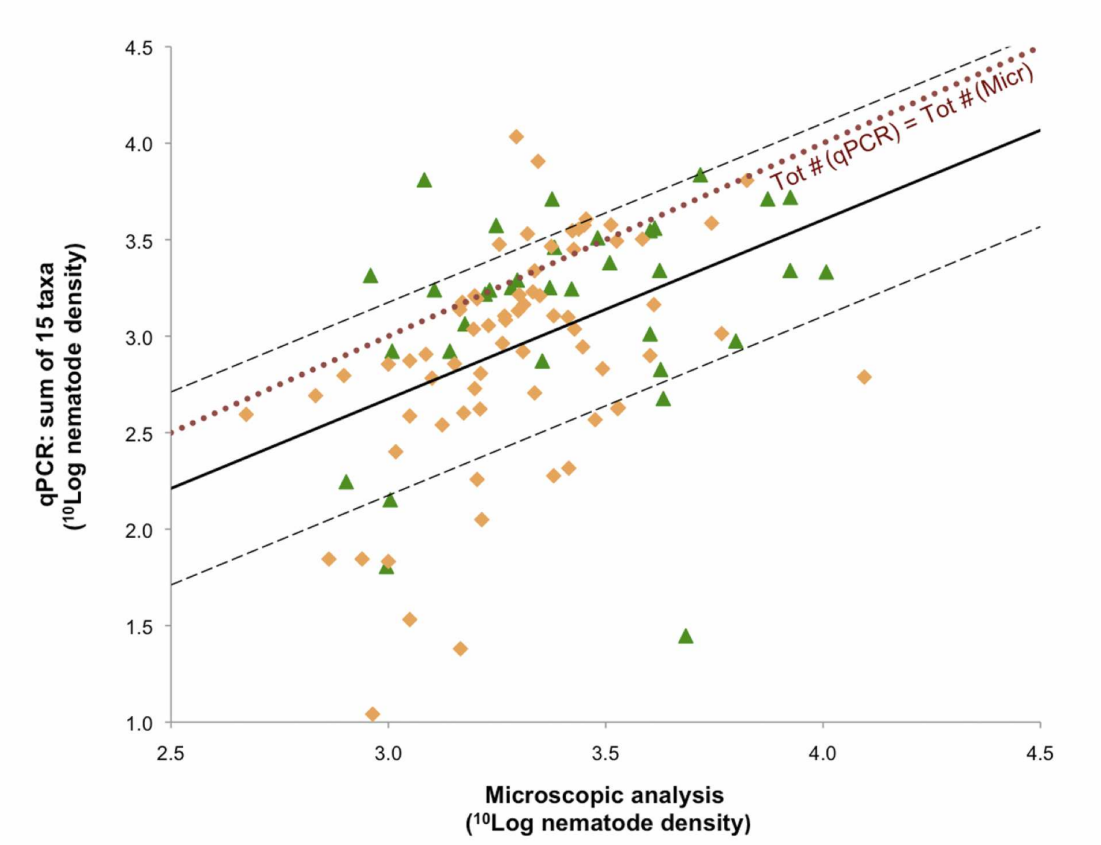


Figure 4: Quantitative coverage of the DNA-based tool using environmental samples. Logarithm of the total of individuals as detected by optical microscopy (x-axis) was plotted against the logarithm of the total of individuals as estimated by quantitative PCR (y-axis). The solid line shows the trend of all data and the two dashed lines show the boundaries of one-order-of-magnitude precision. The dotted line represents an equal amount of nematodes for both methods. The correlations of the quantitative PCR with classical analyses seem to be accurate, while coverage lower than 100 % is expected since not all taxa were included (Vervoort et al*. 2012).*

We are processing additional samples for which the soil nematofauna is analysed by both molecular methods (qPCR) and classical methods (light microscopy). The results will provide information on the comparability of density estimations for separate taxa using qPCR analyses and light microscopy. We expect that the results of this analysis will be available at the end 2012.

Since there is a very large amount of data available of classical determined nematode assemblages, it is important that we can compare the newly generated qPCR data with the classical data. Also, a number of indices have been developed based on classical identified nematode communities to determine the quality of soil systems. Not enough qPCR data is available at the moment to calculate these indices.

4 Main results from nematode barcode assay

Here we present an overview of the results obtained with the DNA barcode-based method during the project. Besides the study on seasonal fluctuations of nematode taxa (Vervoort *et al.* 2012), most of the collected data is still being analysed with most publications expected to be ready early 2013. Nematode datasets were collected to cover the three main topics of our research project. The first study covers seasonal fluctuations of nematode communities as measure for natural variability over time. The second study focuses on the effects of crops and management (bio-fumigation using *Brassica juncea*) on nematodes, while in the last studies effects of GM-crops (pathogen-resistant potatoes and amylase-potatoes) on soil nematodes are determined.

4.1 Nematode communities

Quantification of nematode communities in the field using the DNA-barcode method was firstly performed on the Veluwe (Vervoort *et al.* 2012). As a first field test for this DNA sequence signature-based approach, seasonal fluctuations of nematode assemblages under open canopy (one field) and close canopy (one forest) were monitored. Fifteen taxa representing four feeding guilds at two trophic levels were detected. These four guilds are composed of taxa that developed independently by parallel evolution and we detected ecologically interpretable patterns for free-living nematodes that belong –as basal consumers– to the lower level of soil food webs (Hunt and Wall 2002).

The overall nematode density (whole community) was rather constant over time, however, individual taxa and different guilds showed distinct temporal patterns. Comparison of the abundances of eight bacterivorous taxa during the entire experiment resulted in a very diverse picture: for two taxa no difference was detected between the sites, whereas six differed (4 taxa were consistently more abundant in the field and two were present in significantly higher densities in the forest). Lumping data into the feeding guild bacterivores masks the differences between sites. One of the striking differences between the sites is the high density of Prismatolaimidae in the moder. It is known that members of *Prismatolaimus* (the only genus in this family) have a long filiform tail with a hook-like mucro used for temporal attachment to litter substrates. Such a litter layer was present in the forest and absent in the field. If such a layer is the preferred habitat for *Prismatolaimus* spp., this would explain its abundance in the forest. Another factor that might contribute to this asymmetric distribution is the pH as some *Prismatolaimus* species prefer acidic conditions (Vervoort *et al.* 2012). The acid moder in the forest might constitute an optimal environment for acidophilic bacterivores. Another remarkable distribution was observed for the genus *Anaplectus* (Plectidae – Anaplectinae: Figure 5). The research on nematode assemblages by Vervoort *et al.* (2012) revealed ecological information about the soil food web that had been partly overlooked.

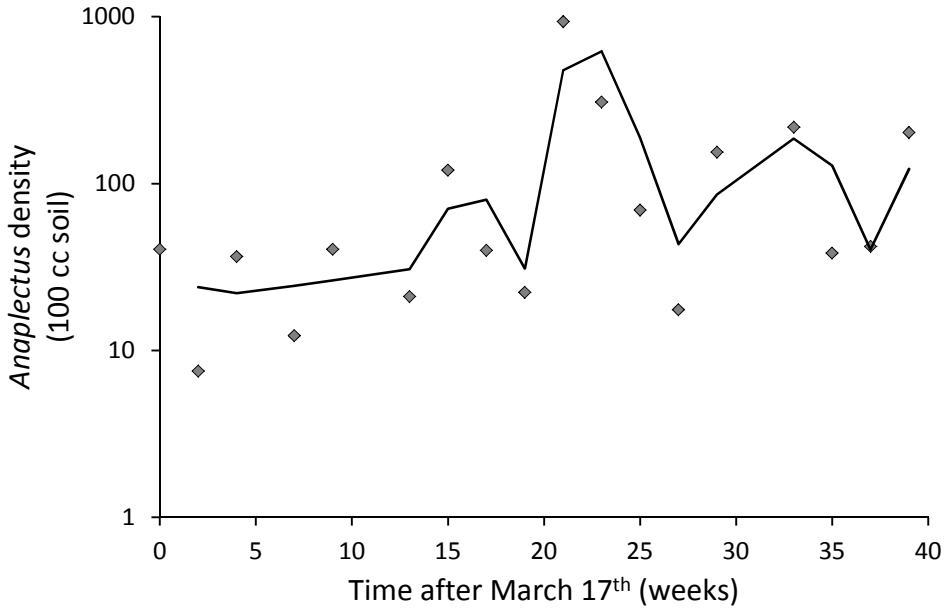


Figure 5: DNA-based determination of nematode densities showed seasonal variations for individual taxa, as shown here for the genus Anaplectus *(after Vervoort* et al*. 2012). Trend is given as two-period moving averages.*

4.2 Effects of non-GM crops on soil nematodes

There are two main routes for compounds produced by (GM) crops to enter the soil (Flores *et al.* 2005, Mulder and Lotz 2009): (1) incorporation of plant residue containing toxic compound into soil organic matter and (2) exudation from roots of living plants. As an example of the first process, we determined the effects of biofumigation, a currently used practice in which *Brassica* plant material containing glucosinolates (GLS) is incorporated into the soil to control soil-borne pests and diseases by releasing isothiocyanates (ITC) (Gimsing and Kirkegaard 2009).

