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COLLABORATIVE PROJECT

Assessing and Monitoring the Impacts of Genetically modified plants on Agro-ecosystems

Deliverable 4.8 – Report on definitions of harm, damage and limits of concern for soil fertility

Workpackage 4

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Summary

In this deliverable we report about limits of concern (LoC) as they were elaborated for soil organisms by results of the experimental studies in the AMIGA project. We explain the concept of functional guilds and their particularities which require consideration for the assessment of measurable endpoints and their interpretation in regard to harm, damage and LoC. For each functional guild we suggest, based on the yet preliminary data analyses of the AMIGA research results, specific LoC, which can be utilized for further statistical analyses by Workpackage 9 of the AMIGA project.

Introduction

A precondition to introduce genetically modified (GM) plants into agricultural production systems is that they do not cause harm and damage to the environment, including their biological and abiotic constituents. Workpackage WP4 of the AMIGA project focusses on the **biological components of soil fertility**. In this report we address the question, what kind of harm and damage could be envisaged in a scenario considering the cultivation of a GM crop for selected soil organisms and make an attempt to define specific limits of concern (LoC).

Target and non-target organisms, direct and indirect effects

It should be noted that the vast majority of the current GM crops introduced to, or considered for agriculture, do not target soil organisms, with a potential exception of plants designed to control e.g. plant-feeding nematodes or soil-borne microbial pathogens. Generally, due to their importance for soil fertility, soil organisms are considered to be **non-target organisms** (NTO), which need to be protected of even supported for their ecosystem services (1). Thus, effects of a GM plant on the biological components of soil fertility would be **unintended**. For the unintended effects these could be **direct**, e.g. by interactions between an NTO and a recombinant protein, or **indirect**, by any means beyond that, e.g. altered digestibility due to a compositional change in regard to fiber-contents, or even due to altered agricultural management practices resulting in different pesticide use, or changing crop rotations from three or five crops to mono-culture.

Harm, damage and limits of concern (LoC)

For the environmental risk assessment (ERA), the whole GM crops can be regarded as **stressors** and not only specific new constituents introduced into a **receiving environment** (2). Organisms are not exclusively exposed to a genetically modified product, e.g. a protein, but to a whole viable plant, which includes the recombinant product, or a fraction of it, which, e.g., remains and decomposes on fields after harvest. "**Harm**" means that organisms would be affected by a GM crop, directly or indirectly, in a negative way (adverse effect). This adversity can be transient depending on the resilience of the affected organisms. "**Damage**" is stronger and indicates that the system is significantly affected and not capable to return easily or relative quickly without any management options to the status before. "**Limits of concerns**" (LoC), as defined in the AMIGA project follows the EFSA ERA guidance document on risk assessment of GM plants (2) where these limits are defined as ranges of variables in which the ecological (or economical) functions of agricultural ecosystems are damaged or about to be damaged. The concept of LoC is emerging in risk assessment studies and differentiates these effects of a stressor (say a GM crop) that simply alter a variable and from those effects that move or keep a variable in an ecological damage or damaging state.

Soil organisms assigned to functional guilds

Based on a recent definition of functional guilds (3) three groups of soil inhabiting organisms were distinguished: *chemical engineers*, *biological regulators*, and *ecosystem engineers*, respectively. Thus, these guilds were selected in AMIGA for further consideration and investigation. For the AMIGA project, *chemical engineers* included all soil bacteria, archaea and fungi; *biological regulators* included nematodes; and *environmental engineers* included earthworms. Due to the different biology and life-styles as well as their environmental abundance and interactions with GM plant material, it was clear that for the studies in this WP4 each group of organisms was studied with a particularly suitable experimental and analytical approach, targeting different measurable assessment endpoints. Depending on the selected group of organisms, studies encompassed literature surveys, laboratory incubations in microcosms, and/or monitoring at the AMIGA field sites where GM maize or potato were cultivated.

