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COLLABORATIVE PROJECT

Assessing and Monitoring The Impacts of Genetically Modified Plants on Agro-ecosystems

Deliverable 4.6 – Protocol for testing potential effects of GM crop residues (maize and potato) on earthworm species

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Summary

A laboratory test system with use for standard testing of the impacts of genetically modified (GM) maize and potato of selected European biogeographical regions (Atlantic, Boreal and Mediterranean) on earthworms as important non-target decomposers was developed as part of the AMIGA Workpackage 4. The species *Lumbricus terrestris* and *Aporrectodea caliginosa* were selected as test organisms because they meet the requirements of focal species, as defined according to the EFSA ERA guidance document (2010). Both species are of significant functional relevance in crop rotations with maize and/or potato within Irish, Swedish and Spanish AMIGA sites (see Deliverable 4.7), exemplarily representing specific conditions of respective biogeographical regions.

For risk assessment, GM effects on performance traits relevant to conclude on potential long-term effects were examined by means of microcosm feeding experiments. In different test phases, impacts on (I) survival and biomass of the initial earthworm generation; (II) the production and hatchability of cocoons; and (III) biomass, maturation and survival of offspring were analyzed. The limits of concern, set for all measurable endpoints, allow a hazard characterization of each detected adverse effect. This way the ecological relevance of effects and their risk potential can be assessed.

A test protocol supplemented by methods and results from a case study gives guidance for setting up the test system, interpreting results and conducting a proper risk assessment of detected impacts.

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1 Background

Earthworms as important decomposers are often considered to represent the "keystone" group of soil invertebrates due to their role in plant litter decay (Lavelle & Spain, 2005; Wall et al., 2012). They contribute to important soil processes, like bioturbation, the formation of organo-mineral complexes during the gut passage, the regulation of nutrient cycling processes and are highly involved in increasing soil fertility (Edwards et al., 1995; Parmelee et al., 1998). Due to their burrowing activity, consumption of leaf litter and promotion of microbial activity they play an important role in soil formation (Tomlin et al., 1995; Wall et al., 2012). Moreover, earthworms represent an important part of the diet of many vertebrates and other invertebrates (Edwards & Bohlen, 1996).

Due to these functional properties, there is a reasonable concern that earthworms may be affected by genetically modified (GM) crops. Impacts might occur via GM-induced expression of specific proteins, like the Cry1Ab protein in Bt maize. The degradation of this protein from litter material, however, is accelerated by earthworm activity (Schrader et al., 2008; Emmerling et al., 2011). Furthermore, GM crops may differ from the near-isoline in the amount of major plant components such as cellulose, lignin, fructose or soluble carbohydrates (Escher et al., 2000; Flores et al., 2005; Poerschmann et al., 2005; Saxena & Stotzky, 2001). These modifications affect nutritional parameters of plant material (Clark & Coats, 2006) and the decomposability of plant residues in soil (Flores et al., 2005; Hönemann et al., 2008; Zwahlen et al., 2007).

Thus, earthworms are, on the one hand, closely associated to GM crops and their compounds and, on the other hand, they contribute to numerous important ecosystem functions and services. According to the combination of these both issues earthworms represent appropriate non-target organisms which should be considered during environmental risk assessment (ERA) of GM crops (EFSA, 2010).

2 Objectives and approach

According to the need for consideration of earthworms during GM crop risk assessment Task 4.4 of Workpackage 4 aims at developing a laboratory test system that serves for standard testing of the impact of GM crops (here: maize and potatoes) of selected European biogeographical regions on earthworms.

To conduct earthworm laboratory studies, some publications give already instructions either in regard to experimental implementations and constraints (Fründ et al., 2010) or about culture requirements and handling of different species (Lofs-Holmin, 1982; Lowe & Butt, 2005). To ensure functioning of the test system for GM risk assessment, applied laboratory conditions (e.g. concerning temperature, substrate, moisture or food supply) and used equipment were chosen according to the respective recommendations.