Four cultivars of *Brassica juncea* (Terrafit, Terratop, Terraplus, and ISCI-99) with expected differences in glucosinolate contents (precursor for ITC) were grown in a field experiment at the Institute for Epidemiology and Pathogen Diagnostics (Julius Kühn-Institut), Münster, Germany. 59 days after sowing, all aboveground material was first chopped using a flail mower and directly afterwards incorporated in the soil up to 15 cm depth using a rotary tiller (bio-fumigation). The effects of biofumigation on the nematode community were analyzed using the DNA-barcode method (Vervoort 2013).

Directly after tillage and mixing of *Brassica* material with glucosinolates into the soil, we observed a decline of around 25 % in the total number of nematodes. For individual nematode taxa, the effects were even larger, as shown in Figure 6. The day after biofumigation, only 20 % of the density of Aphelenchidae remained as compared to the day before. However, no clear pattern between

declining densities of nematodes and glucosinolate-content of the *Brassica* cultivars was observed. It is concluded that the barcode method can detect the effect of a stressor on the nematofauna.

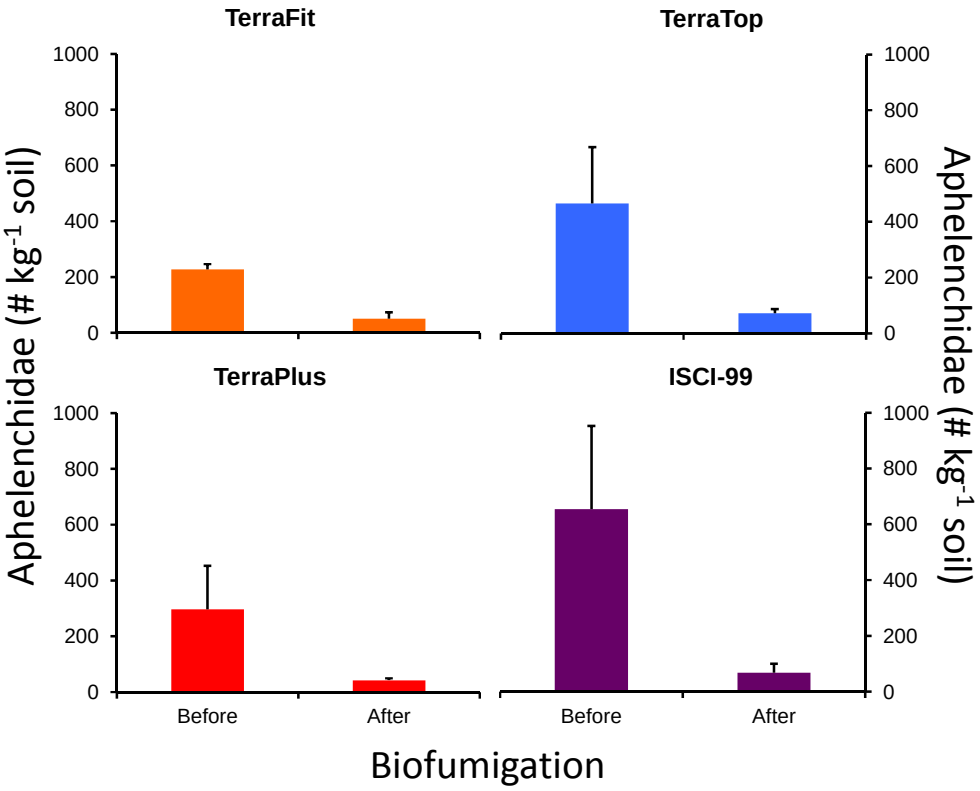


Figure 6: The effect of biofumigation with Brassica juncea *on densities of Aphelenchidae. A decline in nematode density was observed for all 4 cultivars (TerraFit, TerraTop, TerraPlus and ISCI-99), while no clear relation with glucosinulate of the plant was observed (Vervoort 2013). Four replicate samples with Standard Deviation.*

4.3 Effects of GM-crops on soil nematodes

Experiments were performed to determine the effect of GM-crops on soil nematodes. Nematode communities were analysed under potato cultivars with different types of modifications. Analysis of the results on amylase-potatoes (loss of trait) is in progress and pilot results on pathogen-resistant potatoes are presented here. We determined the effects of transgenic modification in three potato cultivars and effects of cisgenic modification in one cultivar on nematode communities. All modified potatoes had an introduced resistance-gen. Nematode densities varied in the field, without clear patterns between original and modified potatoes (Figure 7). The variation in nematode densities between cultivars appeared to be at least as large as differences between modified and original varieties within a cultivar.

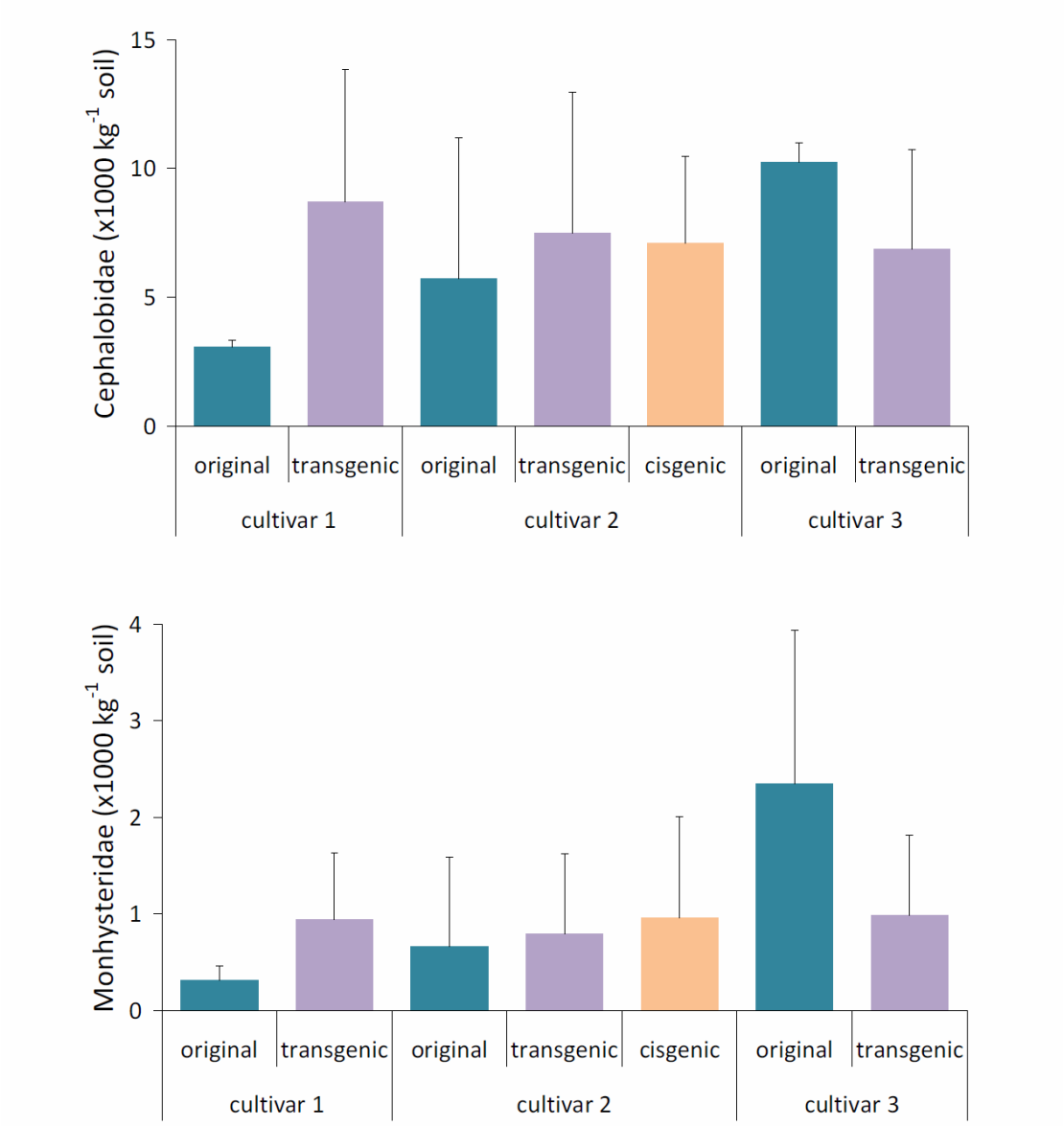


Figure 7: Densities of Cephalobidae (upper histograms) and Monhysteridae (bottom histograms) under original and modified varieties of the potato cultivars (averages with standard deviation). Nematode densities under modified varieties were different (either higher or lower) in comparison to conventional varieties.