Exposure

Exposure is a key issue of environmental risk assessment. Organisms and GM plant material (stressors) may accidentally occur in the same environmental compartment and thus come into contact, e.g. in a litter layer or organisms may in fact actively search and ingest or take up such material, or organisms which previously took up plant material. Thus, the intensity of exposure can vary and depends on habitat and life-style (feeding preferences) of the respective organisms. For *chemical engineers* and *biological regulators*, including soil microorganisms and nematodes, our studies focused, due to the suspected increased exposure, on organisms which were found in GM plant rhizospheres rather than soil not influenced by the plant roots. For *ecosystem engineers*, i.e., earthworms, the exposure was at least equally intense, considering that these organisms ingested plant material, the latter however decomposing with possibly less recombinant product present compared to rhizospheres of viable plant roots.

Chemical engineers – bacteria, archaea and fungi

All agricultural soils are colonized with bacteria, archaea, and fungi. One gram of fertile soil contains more bacterial cells, in the range of 10^{10} and above, than humans on our planet (7.4 x 10^{9}). Archaea contribute with approximately 100-fold lower population sizes. Fungi may occur as spores (resting cell stages), single cells or form mycelia which can connect soil micro- and macro-aggregates with each other.

The rhizosphere and their microbial communities

The rhizosphere represents a soil compartment which typically contains more bacterial and fungal cells than soil not affected by plant roots. This increased cell density is a result of growth on carbon and, sometimes also nitrogen sources, supplied by plant roots, either as exudates or by decaying sloughed-off root cells. The composition of the bacterial and fungal community responds to the composition, i.e., the quality and quantity, of the plant supplied substrates (nutrients). This can be seen by the fact that different plants or even cultivars of the same plants select for differently structured bacterial and fungal communities. The

responsiveness is so high, that the microbial communities even change with plant age (4), and different bacteria have been detected at fine roots and (more mature) coarse roots even from the same plant (5). Thus, the composition of the rhizosphere inhabiting microbial communities can be regarded as an indicator of compositional changes in the root supplied organic compounds.

GM plant varieties differ from conventional ones typically by producing additional or modified proteins, i.e., enzymes or toxins. If existing proteins are only modified, e.g. by alteration of the amino acid sequence, such modified compounds would unlikely trigger a response in the microbial community structure. In contrast, additional proteins would have a potential to modify the community structure, either by exhibiting a direct adverse effect on the microorganisms, or by just providing an additional substrate which can be degraded by certain members of the community, thus resulting in their proliferation and increase in relative abundance.

The detection of differences in the quantity and/or composition of the bacterial and fungal community structure in comparison to the control, i.e. the near-isogenic cultivar, require further consideration. Differences between both cannot immediately be translated to harm or danger, since, as mentioned above, differences also occur between plants of different age and between cultivars. Even minor differences in plant growth between a GM and a comparator may cause a slightly modified microbial community structure. The differences between a GM and the near-isogenic therefore need to be scaled against differences as they occur between conventional cultivars, different years or field sites of cultivation. Thus, in contrast to higher organisms, where e.g. toxicity or other biological effects and be assessed without a comparator, the microbiological approach normally requires side-by-side analyses, for the case of GM plants, this should include in addition to the near-isogenic cultivar also varieties which are utilized in agricultural already. In the AMIGA project this approach was not followed for maize, as it was not the intention to risk assess MON810, but to analyze the importance of different field sites across Europe for interacting with soil organisms. For potato, the trial in Ireland included different cultivars and it was demonstrated that differences between the GM (cis-genic) potato and the isogenic were not beyond those seen with other varieties.

ERA approaches for microorganisms in the AMIGA project

Compared to the detection of indicator functions of microbial community changes in response to the presence of a genetically modified product, it is much more difficult to assess and detect **harm or damage to the ecosystem functions** provided by the *chemical engineers*. Mycorrhizal fungi, for example, are potentially important to support plant growth by facilitating the access to phosphate, but these interactions are inhibited by additional supply of fertilizers, as applied in conventional agriculture. In fact, the AMIGA project data demonstrate that mycorrhizal fungi were not significantly contributing to the fungal community structure of maize and potato as cultivated at the AMIGA field sites across Europe, as reported in the AMIGA Deliverable D4.2 in more detail. For these two crops, conventional agriculture does also not rely on plant growth promoting bacteria, e.g. those which fix atmospheric nitrogen. In a broader context, however, these activities are exploited by using leguminous plants as part of crop rotations. For other ecosystem services there are, due to the diversity and adaptability of soil microbial communities, no simple tools to detect harm or damage in response to the cultivation of a GM crop.