2.1 Focal species approach

Previous risk analyses of GM crops on earthworms mainly relied on common laboratory species, usually *Eisenia fetida* (e.g. Ahl Goy et al., 1995; Clark & Coats, 2006; Liu et al., 2009). However, as these species are often irrelevant in arable field soils, they may only be of limited value for risk assessment. The present test system, by contrast, bases on species with high potential exposure linked to a significant functional importance in soils of a specific bio-geographical region under cultivation of a respective crop (focal species (EFSA, 2010)). This focal species approach avoids that laboratory tests might be classified as "not ecologically realistic" due to the selection of non-target species which may not represent the most sensitive examples from the functional group in the regional food web, an often critical point in previous risk assessment studies (Lövei & Arpaia, 2005). The present test system, therefore, meets the requirements emphasized in the EFSA ERA guidance document (EFSA, 2010).

2.2 Risk assessment based on performance traits

Tests designed to assess acute toxicity over short-term exposure may not predict effects of chronic exposure, like sublethal direct or indirect effects on non-target species over several generations (Birch et al., 2007). To meet this need for risk assessment under chronic exposure conditions, the present test system includes growth, reproduction and survival of focal earthworm species as main components of their fitness and relevant performance

traits, thus allowing to conclude on potential long-term effects and changes in ecological functions (Pey et al., 2014; Violle et al., 2007). GM crop risk assessment, as suggested here, therefore bases on (I) survival and biomass of initial generations, (II) cocoon production and hatchability, and (III) biomass, maturation and survival of offspring as measurable endpoints.

2.3 Selection of focal test species

By means of a literature survey potential focal earthworm species which (1) are of high functional relevance in soil systems at different European AMIGA sites (Ireland, Sweden and Spain) belonging to three biogeographical regions (Atlantic, Boreal and Mediterranean), and (2) fulfill the requirements along with the development of the test system based on performance traits were identified. According to the availability of species, their suitability to testing under laboratory conditions and the sensitivity of different performance traits against GM plant residues, the species *Aporrectodea caliginosa* (endogeic secondary decomposer) and *Lumbricus terrestris* (anecic primary decomposer) were finally selected (see AMIGA Deliverable 4.7). Conditions within the present test system were, therefore, adapted to the respective demands of these both species (Lowe & Butt, 2005), which are generally widespread and very common in arable soils (Lee, 1985).

3. Test protocol

3.1 Experimental equipment

Earthworm test species

For each test procedure, 40 healthy adult specimens of good physiological condition (high turgidity, regular body shape, no constrictions or injuries, high mobility (Fründ et al., 2010)) and with well-developed clitellum of either *L. terrestris* (Fig. 1a) or *A. caliginosa* (Fig. 1b) are required.

Earthworms can be field collected, laboratory bred or purchased from a commercial supplier. Concerning the origin of test specimens, collection site, collection method and season for field collected organisms, or the source of commercial supply should be recorded, as these factors have been reported to potentially influence earthworm productivity (Lowe & Butt, 2007; Fründ et al., 2010).

Prior to starting the test run, earthworms need to be adapted to laboratory conditions and the test substrate at 15°C and permanent darkness for at least 7 days (Fründ et al., 2010) to ensure a complete gut content exchange (Pokarzhevskii et al., 2000). During this period only non-GM control plant material (about 1 g) should be used as food source.

Whenever earthworms are to be transferred, weighed or their physiological state determined, they need to be put into cold tap water to remove adherent material (mucus, adhesive residues and soil) and afterwards carefully dried on a cellulose tissue (Fründ et al., 2010). This method, which shall be used during the whole test system, ensures that earthworm biomasses are generally analysed at optimum turgidity including the gut content.

Fig 1: The focal earthworm test species *L. terrestris* (a.) and *A. caliginosa* (b.) on the test substrate with maize leaf material as food source.