Soil nematodes are known to regulate the population of their microbial resource (bacterivorous and fungivorous nematodes), to fragment plant roots and transport fresh organic matter (fungivorous and plant-feeding nematodes), and to alter nutrient turnover in the rhizosphere (Mulder and Lotz 2009). Hence, seen that taxa are characterized by functional traits that collectively provide information about environmental quality (Mulder and Vonk 2011), ecosystem services are supposed to be dependent upon specific combinations of traits.

The molecular method presented here allows for the analysis of nematode communities without microscopic pre-selection because it is based on a considerably broad (2,400 taxa) full length SSU rDNA database that covers the majority of terrestrial and freshwater taxa. By uplifting restrictions concerning time and expertise, the use of this molecular method allows for intense and frequent sampling schedules. Extensive datasets produced in this manner can contribute to our current knowledge about the influence of seasonality, location and soil characteristics on the nematode community. Furthermore, molecular tools like the DNA-barcode offer the possibility to identify the role of environmental factors in driving soil nematode communities, hereby allowing the indicator value of the nematode community to become more versatile. Finally, a major advantage of this detection framework is its simplicity, as it just requires standard laboratory equipment.

5 Accounting for natural variability in managed agroecosystems

5.1 Natural variability and risk or impact assessment

Important is the distinction between natural biological variation induced by natural conditions on the one hand (reflected as either baseline or Normal Operating Range), and the possible additional effects induced by anthropogenic stressors and land use on the other hand (Mulder and Vonk 2011). As a matter of fact, we are (too often) forced to illustrate different diagnostic methods to detect signals from (unknown) stressors in large datasets without prior knowledge of the effects of a specific stressor. In the case of nematode assemblages, stressed (or not) by GM-cropping or other variables, comprehensive tests on systems that vary in space and time due to natural heterogeneity, variability due to accepted agricultural practices, or both, are not available yet. Hence, appropriate modelling is crucial. Starting from positive experiences, applicability of existing diagnostic methods to our purpose with illustrative examples on the influence of natural or induced variability on risk/impact assessment is briefly reviewed.

There are existing diagnostically-aimed approaches, which have been developed to identify the presence of a possible impact in natural systems, based on existing monitoring data sets. Five major diagnostic methods are: Artificial Neural Network (ANN; Kohonen 1982), River Invertebrate Prediction and Classification System (RIVPACS; Wright *et al.* 1993), Effect and Probable Cause (EPC; De Zwart *et al.* 2006), Weight of Evidence/Weighted Linear Regression analysis (WoE/WLR; Kapo and Burton 2006), and Observational and Simulated Evidence (OSE; Mulder *et al.* 2003). Using one example for each method, we show implications of different mathematical, ecotoxicological and ecological approaches in various ecosystems. Potentials of the methods are discussed in chapter 8, together with their application in datasets originally collected for other purposes. Though none of the methods has been developed using barcode-like monitoring data, there is no fundamental objection which would hamper the use of these diagnostic methods for barcode data. Hence, the examples below illustrate how natural variability, multiple stressors and a possible stressor of interest can be addressed.

5.1.1 Artificial Neural Network (ANN)

ANN uses statistics to identify similarities and differences between sites (Kohonen 1982) and is a non-hypothesis-driven method to structure, describe and summarize data. The Kohonen Self-Organizing Map approach, an unsupervised ANN algorithm, is used for discovery and recognition of patterns, and clustering and visualization of large multidimensional datasets (Chon *et al.* 1996). A SOM provides an alternative to traditional statistics such as Principal Component Analysis and Multidimensional Scaling (Brosse *et al.* 2001). These latter computations are based on the *a priori* selection of suitable functions or algorithms, whereas ANN can adjust its inner structure to provide optimal solutions, given enough data and a proper initialization. This makes SOM suited to analyze non-linear relationships in complex data (Park *et al.* 2003). ANN is a

promising method to highlight relationships between biological responses and environmental pollution (Comte *et al.* 2010). We will illustrate ANN in the section 8.2 with an assessment of biological quality based on Mele and Crowley (2008).

5.1.2 *River InVertebrate Prediction and Classification System (RIVPACS)*

RIVPACS was developed by the IFE (Institute of Freshwater Ecology) to assess the ecological quality of rivers in the UK (Wright *et al.* 1993; Wright 1995) and has been adopted since then by many other countries. (Systems comparable to RIVPACS were developed in Australia, AUSRIVAS: Davies 2000, and Canada, BEAST: Reynoldson *et al.* 2000.) In RIVPACS, monitored sites are compared to pre-defined reference sites with desirable conditions (Wright *et al.* 1993). The philosophy is to develop relationships between meiofauna and environmental characteristics of a large set of reference sites, which is then used to predict the expected (E) fauna at any site in the absence of pollution or other recognized environmental pressure (Clarke *et al.* 2003). From the possibly affected sites, the observed (O) fauna is compared with the fauna from the reference sites to determine the relative impact ($^{O}/_{E}$). RIVPACS has not only been applied to aquatic systems, but there have been some efforts to develop a comparable approach for European soil systems (*e.g.,* Spurgeon *et al.* 1996; Ruf *et al.* 2003; Breure *et al.* 2005).

5.1.3 *Effect and Probable Cause (EPC)*

EPC is an extended method of RIVPACS-type of modeling that can additionally identify relative impacts of local stress that probably caused deviations from a reference condition (De Zwart *et al.* 2006). The first step is a RIVPACS-type analysis (*i.e.* the dataset is divided into a subset of reference or minimally disturbed sites and a subset of possibly affected sites). This step yields the total impact at each sampling site, representing the species expected but absent, defined as ($^{O}/_{E}$). In the next step, this total impact is associated with probable causes (measured stressors). The results of the EPC method are presented on maps, with pie size representing the magnitude of impact (species expected but absent), and slice sizes representing the relative contribution of certain identified stressors to the impact. By merging multiple substances together into a single proxy for toxic pressure of mixtures (the multi-substance Potential Affected Fraction of species, msPAF) to reflect the environmental pressure, a better separation between types of stressors was achieved in different regions (Posthuma and De Zwart 2006; De Zwart *et al.* 2009). In this approach, the aforementioned issue of statistical power was again of great importance.

5.1.4 *Weight of Evidence (WoE)*

WoE considers the issues covered by the previous method in a geo-referenced way, *i.e.* it considers the location of sampling sites, and not all sites individually as in EPC, in order to identify regions which are affected by certain stressors or are characterized by certain communities (Kapo and Burton 2006). WoE has been developed to analyze data at landscape-level in order to quantify firstly the occurrence and magnitudes of local impacts, again compared to a set of reference sites, and secondly probable causes associated to those impacts. To determine cumulative stressor influence, the spatial patterns of all potential stressors are integrated in a logistic regression model. Results of the WoE-method can be displayed and queried in a GIS interface, whereby similarly

colored parts of a landscape identify sites with similar degree of impact and whereby associated stressor combinations may be queried and plotted as well. WoE has been applied in various regions (*e.g.,* Kapo *et al.* 2008; Van Wijnen *et al.* 2012).

5.1.5 Observational and Simulated Evidence (OSE)

OSE illustrates the value of enrichment of an existing monitoring dataset to detect differences between sites and relate these changes to independent predictors. Large unexplained variance indicated that there were additional stressors acting on the communities, showing that data enrichment can strongly enhance diagnostics. The first part of OSE consists of the application of procedures like the General Linear Model approach to Analysis of Variance and the Generalized Linear Model, or the multiple regression and smoothing techniques under the Generalized Additive Model. Non-parametric locally weighted scatterplot smoothing (LOWESS) can be further used to reduce the influence of exceptional values and outliers (Legendre and Legendre 1998). The second part of the OSE-approach continues with Monte Carlo simulation for validation (*e.g.*, Mulder *et al.* 2003). Validation steps are critical components of the development of a reliable model.

5.2 Overview changes in nematode taxa

The relative importance of taxa within nematode assemblages is influenced by their body size and abundance. With the DNA barcode-based tool, we determined nematode abundances, assuming that the amount of cells in individual nematodes does not show large changes over different life-stages (Vervoort *et al*. 2012; cf. Sin and Pasternak 1971, De Cuyper and Vanfleteren 1982). Therefore, it was possible to relate the quantitative PCR data to the number of individual nematodes without knowing their life-stage distribution.