Generally harm to soil microorganisms can be regarded as a change of the soil microbial community so that they would not carry out their ecosystem functions as efficiently as with a conventional crop. This could be a result of a decline in microbial diversity, since generally communities with higher diversity are regarded to be more efficient than lower diversity communities (6-8). Harm could also be a change of enzymatic activities, e.g., to reduce nitrite in context of denitrification. This could be indicated by quantitatively less, or less diverse organisms contributing to these functions. A damage would exist, if such alterations would not return to the normal range after the stress is gone, e.g. after the GM crop has been harvested.

Structural vs functional diversity

The potential of molecular approaches for characterizing microbial communities in context of GM risk assessments was explored in the AMIGA project. The analyses were based on characterization of PCR products amplified from directly extracted soil (rhizosphere) DNA. Depending on the targeted genes utilizing specific primer systems, communities can be

characterized for structural diversity or for the functional diversity. The structural diversity tries to include all organisms of a defined taxonomic rank, e.g. at the domain-level, all members of the *Bacteria* or the *Archaea*, at the phylum-level all *Proteobacteria*, or at lower ranks, e.g., the diversity of *Pseudomonas* and relatives as a specific subgroup within the *Gammaproteobacteria*. Structural diversity of bacteria and archaea is commonly assessed by analyzing the 16S ribosomal rRNA genes (16S rRNA), as these occur in all organisms and the similarity of the encoding genes is the best known estimate of a phylogenetic neighborhood based on a single gene. The 16S rRNA gene is used to search for sequence identity in databases and assess diversity. Operational taxonomic units (OTUs) are an estimate of species-level, if they show at least 97 % sequence identity (9, 10). It should be noted that this OTU-species definition, is however only a rough estimate, especially keeping in mind that only partial and not full-length 16S rRNA genes are compared with each other). To assess the fungal community structure, recent studies revealed that the ITS1 region was found to be highly useful (11, 12).

The functional diversity of a microbial community is assessed in order to answer the question, "who is doing a specific job?" Molecular approaches can target by PCR specific genes which encode for specific functions, normally i.e. enzymatic reactions. In the AMIGA project, the nitrite reductase was selected: An enzyme mediating a key metabolic step in the denitrifying section of the nitrogen cycle. As for most enzymes, these have not evolved homogenously from one single ancestor. In fact, for the nitrite reductase, two different versions of the genes (*nirS*, *nirK*) are abundant in the environment, and they require different detection systems (13, 14). A change in the ratio of the enzymes encoded by the different genes would not necessarily indicate harm, but it could indicate in a comparative approach that the GM plant would generally affect the organisms carrying out this specific step in the denitrification pathway. Other functions, i.e. most of the carbon cycle are represented by many more different enzymes, making it difficult to utilize a similar approach to assess the effect of the GM e.g. on the decomposition of plant material. On the other hand, it is possible to assess the effect on the chemo-lithotrophic process of ammonium oxidation, as there are, by current knowledge, only two versions of the enzyme ammonium monooxygenase, one provided by bacteria and one by archaea (15, 16).

Measurable endpoints

Measurable endpoints for assessing the GM plant effects on soil microorganisms include a quantitative and a qualitative aspect, to determine the abundance and the diversity. Abundance is assessed by qPCR and data are reported as copy numbers per ng DNA (DNA refers to the total DNA extracted from rhizospheres). Structural diversity is assessed by copy numbers of rRNA genes or ITS1 regions (for fungi), and functional diversity in AMIGA was assessed by copy numbers of *nirS* and *nirK*.