Test substrate

To ensure that the test conditions are as close as possible to natural field conditions, a soil commonly present in arable fields and widely distributed in the respective biogeographical region of interest (Atlantic, Boreal or Mediterranean) shall be used as test substrate. To meet the demands of the earthworm species, a loamy soil (Butt, 2011) with a pH value between 4.5 and 7 should be selected (Lowe & Butt, 2005). Prior to the start of the test, the soil should be air dried, macroscopically cleared of plant residues and stones and then sieved using a mesh size of 2 mm. To avoid confounding factors due to present soil fauna (Fründ et al., 2010), the test soil must be defaunated (Lowe & Butt, 2005) by a three times repeated freezing (-20°C, 24h) and thawing (room temperature, 24h) cycle, which significantly reduces the number of microarthropods and annelids (Huhta et al., 1989). Finally, the soil should be adjusted to moist conditions, depending on soil texture comprising a range of about >14-30 % wet soil mass (Lowe & Butt, 2005). According to Lowe & Butt (2005) loamy soils with moisture contents of about 25% and a pH of 6-7 are most qualified to culture numerous earthworm species. If necessary, the soil can be stored at 4°C until further application. During incubation in microcosms, the soil properties are inevetibly to some extent changed compared to those found in the field, and therefore, detailed knowledge and description of test substrate with regard to soil type, land use, soil horizon, texture, water holding capacity (WHC), pH and carbon and nitrogen content are essential (Fründ et al., 2010).

Food – Plant material

Maize leaves or potato tuber material from Atlantic, Boreal or Mediterranean regions may be used as food sources during the present test system. As described by Vercesi et al. (2006) and Emmerling et al. (2012), GM crop material shall, thereby, be tested against material of the respective non-GM near-isogenic cultivar, representing the control treatment. Plant material should be harvested, washed and air-dried. To feed adult *L. terrestris*, potato tubers should be chopped to approximately 0.5 cm x 1 cm large fragments (Emmerling et al., 2012), maize leaves shall be cut to a length of about 1 cm (Lowe & Butt, 2005). As a diet for adult *A. caliginosa* plant material of both crops should be cut to fragments nearly half the size as compared to those for *L. terrestris* or coarsely crushed. To feed hatchlings and juveniles of both species maize leaves and potato tubers must be ground to about 1-2 mm, ideally using a rotary mill. Food material is recommended to be surface applied for the species *L.*

terrestris, but mixed into the soil for *A. caliginosa* (Lowe & Butt, 2005). After adding food material to the test substrate in the microcosms, it should carefully be moistened by spraying with tap water.

Experimental vessels

Microcosms

According to the different body sizes and burrowing activities of anecic *L. terrestris* (vertical burrows) and endogeic *A. caliginosa* (horizontal burrows) specimens, microcosms of different size and height should ideally be used for both species (Figs. 2a and b). Whereas *L. terrestris* requires a soil depth of approximately 10 cm for completion of full life cycle (Butt et al., 1994), for *A. caliginosa* a soil depth of about 5 cm is sufficient (Lowe & Butt, 2005).
Typically stackable plastic vessels of at least 1-2 L volume with sealable lids to prevent exceed loss of soil moisture and escaping of test specimens have been found suitable in practice (Lowe & Butt, 2005; Emmerling et al., 2012). Smaller test vessels should not be used as they could reduce growth rates of both earthworm species (Eriksen-Hamel & Whalen, 2007). Because the experiments are conducted under permanent darkness (Lowe & Butt, 2005), vessels with opaque sides are not necessarily required.