Soil type and soil abiotic conditions may influence the size of nematodes. Therefore, the body sizes of nematodes occurring in arable fields and grasslands on different soils and under a range of soil nutrient conditions were compared. The variation in average body mass of the most abundant nematode taxa was always within 50 % between clay and sandy soils (Table 2). The influence of soil nutrient ratios on the average size of individual nematodes was even smaller than the influence of soil type: no significant relations were observed between nutrient ratios and nematode size in arable fields and grasslands on clay and sand (example for soil carbon-to-phosphorus ratio in Figure 8). In contrast to body size, nematode abundance was determined by the cross-product of soil type and ecosystem type (Mulder *et al*. 2012). Differences in total abundance of nematodes between soil and ecosystem types largely determined the total nematode biomass in agroecosystems. The importance of ecosystem type as predictor of soil nematodes and community structure is shown in Figure 9. The soil log[C]–log[P] as predictor of nematode mass–abundance regression slopes indicates the importance of phosphorus for abundance.

Table 2: Density and mean size of dominant nematodes in clay-rich and sandy soils.

	CLAY		SAND		Delta
	total ind.	body mass	total ind.	body mass	body mass
Taxon	#	µg	#	µg	(± %)
Acrobeloides	136	0.101	599	0.092	-10.4
Aglenchus	91	0.118	457	0.117	-0.9
Anaplectus	112	0.370	361	0.397	+6.9
Aphelenchoides	260	0.058	791	0.054	-7.9
Dorylaimoidae	126	0.463	527	0.455	-1.7
Eucephalobus	899	0.150	2929	0.162	+7.3
Helicorylenchus	198	0.191	912	0.216	+11.8
Meloidogyne	284	0.076	353	0.065	-18.2
Panagrolaimus	137	0.192	708	0.169	-13.2
Plectus	137	0.454	957	0.304	-49.4
Pratylenchus	308	0.091	467	0.076	-19.5
Rhabditidae	848	0.197	3065	0.342	+42.3
Tylenchidae	1381	0.090	1914	0.067	-35.2
Tylenchorhynchus	526	0.187	1753	0.302	+38.0

We are writing an article to determine the relative effects related to crops, management practices and environmental conditions on nematode densities in arable fields (Vervoort *et al.* in progress). We will quantify the variation in densities for different nematode taxa, with special focus on taxa that are included in the DNA-barcode method. Using this literature overview, we aim to determine which nematode taxa are suitable for analysis as well as to identify currently not included taxa which can provide additional information.

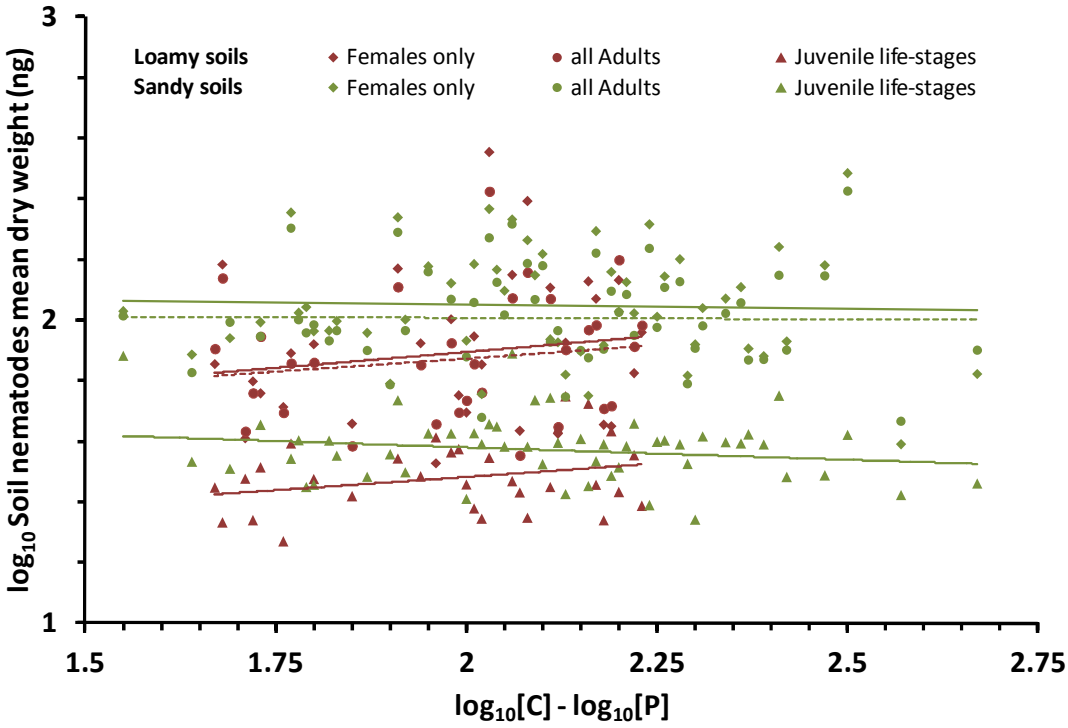


Figure 8: The soil types (ST) influence the size of individual nematodes more than nutrient ratios do. Arable fields and grasslands were consolidated for molar C:P ratios and log-transformed. Log–log linear regressions are plotted although they are not significant: from top to bottom, regressions for all females (upper solid lines), all adults (dotted lines) and all juveniles (lower solid lines). The cross-product soil type (ST) versus ecosystem type (ET) determines the total abundance of individuals (i.e. total biomass). Figure from Mulder et al. *(2012).*

5.3 Normal Operating Range (NOR), references and baselines

State variables are the variables that characterize the (eco)system and the total of possibilities of the state variables of a system comprise the multidimensional state space (Kersting 1984). This region in the state space can be called the normal operating range (NOR), and was calculated as the 95 % tolerance ellipse of state variables in a reference ecosystem.

Besides NOR, selected reference locations can be used to determine maximum deviation for environmental parameters. Rutgers *et al.* (2008) provides an example for this method using a number of agroecosystems in the Netherlands. These authors determined ecosystem profiles and the ten biological soil quality references for: arable land on clay, cattle or dairy farms on clay, cattle or dairy farms on Loess, cattle or dairy farms on peat, arable land on sand, cattle or dairy farms on sand, semi-natural grasslands on sand, heathlands on sand, mixed woodlands on sand, and municipal parks (Rutgers *et al.* 2008). In a so-called EXCEL amoeba chart the national average of each parameter is shown, and comparison made with the absolute deviation from the reference (based on 100 %; the circle). The differences in biological, chemical and physical soil

characteristics between the Dutch average(s) and the selected reference were not evaluated.

A wide range of statistical and mathematical computational approaches for crop protection (and, hence, ERA) is used to detect unexpected effects from (unknown) stressors (see also examples in chapters 5 and 8). It is even so, that such methods are operational in a continental context, as in the case of the Water Framework Directive (EEC 2000). Within this European context, diagnostic methods were developed to detect deviations from Good Ecological Status (GES) of water bodies and to identify which stressors may locally cause these deviations. Important in this respect is the need for the distinction between natural biological variation induced by natural conditions on the one hand (reflected as either baseline variability or the so-called Normal Operating Range [NOR]), and the possible additional effects induced by anthropogenic stressors and land use on the other hand (Mulder and Vonk 2011).