Diversity, including "species" richness is determined by DNA sequencing of PCR amplicons. A decade ago this approach would have been impossible but since the advent of highly efficient and fast DNA sequencing technologies, it is now feasible to sequence millions of PCR products for a reasonable amount of money. In the AMIGA project, 15.7 million sequences were obtained for characterizing the diversity of bacteria from maize and potato, and for the function diversity (*nirS*/*nirK*) 3.9 million were obtained, as reported in detail in the reported provided as Deliverable D4.1 to the AMIGA consortium. Fungal diversity was assessed by sequencing of 25.5 million ITS sequences; for more details see Deliverable D 4.2. Species richness is reported as the number of different OTUs. Diversity is expressed by the Shannon Index (H'). The corresponding data from the AMIGA project are reported for maize in Table 1, and for potato in Table 2.

The compiled data in Tables 1 and 2 were calculated from both, the GM and the comparators, since the statistical analyses (see Deliverables D4.1 and D4.2) did not reveal significant differences: Thus, the data give a good estimate of the range as it may occur across different European regions on agricultural fields. Clear differences are indicated between maize and potato, e.g., the number of bacteria in the total DNA extracted from potato rhizospheres is higher than for maize, but the number of archaea and fungi lower. For both, maize and potato, the diversity of bacteria was higher than for fungi. Interestingly, the Shannon diversity indices (H') for bacteria from maize and potato were very similar, and the same was true for fungi.

Table 1 Maize: Assessment endpoints and suggestions for limits of concern (LoC) for soil microorganisms, including bacteria, archaea and fungi, based and a comparative approach

	Measurable assessment endpoints	Method	Ranges[2]: Mean (min – max)	**LoC (<, >)[3]**
Structural diversity				
Bacterial abundance	Quantity of domain-specific 16S rRNA genes	qPCR (copies per ng DNA)	9.8×10^5 ($3.8 – 21 \times 10^5$)	**0.49×10^5, 98×10^5**
Archaeal abundance			2.1×10^4 ($0.8 – 5.4 \times 10^4$)	**0.11×10^4, 21×10^4**
Fungal abundance	Quantity of genomic ITS1 sequences		5.6×10^4 ($0.7 – 36 \times 10^4$)	**0.06×10^4, 112×10^4**
Bacterial richness[1]	Quantity of different bacterial "species" (OTUs)	Illumina PCR amplicon sequencing and bioinformatic analyses including statistical tools	2,933 (1,464 – 4,706)	**733, 5866**
Fungal richness	Quantity of different bacterial "species" (OTUs)		500 (347 – 640)	**125, 1000**
Bacterial diversity[1]	Shannon Index 16S rRNA genes		5.6 ± 1.1	**3.4, 7.8**
Fungal diversity	Shannon index ITS1 sequences		3.9 ± 0.4	**3.1, 4.7**
Functional diversity				
Abundance of nitrite reductases	Quantity of *nirK* genes	qPCR (copies per ng DNA)	1.3×10^6 ($0.1 – 8.7 \times 10^6$)	**0.01×10^6 26×10^6**
	Quantity of *nirS* genes		3.6×10^3 ($0.4 – 12 \times 10^3$)	**0.04×10^3 72×10^3**
Richness of nitrite reductases	Number of different *nirK* sequences (OTUs)	Illumina PCR amplicon sequencing and bioinformatic analyses, including statistical tools	178 (73 – 349)	**53, 356**
	Number of different *nirS* sequences (OTUs)		Not determined	**-**
Diversity of nitrite reductases	Shannon Index *nirK*		Not yet analyzed	**-**
	Shannon Index *nirS*		Not determined	**-**

[1], with the selected molecular approaches, archaea represented approximately a maximum of 1 % of all prokaryotic sequences, while bacteria represented at least 99 % . Thus these data are a good estimate for bacterial abundance
[2], estimated by preliminary analyses of data from the AMIGA project
3, values indicate the lower limits and the upper limits, values beyond those would be of concern

Table 2 Potato: Assessment endpoints and suggestions for limits of concern (LoC) for soil microorganisms, including bacteria, archaea and fungi, based and a comparative approach