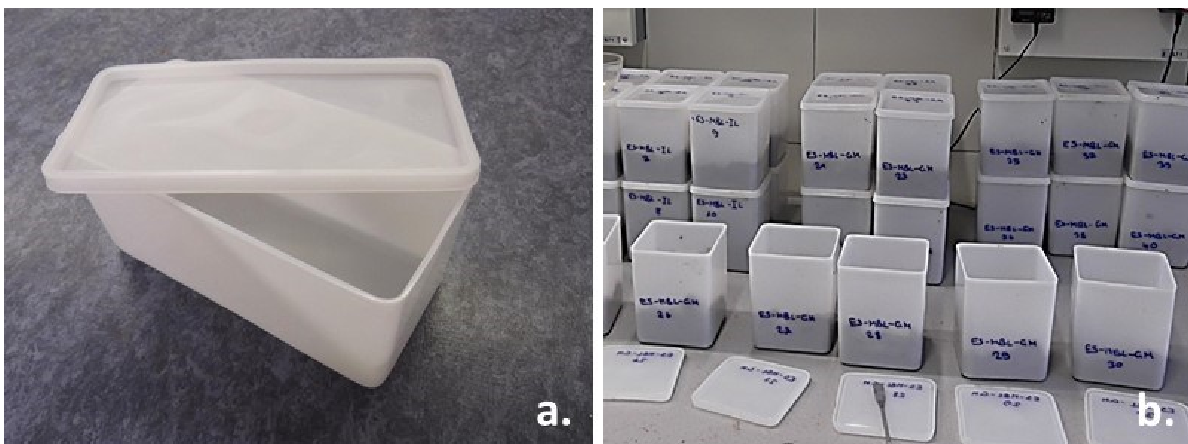

Fig 2: Microcosms (1-2 L) suitable for the focal earthworm test species *L. terrestris* (a.) and *A. caliginosa* (b.).

For culturing hatchlings, smaller test vessels ensuring the capacity for the required amount of 200 g of substrate can be used (volumes of about 0.4 L are suitable). Vessels must be tightly sealable, as hatchlings, due to their small body sizes may otherwise easily escape.

Petri dishes for cocoon incubation

It is recommended to incubate cocoons in small petri dishes (5 x 1.2 cm, diameter x height) placed in larger ones (9 x 2 cm, diameter x height), to inhibit escaping of the hatchlings (Fig. 3b). To prevent cocoon dehydration, which can retard embryonic development (Holmstrup, 1994), each dish must contain a filter paper and be maximum half filled with distilled water (Butt, 1993) (Fig. 3a). Filter papers, which can, if necessary, temporarily also serve as an inert food source, should be rehydrated as required (Lowe & Butt, 2005). As microbial contamination can be a problem during cocoon incubation (Lowe & Butt, 2005), filter papers should frequently be replaced (Holmstrup et al., 1991), and the use of tap water to moisten cocoons and filter papers should be avoided (Lowe & Butt, 2005).

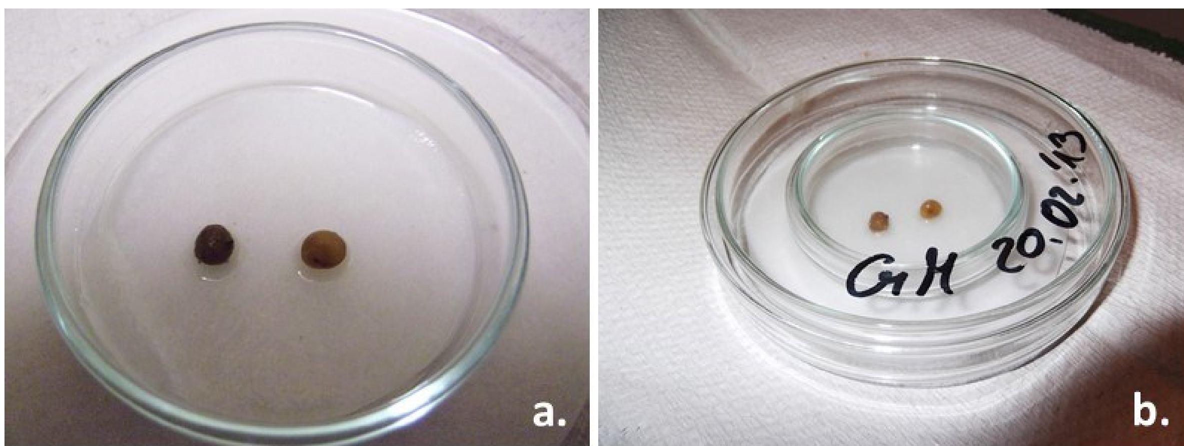

Fig 3: Cocoon incubation on moistened filter papers (a.) in two nested petri dishes (b.).

3.2 Experimental design and procedure

The test system comprises three test phases of GM effects on:

(I) survival and biomass of initial earthworm generations
(II) cocoon production and hatchability
(III) biomass at hatching and at maturity, maturation period and survival of offspring

All three test phases (I-III) require a climate chamber to ensure permanent darkness and a temperature of 15°C (±1°C), representing optimal conditions for culturing adult earthworms, cocoon production and maturation of hatchlings of both focal species (Lofs-Holmin, 1982; Butt, 1993; Lowe & Butt, 2005; Vercesi et al., 2006).