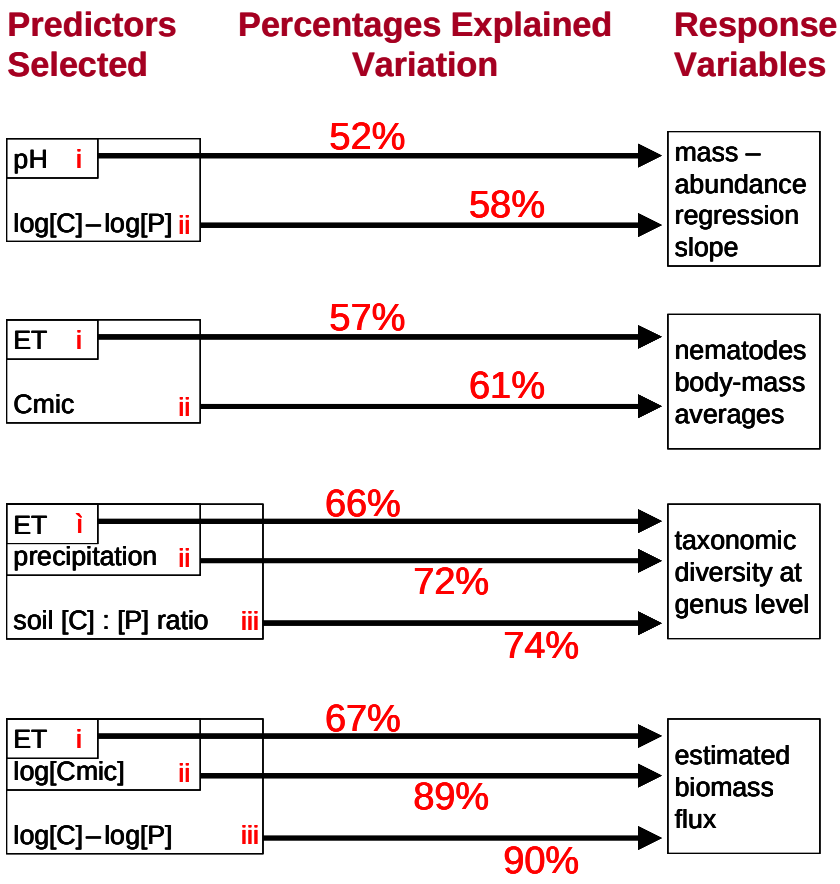


Figure 9: Since Ecosystem Type (ET) was an important predictor to forecast the abundance of soil nematodes and whose community structure, we summarize the mean values of relevant abiotic (elemental and climatic) and microbial descriptors by ET, and provide stepwise statistical ranks of the ET for each descriptor (multiple regression as shown in Reuman et al*. 2008, 2009).*

6 Relating nematode community and soil functions

Nematodes constitute a diverse group within the soil fauna, feeding on bacteria, fungi, other nematodes and plant roots. The feeding relationships and their direct contact with plant roots and residues imply that possible GM-crop effects in the rhizosphere and on the decomposer community (Verbruggen *et al*. 2012 and Vervoort 2013, respectively) will be reflected in the nematode community. We aim to link perturbations in the soil nematode community structure to biological soil fertility, since invertebrates *per se* might not represent a 'valued characteristic' in ERA or Farm Scale Evaluation (Firbank *et al*. 2003; Perry *et al*. 2003; Squire *et al*. 2003, 2005). Soil fertility includes amongst others organic matter decomposition and nitrogen mineralization. Seeking for correlations between nematodes and soil functions is necessary to evaluate if GM-crops cause effects on the soil ecosystem that are relevant for risk management and are evaluated as adverse by society.

Nematode communities can have large effects on soil nutrient cycling. Nematodes have been shown to stimulate the growth and turnover of microbial populations. This enhances of mineralisation and decomposition rates and increases nutrient turnover in soils (Hunt *et al*. 1987). Grazing of nematodes (C:N ratio ~10:1) on bacteria (C:N ratio ~5:1) results in a release of nitrogen, which becomes available for plants (Ingham *et al*. 1985). GM crop-induced adverse effects include reduced N availability during plant growth, increased N leaching outside the growing season, and enhanced organic matter decomposition.

The feeding strategy of nematodes (bacterivores, fungivores, omnivores) reflects their food resources (Yeates *et al.* 1993) and their role in the main energy pathways in the soil. For instance, predatory nematodes feeding on plant parasitic nematodes can reduce the negative effects of the latter group. The relative importance of the bacterial pathway compared to the fungal pathway indicates the actual effects of human pressure on the numerical abundances of soil organisms (Hunt and Wall 2002; Mulder *et al*. 2011; Verbruggen *et al.* 2012). From their position in the soil food web, the influence of nematodes on soil functions can be derived. Besides the well-studied negative effects of plant parasitic nematodes on crop production, most free-living nematodes have positive effects on soil productivity and support healthy soil systems.

6.1 Experimental results using DNA barcode assays

Most of the results obtained within the ERGONema project on soil functions are not yet fully analysed. Microbial activity profiles were obtained using the Microresp. methode while also soil respiration and nitrogen mineralisation rates were determined (Brolsma 2014).

Plant genotype had a significant ($P < 0.05$) effect on the structure of the nematode community (Figure 10) and a marginally significant effect ($P < 0.06$) on the substrate induced and basal microbial respiration. Figure 11 indicates that the relationship between the 13 nematode taxa and the microbial respiration was taxon specific, with some taxa showing a negative (*e.g.*, Prismatolaimidae, Alaimidae, Monhysteridae, Cephalobidae) some a positive (*e.g.*, Dorylaimida D3) and some a neutral (*e.g.,* Mesorhabditidae, Plectidae)

relationship with microbial respiration. Results indicate that the nematofauna and the carbon mineralization were both affected by plant-induced effects, and that nematode response was taxon-specific.

Furthermore, a positive correlation between nematode abundance and potential nitrogen mineralization, an indicator for soil fertility, was observed in the field experiment testing the effects of *Brassica* cultivars on the nematode community (Figure 11). A detailed comparison of the molecular nematode analyses and microbial activities will become publicly available when all analyses will be complete and peer-reviewed.

6.2 Field monitoring results

Besides the observed correlations between nematodes and soil functions from our experimental work, we also analysed long-term monitoring data (DSQN; Mulder *et al*. 2011). From this analysis, a significant correlation between the (log-transformed) nematode density and microbial activity, here presented by the Metabolic Quotient (qCO_2/qO_2: Oberholzer and Höper 2000; Mulder *et al*. 2005). Although qCO_2 and qO_2 values highly correlate, the Metabolic Quotient shows important divergences between management regimes regarding the redox state and energy content of the respirable substrates (Mulder *et al*. 2005). As we may assume that a higher qCO_2 indicates environmental stress, the increasing variation in the measured qO_2 supports also an alternative hypothesis. Regardless of the kind of soil, pioneer, immature bacterial populations, as in the case of croplands and other arable fields in Figure 12 (open circles), show in fact that their Metabolic Quotients were much lower than in the case of more respirable substrates from our low-intensity managed grasslands (Mulder *et al*. 2005; Vonk *et al*., in progress).

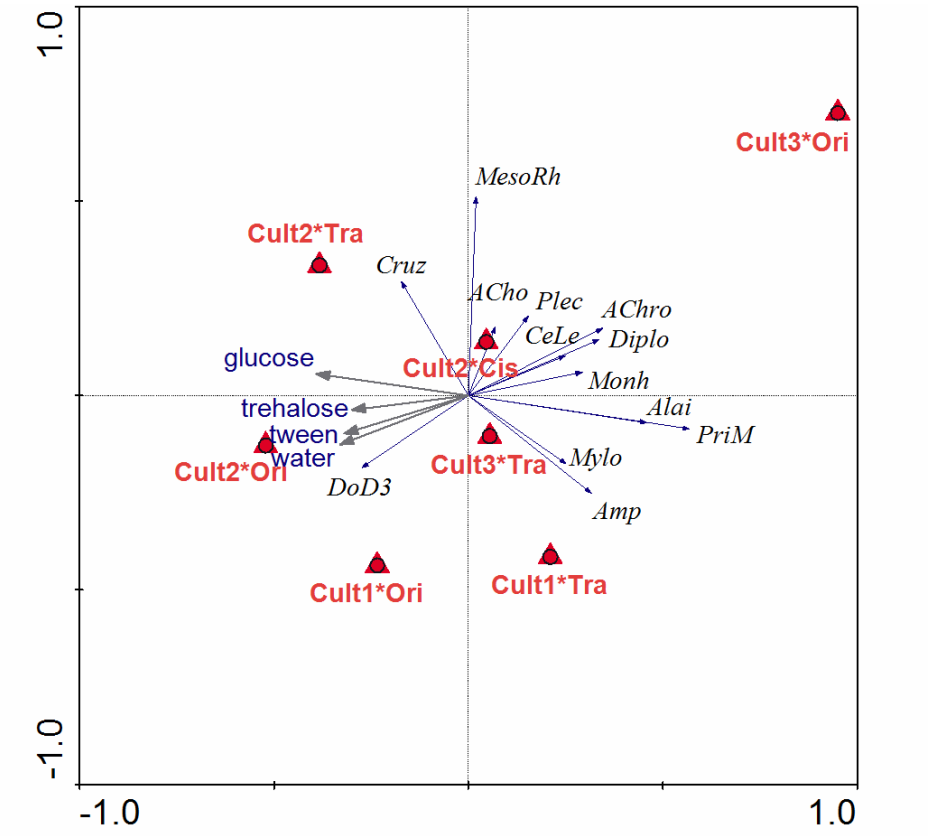


Figure 10: Correlation biplot based on the Redundancy Analysis of the composition of the nematode community in a field experiment with three potato cultivars (Cultivar 1, 2 and 3) and their modifications (Ori, original mother plant; Tra, transgenic; Cis, cisgenic) displaying 14.1 % of the variation in the nematode abundance (13 taxa quantified by DNA barcoding; blue arrows) and 63 % of the variance in the fitted abundances. The experimental model (cultivar x modification; triangles) explained 22.5 % of the total variance in nematode community data (P < 0.05). Substrate induced and basal respiration are included as supplementary variables (grey arrows). The model explained 23.6 % of the total variance in carbon respiration (marginally significant at P = 0.0560).