	Measurable assessment endpoints	Method	Ranges[2]: Mean (min – max)	**LoC (<, >)**
Structural diversity				
Bacterial abundance	Quantity of domain-specific 16S rRNA genes	qPCR (copies per ng DNA)	3.3×10^6 ($0.3 – 28 \times 10^6$)	**0.2×10^6, 33×10^6**
Archaeal abundance			6.8×10^3 ($0.8 – 21 \times 10^3$)	**0.4×10^3, 68×10^3**
Fungal abundance	Quantity of genomic ITS1 sequences		4.8×10^4 ($0.1 – 35 \times 10^4$)	**0.05×10^4, 96×10^4**
Bacterial richness[1]	Quantity of different bacterial "species" (OTUs)	Illumina PCR amplicon sequencing and bioinformatic analyses including statistical tools	2,042 (630 - 3491	**511, 4,084**
Fungal richness	Quantity of different bacterial "species" (OTUs)		385 (223 – 555)	**96, 770**
Bacterial diversity[1]	Shannon Index 16S rRNA genes		5.6 ± 0.7	**4.2, 7.0**
Fungal diversity	Shannon index ITS1 sequences		3.7 ± 0.6	**2.5, 4.9**
Functional diversity				
Abundance of nitrite reductases	Quantity of *nirK* genes	qPCR (copies per ng DNA)	1.5×10^6 ($0.1 – 5.7 \times 10^6$)	**0.02×10^6 30×10^6**
	Quantity of *nirS* genes		3.7×10^3 ($0.4 – 61 \times 10^3$)	**0.04×10^3 74×10^3**
Richness of nitrite reductases	Number of different *nirK* sequences (OTUs)	Illumina PCR amplicon sequencing and bioinformatic analyses, including statistical tools	180 (91 – 374)	**54, 360***
	Number of different *nirS* sequences (OTUs)		Not determined	-
Diversity of nitrite reductases	Shannon Index *nirK*		Not yet analyzed	-
	Shannon Index *nirS*		Not determined	-

[1], [2], [3], - see footnotes of Table 1

Limits of concern (LoC)

The ranges reported in Tables 1 and 2 were utilized to suggest LoC values. These are preliminary judgements, considering the limited sample size and the variability which may be introduced by not using the same detection protocols. This is especially important for qPCR, where each PCR cycle theoretically doubles the number of copies detected. Thus, for further studies, PCR efficiencies and detection systems have to be carefully evaluated, to add more data to those reported here. Based on the distribution of values, mean values and minimum-maximum values we suggest for the qPCR data to utilize as an estimate a 95 % reduction and a 10-fold increase as the limits of concern for bacterial and archaeal 16S rRNA genes. For fungi, as well as for the functional genes *nirS* and *nirK*, the ranges of observations were higher, making it necessary to adjust the corresponding values to 99 % reduction and 20-fold increase. The values for the Shannon Index (H') were more stable and, thus, we decided to define the LoC value by doubling the standard deviation. For the species richness, a decline of 70 % and an increase by two-fold were set as the LoC. Based on this definition, one value (maximum of *nirK* from potato) was beyond these limits. Values outside of the LoC should trigger further analyses about the consistency of such values, which can be tested with site and annual replication. In this specific case, the extreme values were not reproducible and, thus, probably associated to field heterogeneity or technical/analytical problems.

Concept of the core rhizomicrobiome for GM plant risk assessment

The comparative analysis of the bacterial and fungal diversity, as assessed by identification of OTUs from the plants collected at the different field sites revealed that a relatively low number of OTUs, which however was highly abundant, consistently occurred independently of the sites. E.g., for maize, 87 bacterial OTUs, representing 0.7 % of all OTUs which were found with maize, were detected with all individual plants collected during the three years in Sweden, Denmark, Slovakia and Spain. These OTUs however represented 37 % $\pm$ 12 % of all sequences (for more details see D4.1). Similarly, for fungal sequences from maize there were 13 OTUs which represented on average 29 % of all sequences. These OTUs can be regarded as part of the inheritable microbial variation of the maize (17). Their abundance, but not

their presence may be affected by environmental conditions. Similar results were obtained with potato, with less strong conclusions, because of data from only two fields sites and only one site with annual replications.