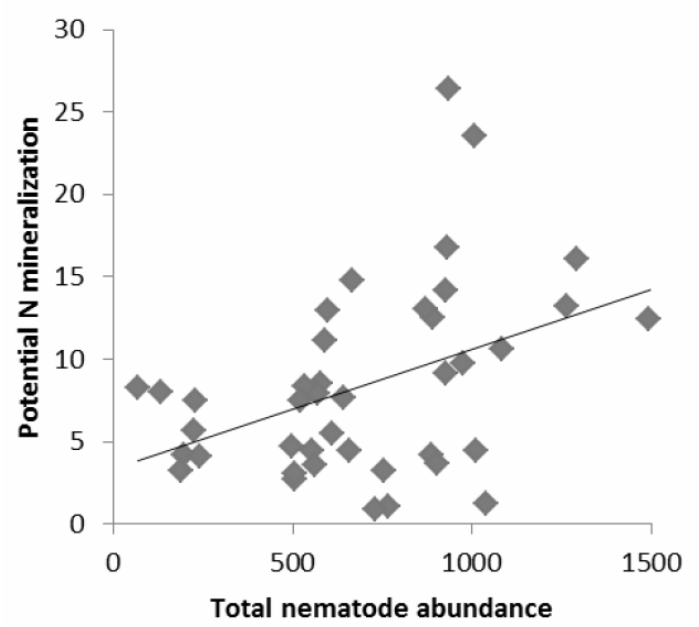


Figure 11: Relationship between the total abundance of nematodes (100 g^{-1} dry soil) and potential nitrogen mineralization (µg N g^{-1} 5 wk^{-1}) in arable soil grown with wheat and two varieties of Brassica juncea *(R^2 = 0.174, n = 41, P < 0.01).*

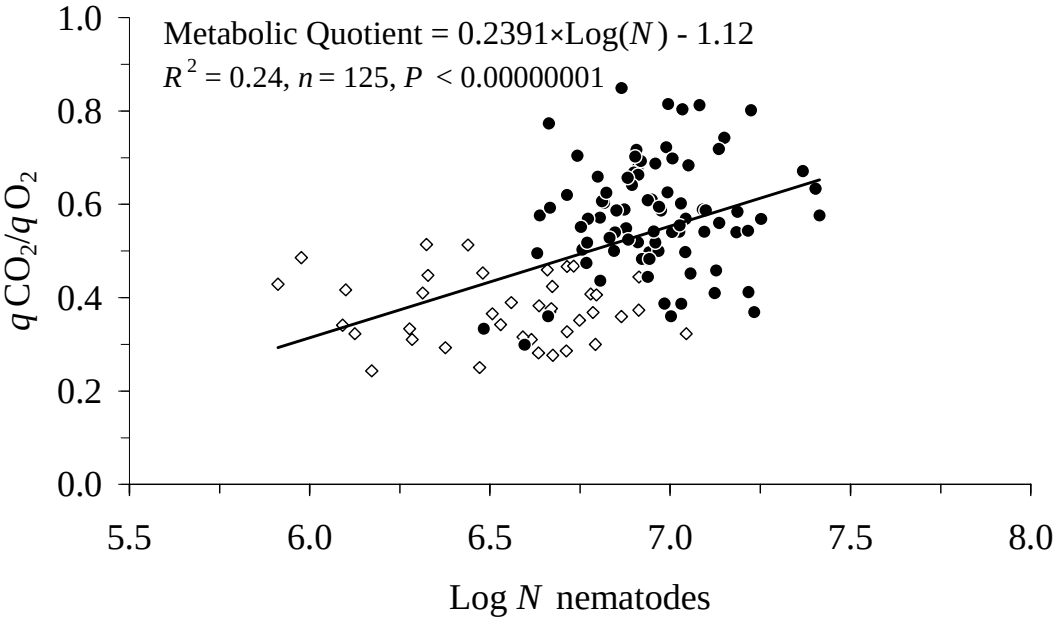


Figure 12: The so-called microbial Metabolic Quotient (Oberholzer and Höper 2000; Mulder et al*. 2005) was positively correlated to the nematode abundance (Log* N*; individuals m^{-2}) for two ecosystem types (arable fields [open diamonds] and managed grasslands [closed circles] under organic and conventional management). Four different soil types (sand, clay, peat and Loess) were included in this analysis (modified from Vonk* et al.*, in progress).*

7 Nematode DNA barcode assessment and GM-crop risk

In this chapter we aim to merge general principals of risk assessment with our nematode DNA barcode approach to develop an ERA tool to explore possible undesired effects of GM-crops on soil systems. The method can be useful in the context of pre-market assessment and post-market case specific monitoring (this section), as well as for GS (next section). We provide proposals and issues for risk assessment, in the shape of a preliminary formulation of guidance on how to use this method from the development of a new GM-crop up to the possible introduction in agroecosystems according to existing regulations or concerns.

Any guidance for using a nematode DNA-barcode tool is supposed to proceed as follows:

1. Define a possible hazard: how should a GM-crop pose any hazard to soil organisms?
   For instance, exposure via root exudates or through other plant remains ploughed into the soil after harvest. If there is no actual concern for exposure, barcodes can be used to determine if impacts are really absent, as in ERA to verify the absence of risk.
2. Determine the appropriateness of using the nematode DNA-barcode tool to address more issues of hypothesized hazards, and to define *a priori* which response endpoints (specific taxa, or certain traits) will be evaluated, and against which "no unacceptable impact benchmark" the effects are tested.
3. Collect sufficient soil samples, including the nematode assemblage, to run experiments with (GM) crops. Exposure tests that reflect field situations can be:
   a. Mimic exposure differences via root exudates in a range of increased final exudate concentrations to affect the nematodes
   b. Idem via different levels of ploughed-in plant remains
   c. Execute an exposure-difference study, comparable to laboratory tests with chemicals, and when necessary, explore variations in the rhizosphere structure
4. Collect the nematode DNA barcodes that are relevant, and explore the exposure-impact relationship or the difference between treatments. This step again needs to be compared to the pre-set level where impacts are considered to be unacceptable.

This order of proceedings in guidance is applicable to both pre-market assessment and post-market CSM (*e.g.*, General Surveillance). There are two major points in which the nematode DNA-barcode tool differs from common approaches in the risk assessment of chemicals. The first major difference is that chemicals commonly need be tested using various sentinel species, after which the predicted no-effect concentration (PNEC) is derived by extrapolation from the known test species data. The PNEC is a measure to protect the whole ecosystem against effects which are considered unacceptable. For example, test data are often required for algae, daphnids and fish to derive a PNEC according to adopted guidance, or – when test data are available for more than a few species – species sensitivity distributions are considered to statistically derive so-called hazardous concentrations (HC-values).

In the case of DNA barcodes, the risk assessment process can consider either the whole assemblage or certain nematode taxa known to be sensitive to the type of stress induced by a specific GM-crop. Effects on nematodes are

extrapolated to effects on soil functions. The second major difference is that in the domain of chemical testing often artificial soil (OECD) or highly-controlled laboratory strains are used, while for the DNA-barcode tool nematode assemblages from agricultural soils were used. The DNA-barcode test might develop in the direction of standardizing and testing in laboratory conditions. Whether such technical developments are possible or desirable, is beyond the scope of this study. Note that crops can be grown on a variety of agricultural soils with different nematode assemblages. Hence, the implications for effects of a certain crop on nematode assemblages can also be soil-dependant.

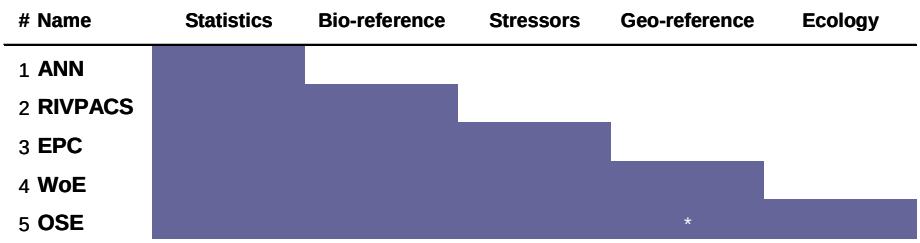
8 (Post-market) General Surveillance

The nematode DNA-barcode approach to study impacts of stress on soil systems can potentially be used in the context of General Surveillance. In this case, nematode assemblage data are collected in the field including data on the values of local (potential) stressor variables, like pH, organic matter content, pesticides, et cetera, so that a large (bio)monitoring data set is composed. Parts of such data sets are collected due to scientific or policy decisions taken in the past, but it is also possible to design a specific (bio)monitoring network for GS that includes the DNA-barcode method. Whichever is the case, the key issue is that data which are collected at high investment can be analyzed so that impacts that are locally present and which are beyond the natural variability and/or the variability induced by the (combination of) other variables can be recognized and (eventually) be attributed to the possible influences of GM-crops.

Defining impacts requires definition of reference conditions or reference status, as discussed in chapter 5. An example of using references is the concept of Good Ecological Status (GES) in the water management policy framework of the EU, the Water Framework Directive. GES is defined for different water bodies, which acts a spatially variable set of points of departure to quantify impact and its probable causes. An impact is considered present when *e.g.,* species are missing from a site while expected. When working with (bio)monitoring data, a conceptual limitation is always the issue of cause and effect: by virtue of the type of data and analyses, diagnostic approaches unveil possible statistical associations – not causation.

8.1 General issues in GS

Over the last couple of decades, large monitoring datasets have been compiled for a wide range of ecosystems. The aim of collecting data was often to identify ecological processes, including problems that might have been induced by an environmental pressure or by mixed pressures, essential for understanding complex ecological systems (Green *et al.* 2005). Since the early days of stress ecology, many stressors are analyzed to identify their importance in altering ecosystem services and human well-being (Perrings *et al.* 2011). The aim is to illustrate the very presence and availability of different diagnostic methods to detect signals from unknown stressors in large datasets without prior knowledge of the effects of a specific stressor. Starting from positive experiences, we briefly explore the applicability of existing diagnostic methods to the purpose of GS for GM-crops. We have already shown in Section 5.1 some implications of different mathematical, ecotoxicological and ecological approaches in various ecosystems. Potentials of the methods are discussed, together with their application to identify stressors using datasets that were originally collected for other purposes. General Surveillance (GS) implies the presence of (bio)monitoring data. As soon as all criteria are fulfilled, the five diagnostic methods previously described (and summarized in the upper Figure13) can be used to identify sites that are disturbed beyond observed natural variability.



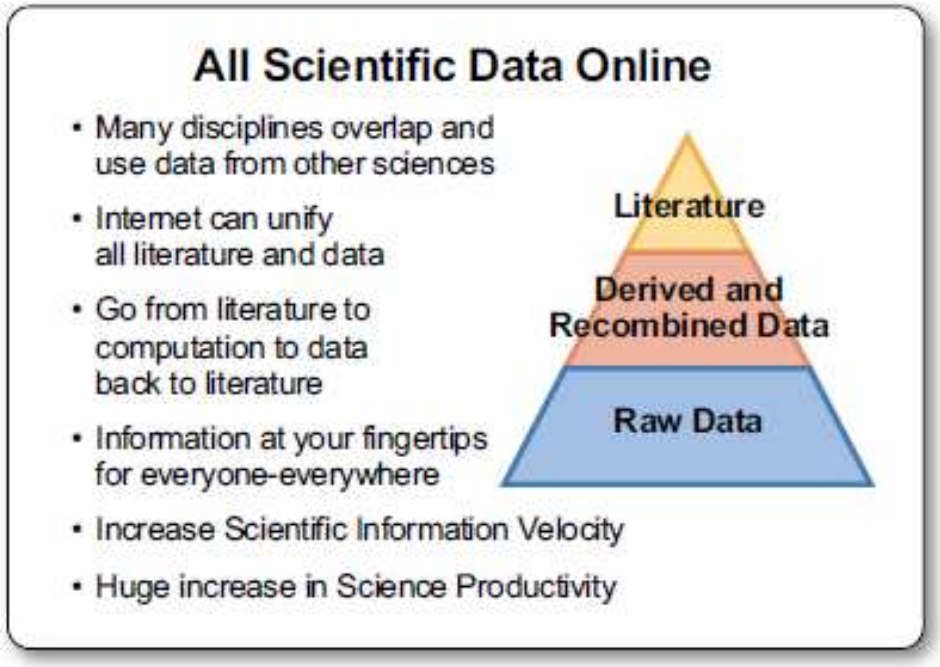


Figure 13: Above: overview of examples used in this report to identify effects of unknown stressors, in decreasing order of information required to apply the model. ANN = Artificial Neural Networks; RIVPACS = River Invertebrate Prediction and Classification System; EPC = Effect and Probable Cause; WoE = Weight of Evidence; OSE = Observational and Simulated Evidence. (Sites are geo-referenced in OSE, albeit only for geographical purposes and not included into the model itself.) Below: several ecological disciplines overlap and typically (re)use data, as foreseen by Jim Gray who described one world in which data and literature interoperate with each other (Hey* et al. *2009). Main criteria for the suitability of data derived from general monitoring networks are: (1) data collected objectively and according to protocols; (2) data collected on large spatial scales; (3) data collected on a regular basis; and (4) data generated in a way useful for modeling. Overall, models relevant for monitoring data range from statistical methods up to approaches that include enrichment of data by theory.*

8.2 Comparing approaches for GS

Mele and Crowley (2008) collected data from ten paired sites in Victoria, Australia, in which they measured twelve biological, fourteen chemical, and one management variable. These variables were incorporated in two separate parts of the SOM display: the unified (U)-matrix and the component planes for individual variables. The U-matrix allowed examination of the overall cluster patterns in the input dataset after the model had been trained. The neurons were drawn into distinct clusters during model training and relative distances between neuron clusters were displayed. SOM results indicated that most of the input variables were co-varying in one direction in one *n*-dimensional space (where *n* is the number of input variables). ANN can identify similarities and differences between sites based on monitoring data using the principles of statistics only. No additional ecological knowledge of the soil systems is required, though data enrichment (as in OSE) may happen. No reference locations have to be added in this method, which can be especially useful when the monitoring dataset consists of a limited number of sites. Since all sites are put together in a single analysis, this is the least data demanding method described here.

The family of RIVPACS-based approaches depends upon the use of a set of reference sites (Reynoldson and Wright 2000), *i.e.* locations considered to be of high ecological and chemical quality and chosen as representative of a particular type (*e.g.,* small rivers, larger rivers, lakes) with their expected fauna (Clarke *et al.* 2003). A set of appropriate environmental predictors is measured at any site type and the RIVPACS algorithms then calculate an expected (E) community composition for any site based on these measured local abiotic variables (Clarke *et al.* 2003). For all non-reference sites, the expected value can be compared with the actually observed (O) communities. The ratio between O and E determines the "ecological quality" at the site: in an ideal case, O equals E (ratio equals 1), and the studied site is concluded to be non-deviant from the reference site of the same type. When $O \neq E$, the conclusion will be that the site ecologically deviates from expected conditions, thus the site is either "enhanced" ($O > E$) or "affected" ($O < E$) by environmental and biological predictors. The deviation from expected conditions gives an indication of the severity of environmental pressure at the monitored site. There are possible pitfalls. Clarke *et al.* (2003) listed that errors in the estimates of the observed and expected fauna and observed and expected values of biotic indices are due to (a) inadequate set of references site, (b) weak statistical method to predict the biota from the environmental variables, (c) missing relevant variables as environmental predictors, and (d) sampling errors or methodological bias for a site. Although the latter two errors are pitfalls in all diagnostic methods, this indicates that the main problems of RIVPACS are methodological (Ostermiller and Hawkins 2004). These aspects are universal and have to be taken into account when RIVPACS-like modeling is applied to detect possible changes (this example holds for EPC and WoE as well). However, the RIVPACS software is widely used to assess biological quality of rivers and streams in United Kingdom, and equivalent software have been developed in other countries where RIVPACS (*i.e.* the reference condition approach) has been applied successfully.

The previous method commonly stops by identifying the total deviation at sites and does not address the relative association of individual stressors. This issue was the trigger to develop EPC, which was first applied to data for rivers in Ohio. From the total of 600 sites, 100 were selected as references; 35 environmental

and chemical stressors were included and the toxic pressure of all substances was put together into one proxy (Posthuma and De Zwart 2006). EPC can be applied to identify the magnitude and the (most probable) causes of biological impairment, given the variability in species composition and species abundances that occurs naturally. Although a set of statistical analyses is required, a final product consists of effect-and-cause pie charts which facilitate interpretation and communication. The most innovative aspect of EPC involves the linking of different types of models, all of which have been individually applied in the past for many purposes. The first step is a qualitative analysis. Focusing on native species biogeographically expected but locally absent, the missing species can be quantified as fraction of the species expected to plot pie sizes for each non-reference site in GIS. The second step is the quantitative assignment of species expected but absent to causes by a set of steps, like Generalized Linear Models between each species abundance and measured abiotic predictors (De Zwart *et al.* 2006). In this way, filling out the Generalized Linear Models with the values of (only) relevant variables results in a list of 'negative terms' per species, based on which the slice sizes of the local EPC are made. A 'negative term' is present for those variables which potentially cause a local reduction in abundance in comparison to reference.