We hypothesize that GM-plant triggered changes in the OTUs which occur at single sites may not be that risk relevant *per se*, as changes, e.g. losses of OTUs from the core community. In order to transpose such data to the level of harm or damage, more research would be required to identify functional properties of the "lost" or newly gained OTUs, e.g., whether they could be plant pathogens, or provide beneficial functions, i.e., nitrogen fixation or mycorrhization. A better understanding of the identity and functions of core rhizomicrobiome could give leverage to distinguish changes which would be of concern due to an unintended genetic modification of plant properties, from those which would have no impact on plant health or ecosystem services. More research in this field is required.

Biological regulators - nematodes

Nematodes promote important soil processes such as decomposition, mineralisation and nutrient cycling. Alterations in the nematode community structure may have the potential to influence ecosystem functioning (18). Widespread and highly diverse, nematodes form part of the food web of soil by occupying primary, secondary and tertiary positions in multiple trophic groups: bacterial feeding (BF), fungal feeding (FF), predators (PR), omnivorous (OM) and plant feeding (PF) (19), making them excellent indicators of fluctuations in soil composition arising from for example, plant genotype and/or type of soil management and environmental conditions in the rhizosphere (20, 21). With the ability to provide insightful information on soil food web dynamics (22), it can be hypothesised that nematodes are important NTO and useful bio-indicators in monitoring more generally the potential impacts of genetically modified plants on agro-ecosystems.

As part of AMIGA, a previous deliverable (4.3) detailed the analysis of maize and potato rhizospheric samples in order to quantify nematode abundance and community structures, following the cultivation of GM maize and GM potato versus their respective comparators.

Generated datasets for maize were derived from samples taken in Slovakia, Denmark, Sweden, Spain in 2013 and Slovakia, Denmark, Sweden in 2014 while potato datasets were derived from Ireland (2013, 2014 and 2015). To objectively assess the nematode community baselines for each year, crop and location, nematode structure and diversity were measured through established indices, accounts and taxonomy.

Methods applied to study nematode communities

This process of characterizing nematode populations can be achieved morphologically through microscopy or via the sequencing of nuclear, e.g., 18S rRNA genes, and/or mitochondrial genes, e.g. encoding for a cytochrome c oxidase subunit. Of the targets listed the 18S rRNA gene (SSU rDNA) has proven to be most informative for investigating nematode populations considering the semi-conserved and variable regions within the sequence which provides opportunity to identify down to the species level. From this, taxonomic conclusions along with absolute values and respective indices, that integrate the responses of different nematode taxa and trophic groups to soil perturbations, can be calculated as a means to measure environmental impact on the soil ecosystem.

The data on which the evaluations in Tables 3 and 4 are based, were generated by microscopic analyses of rhizosphere samples from maize, and in the case of potato, through the sequencing of 18S rRNA gene fragments amplified by PCR from the rhizosphere extracted total DNA.

Evaluation of data and estimates for limits of concern

The analysis of nematode communities from the maize sites previously indicated that the occurrence of nematodes, their abundance, proportion of feeding types and selected ecological indices did not depend on the type of maize (GM or non-GM).

For potato, there was similarly no significant difference recorded for nematode abundance or diversity between cropping systems. Consequently the LoC values were calculated from both the GM and non-GM crop genotype datasets. The basic ecological and functional indices listed below are key indicators of the status of a soil ecosystem as per rhizospheric nematode communities and provide insight into the impact of a 'disturbance' on multiple

aspects of nematode community structure. They include the Maturity Index (20), the Plant Parasite Index, the Chanel Index, the Basal Index, the Structure Index and the Enrichment Index.