While the previous method considers sites as separate codes (*i.e.* unrelated to each other spatially), WoE evaluates sites in a spatial context, incorporating geographic area and sampling probability into all its computations. The similarity between the EPC and WoE is the use of the reference concept and the ability to attribute the relative stressor influence. WoE utilizes a spatial analysis approach developed for minerals exploration (Sawatzky *et al.* 2009). The first step in WoE is comparable to RIVPACS and EPC. Two training data subsets based on biomonitoring data are prepared, one dataset representing minimally disturbed sites and another containing other sites. Per site, the probability of occurrence is computed within the hydrologic study area (catchment/watershed, river network, or water body). WoE determines how the spatial patterns of individual environmental variables alter this probability, and variables which significantly increase the probability of disturbance are selected as potential stressors. To determine cumulative stressor influence, the spatial patterns of all stressors are integrated in a logistic regression model. This results in a probability map displaying predictions over the study area as a function of the variable and model coefficients which enable rankings of stressor influence for the entire study area.

National surveys are very suitable for the OSE method. Data enrichment steps show that the dataset originally collected can be re-shaped (Mulder and Vonk 2011) into more robust, derived parameters, based on ecological knowledge of the studied organisms as well as introduction of more meaningful endpoints in terms of the ecological interpretation (*i.e.* a variable directly related to an ecosystem service). After the data enrichments, the first part of OSE consists of the application of Generalized Linear or Additive Models to fit data to continuous abiotic gradients, often using data from a neighborhood around one specific abiotic predictor-value and revealing the underlying pattern without any preconception of what the underlying relationship is. The second part of OSE continues with model simulation for validation, a critical component in the development of a reliable model. Mulder *et al.* (2003) performed such an analysis for soil nematodes by means of a Monte Carlo simulation with 10,000 random permutations. The adequacy of the fitted model was checked by plotting the standardized residuals. This diagnostic tool showed the serial independence

of the observations and a linearity of relationships between the function-related response variable (trait) and the potential stressor variables (soil pH, climate variables, and livestock density), but a non-linear relationship between nematode traits and nutrient availability. The simulation was repeated twice to compare the mean distribution and the percentiles in the results, so as to check robustness of the modeling procedures. GIS-maps were made to illustrate the presence of "response" (variability across sampling sites) of the functional traits to be analyzed in relation to any potential stressor variable. These analyses showed that there was a statistical association between the farming regime and the cattle pressure, superimposed on the influences of the other predictors, and the nematode traits (Mulder *et al.* 2003). OSE resulted in the identification of an unanticipated stress variable (livestock) on the nematode community, including a shift in functional characteristics of some soil biota.

8.3 Data enrichment and comparison of uncertainties

Binary data (presence/absence) are case-sensitive, given that the "presence information" has in statistics a different weight than the "absence information". Aggregation of data using ecological knowledge, for example merging species together by traits, may enhance modeling and reduce the "noise" in a dataset. Data enrichment has large implications on the results of the analysis, uncertainties and the need for extensive sampling efforts. Encountered outliers are a typical problem during analysis of datasets and can disturb or even influence analysis, albeit the LOWESS approach, as described here as part of OSE, reduces their influence. Another problem is related to the type of relationships assumed. The LOWESS approach as well as ANN will reveal the underlying patterns in a dataset without any preconception and both are suitable methods to detect different types of unexpected effects. Concerning the sensitivity of the models to detect possible effects from unknown stressors, it can be stated that, in general, if more information is available on the studied systems, both from sampling efforts and additional studies, the data analysis will result in a more sensitive model with a higher statistical power to detect signals when present. One should always take into account that the balance between the number of environmental variables and the number of sampling sites has to be correct. Various rules of thumb are in use on the minimum required numbers of samples (see Sokal and Rohlf 1995), but in general higher (policy) required sensitivity demands more sampling sites. In any interpretation of results, there is a difference between the *presence* of results indicating significant deviation and the *absence* of results indicating deviation. At low sampling sizes or under large influences of other variables, responses might be unnoticed. Data sensitivity can be determined, but due to the specificity of techniques for studies and models, it is beyond our scope to expand our examples with analyses of statistical power and we refer to the original studies themselves.

To enable a standardized comparison of biological conditions under different crops across ecosystem types and (eco)regions, there is a need to summarize the differences between observed and expected ecological situation. In RIVPACS, sites are classified into a small number of biological quality grades. This is done by calculating ecological quality indices, mostly defined as the ratio of observed to expected values of each biotic index being used in the grading process (a kind of standardization). A particular value of such a ratio implies the same ecological quality for that index, no matter what type of river, stream, or soil system. It is because of the success of RIVPACS and its acceptance as a robust tool for standardization for freshwater quality assessment that the

calculation and use of these ratios became so widespread. Also EPC and WoE use this same comparison between observed communities at a possible affected site relative to expected reference values from minimally disturbed or reference sites, in contrast to ANN. Although the SOM-presentation derived from ANN does not specifically highlight a site where there is an adverse effect, there are SOM-based methods which are applied to find deviations from a Good Ecological Status. In that case, ANN can be applied to generate a SOM from a training set of data, after which a *new* sampling site can be tested for similarity to – or difference from – the reference set. In this case, the ANN-set is trained on common variables.

Both similarities and differences in design and output found during the comparison of WoE and EPC show the benefits of applying multiple methodologies, whereby results in agreement from multiple methods have a higher degree of confidence for subsequent regulatory practices, and results that disagree may be evaluated to reduce uncertainties. Only compilations of large datasets will facilitate detailed comparisons between methods. Data mining is often hampered by restrictions and too many publicly available datasets remain digitally unexplored. As a result, current datasets remain small and their signals hidden in the large natural variation due to low statistical power.

9 Conclusions, prospects and future research

In conclusion, there is no principal issue that would limit the use of the nematode DNA-barcode assay in pre-market or post-market (CSM and GS) risk assessment. The tool should be added to the existing, regulatory-induced evaluations. Since the amount of data generated with this new method is still limited, the suitability of this tool to replace current methods used for risk assessment needs to be determined based on further experience. When the tool would be adopted, appropriate refinement of the preliminary guidance given above is needed, before the test can function in a standardized regulatory framework of formal risk evaluations.

Based on experimental progress and this evaluation of the nematode DNA-barcode project within ERGO, the following conclusions and topics for further research can be drawn:

- *Principal improvements on the DNA-barcode technique*

  The DNA-barcode technique has been strongly improved, seen the huge differences between the research draft at the start of the project (2007) and nowadays. The SSU rDNA framework was extended from 1,215 sequences (Van Megen *et al*. 2009) to currently more than 2,400 sequences, allowing for robust primer design and expanded target ranges. We have tested the stability of our method while applying it on different soil types. Even DNA extracts from soils high in organic matter and humic acids, which often introduce inhibition in PCR, provided statistically significant results (Vervoort *et al*. 2012). We show that the nematode DNA-barcode tool is sufficiently sensitive to detect temporal fluctuations of specific taxa (Vervoort *et al*. 2012) and changes related to agricultural management. Overall, the DNA-barcode technique is suitable to quantify a range of disturbances on nematode communities. A main aim for the DNA-barcode tool includes the improvement of taxonomical resolution in future research. As resolution at genera level can be more informative, the goal is to move towards the development of genus-specific primers.

- *Our recommendations for ERA and molecular ecology*

  Soil systems are one the most diverse ecosystems and the relations between nematode communities and soil functions need further attention. Hence, we aim to develop an overview of GM-crop traits and functions that might be affected by these traits. Such an assessment of GM-crops on soil functions can be done by molecular ecology, like the nematode DNA-barcode tool described here, but need also to be compared to applied methods for risk assessment. Only in such a way, in fact, the actual sensitivity of the barcode technique and the detection of effects due to GM-crops (and related traits) is warranted. The DNA-barcode tool provides opportunities to collect additional data with a standardized high-throughput method to study these relationships in more detail. It should also be clear that the extent to which GM-crop effects on soil systems can be detected, is related to what framework

has been used. Besides such a framework, not only GM-crop effects on soil ecology, but also the positive effect of the GM cultivar over existing cultivars must be taken into careful account, as discussed in chapter 2. This report addresses both the functionality of the current nematode DNA-barcode tool as well as some proposals for a refined guidance on risk assessment to be used in the future.

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