Table 3 Maize: Assessment endpoints and suggestions for limits of concern (LoC) for soil nematodes based on a comparative approach

	Measurable assessment endpoints	**Method**	**Ranges[2]: Mean (min – max)**	**LoC (<, >)**
Ecological Succession Indices				
Maturity Index	Value of the colonizer/ persister scale for free-living nematode taxa	Light microscopy for identification of nematode taxon used for calculation indexes	1.9 (1.6 – 2.2)	**1.2, 2.6**
Plant Parasite Index	Value of the colonizer/ persister scale for plant parasitic nematode taxa		2.6 (2.5 – 3.14)	**2.0, 3.8**
Food Web Analysis				
Enrichment Index	A measure of opportunistic bacterivore and fungivore nematodes that constitute the food web	Light microscopy for identification of nematode taxon used for calculation indexes	69.3 36.7 – 76.5	**29.3, 91.8**
Structure Index	Indicator of food web state affected by stress or disturbance that constitute the food web		35.2 (14.4 – 42.8)	**11.5, 51.4**
Functional Diversity				
Shannon Index	A measure of diversity based on abundance of individuals within each genus	Light microscopy for identification of nematode within each genus used for calculation indexes	2.0 (1.7 – 2.3)	**1.3, 2.7**

Table 4 Potato: Assessment endpoints and suggestions for limits of concern (LoC) for rhizospheric nematodes based on a comparative approach

	Measurable assessment endpoints	**Method**	**Ranges[2]: Mean (min – max)**	**LoC (<, >)**
Ecological Succession Indices				
Maturity Index	Quantity of free-living nematode taxa	Sequencing of the 18S rRNA gene	2.1 (1.8 – 2.3)	**1.4, 2.7**
Plant Parasite Index	Quantity of free-living, plant parasitic nematode taxa		2.4 (0.0 – 3.6)	**--, 4.3**
Enrichment Index	Quantity of nematodes from fungal /bacterial feeding species that constitute the food web	Sequencing of the 18S rRNA gene	58.5 (4.2 – 95)	**3.3, 100**
Structure Index	Quantity of nematodes from fungal /bacterial feeding species that constitute the food web		48.1 (15 – 89)	**12, 100**
Functional Diversity				
Shannon Index	A measure of diversity based on abundance of individuals within each genus	Sequencing of the 18S rRNA gene	2.3 (1.3 – 2.8)	**1.0, 3.4**

The limits of concern (LoC) reported in Tables 3 and 4 are initial assessments based on the sample sizes, methodologies and geographical locations employed to assess nematode diversity and community structures in the potato and maize rhizosphere. In regard to the maturity index, this is calculated as abundance x coefficient (coefficient is CP value 1-5) thus the MI value can range (theoretically) from 1 to 5. However, these values would be extreme and would not be reflective of natural ecosystems, where MI is typically 2.0 – 2.5 (field) and in more natural ecosystems (e.g. forest, meadow etc…) an MI = 3.5 can be recorded. In determining the LoC for maize and potato, this was therefore based on the recorded min/max values -/+ 20%. For the Plant Parasite Index, this is calculated as abundance x coefficient (coefficient is CP value 2-5), thus the PPI index typically varies between 2 and 5.

However, if abundance is <1 then it is possible for the PPI <2, as occurred with potato from the Irish study. For maize therefore, the LoC for the PPI is calculated as the recorded min/max values -/+ 20%, while for potato, the PPI LoC is only provided as the recorded max values + 20%. The Enrichment and Structure Indices are descriptors of food web condition and values range from 0 – 100. In the case of maize, LoCs were determined based on the min/max values -/+ 20%. For potato, the min-max variability recorded for both the EI and SI impacts on establishing the LoCs. While the lower LoC is calculated as the min-20%, the max value is set at 100 as this is the maximum possible value for both indices. The variability associated with the Shannon diversity index (H) was more stable in maize and potato hence was determined as min/max values -/+ 20%.

In conclusion, the maize LoCs are based on datasets taken from 5 separate locations across Europe and hence the variability captured here in the min/max values should represent a good first estimate of the level of biological variation that can be expected across different biogeographical ecosystems. In contrast, for potato the degree of variability was significantly higher. While the potato data was taken from three successive years, it is clear that for specific indices, the establishment of an LoC is challenging. As a result, this should be re-examined in the event more locations are included in future assessments for potato.

Ecosystem engineers - earthworms

Earthworms contribute to soil formation and strongly affect water availability for other organisms, including plant roots. In arable soil systems, earthworms feed on crop residues and are thus intensively exposed to the GM crop as a potential stressor. Thus they provide important ecosystem services and by their activity they affect the living conditions for many other organisms in soil (23). In contrast to the microbial groups, focal species can be clearly defined and strategies for assessing the implications of their interactions with GM plant material are different.

Following the demands of the EFSA Guidance Document on ERA of GM plants (2010), limits of concern (LoC values) were developed and finally set for life-history traits of earthworms.

In a first step, *Aporrectodea caliginosa* (endogeic) and *Lumbricus terrestris* (anecic) were selected as focal earthworm species (24); see also Deliverable 4.7. The selection process was based on an in-depth literature survey and followed the selection matrix proposed by EFSA (2010). In a second step, an ERA laboratory test system was designed for life-history traits of both focal species (*A. caliginosa* and *L. terrestris*). This test system has been successfully applied to GM maize and GM potato and documented in a test protocol (see Deliverable 4.6).

In sum, eight measurable assessment endpoints (here i.e. life-history traits) were compiled which are known to be sensitive against environmental impact. These endpoints were assigned to two subsequent generations: (1) initial generation: survival, change of biomass, cocoon production; (2) offspring: cocoon hatchability, biomass at hatching, biomass at maturity, maturation, survival (Table 5).

Table 5 Limits of concern (LoC) of measurable assessment endpoints (here i.e. life-history traits) for two subsequent generations of focal earthworm species (24); Deliverable 4.7, and ranges of GM effects detected in a case study conducted according to the protocol of an ERA test system (Deliverable 4.6). Limits and ranges refer to the relation of results for GM vs. non-GM crops.

Generation	Measurable assessment endpoints	LoC	Ranges of GM effects
Initial generation	Survival [%]	**- 50%**	-5% to +31%
	Change of biomass (final vs. initial) [mg]	**- 30%**	+3% to +128%
	Cocoon production [n ind.$^{-1}$ month^{-1}]	**- 50%**	-15% to +56%
Offspring	Cocoon hatchability [%]	**- 50%**	-6% to +17%
	Biomass at hatching [mg]	**- 30%**	-18% to +4%
	Biomass at maturity [mg]	**- 30%**	-4% to +8%
	Maturation [d]	**+ 50%**	-14% to +17%
	Survival [%]	**- 50%**	-2% to +13%

The LoC values as assessment endpoints as measured in the course of the ERA test system (**Tab. 5**, Deliverable 4.6) were set considering the natural variation of respective parameters

and according to current literature data on criteria that should be met for validity of experimental results (25). The presented limits of concern for decreases or increases are not to be understood as ranges of absolute effects, but as differences between GM compared with non-GM effects on respective life-history traits. Thus, it should be noted that detected absolute GM effects, even if significant, do not automatically reflect potential harmful impacts (see case study in Deliverable 4.6).

Results for each assessment endpoint of the GM treatment should therefore be compared to those of the non-GM control treatment. Exemplarily, **Tab. xy** shows ranges of GM effects on earthworm life-history traits. These ranges have been merged and integrated from results of a case study with both focal species (*A. caliginosa* and *L. terrestris*) exposed to GM maize and GM potato (see Deliverable 4.6). The case study has demonstrated crop residues of GM maize and GM potato to be non-hazardous for focal earthworm species with respect to the LoC values (**Tab. 5**). If differences between GM and isogenic non-GM effects are within the limits of concern, no harmful impact of GM crop on the respective non-target decomposer species is be expected. If single or various values lie outside the limits of concern, harmful effects of GM crops on the fitness of the earthworm species are possible. In that case, further studies are needed.

Finally, it is stated that the limits of concern are defined on the current state of knowledge. As previously concluded (24), region-specific conditions and a region-specific composition of an earthworm community might require the need to select an endemic species additionally, if *A. caliginosa* or *L. terrestris* are missing or less abundant. Consequently, such a species has to undergo the ERA test system for focal earthworm species (Deliverable 4.6), which might require a modification of one or more limits of concern. It is concluded that limits of concern for life-history traits of focal earthworm species deliver useful information for (1) risk assessment of GM crops on non-target organisms and (2) modelling purposes to put endpoints in a larger context of a whole agroecosystem or to extrapolate endpoints to an agricultural landscape.

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