Test phase I - Survival and biomass of initial earthworm generations

Test stage "Single 1"

Forty microcosms (n = 20 for each treatment (GM and non-GM)) are to be prepared according to the indications given in the first row (Test stage "Single 1") of Table 1 for the species *L. terrestris* or of Table 2 for the species *A. caliginosa*. For that purpose the respective amount (700 or 400 g wet weight) of the appropriately moistened test substrate should be filled into each microcosm.

After that, 40 mature individuals of the selected species should be watered, dried on cellulose tissue, and their initial biomasses determined by weighing (minimum required precision: 0.001 g). These "starting weights" represent the references to which any biomass changes arising during testing refer (Fründ et al., 2010). Test individuals need to be unambiguously termed (for instance by consecutive numbering) for identification during further procedure. Finally, one earthworm should be introduced into each of the microcosms by placing individuals on the soil surface and rewetting by spraying with tap water. The allocation of specimens to both treatments should be randomly. However, care should be taken that the average initial fresh weight within treatment groups is reasonably balanced.

When all earthworms have entered the soil, the appropriate amount of respective food material shall be added either by surface application (*L. terrestris*) or by mixing into the soil (*A. caliginosa*) (Lowe & Butt, 2005). One set of 20 microcosms can then be applied with GM plant material, the other one with non-GM isogenic material, representing the control treatment. After application, the plant material should slightly be moistened by spraying with tap water.

The microcosms should clearly be marked to ensure a reliable identification of treatment and containing individual (e.g. non-GM 1-20; GM 21-40). Vessels of both treatments should be randomly placed in a climate chamber at permanent darkness and 15°C. The duration of this first test stage comprises 10 days. During incubation, microcosms without ventilation holes need to be opened once a week for aeration. To compensate for potential water losses, the substrate could be remoistened by spraying if necessary.

Table 1: Test stages, individuals per microcosms [n], amount of substrate (wet weight) [g], amount of food (either potato tuber or maize leaves) [g], time [d] and procedure after completion of respective test stages for the species *Lumbricus terrestris*.

Test stage	Individuals [n]	Substrate [g]	Food 1 Potato tuber [g]	Food 2 Maize leaves [g]	Time [d]	Procedure after completion
Single 1	1	700	3	2	10	Biomass determination
Mating	2	1000	8	5	14	Biomass determination Cocoon extraction
Single 2.1	1	700	6	4	21	Biomass determination Cocoon extraction
Single 2.2	1	700	6	4	21	Biomass determination Cocoon extraction
Single 2.3	1	700	6	4	21	Biomass determination Cocoon extraction
Single 2.4	1	700	6	4	21	Biomass determination Cocoon extraction

Table 2: Test stages, individuals per microcosms [n], amount of substrate (wet weight) [g], amount of food (either potato tuber or maize leaves) [g], time [d] and procedure after completion of respective test stages for the species *Aporrectodea caliginosa*.

Test stage	Individuals [n]	Substrate [g]	Food 1 Potato tuber [g]	Food 2 Maize leaves [g]	Time [d]	Procedure after completion
Single 1	1	400	2	1	10	Biomass determination
Mating	2	500	5	3	14	Biomass determination Cocoon extraction
Single 2.1	1	400	4	2	21	Biomass determination Cocoon extraction
Single 2.2	1	400	4	2	21	Biomass determination Cocoon extraction
Single 2.3	1	400	4	2	21	Biomass determination Cocoon extraction
Single 2.4	1	400	4	2	21	Biomass determination Cocoon extraction

At the end of the 10 days interval, earthworms should be removed from the microcosms, weighed and examined for their physiological condition based on hydration, body shape and mobility. Any remarkable or abnormal features (as dehydration, injuries, resting stages or dead) must be recorded.

Finally, soil samples of about 20-30 g should be taken from at least 3 microcosms of each treatment. These samples should be weighed (precision: 0.01 g), dried at 115°C overnight, and then again weighed to determine the soil moisture at the end of this test stage and the water loss throughout the 10 days period. Remaining substrate from microcosms can be discarded. Viable test individuals should immediately be introduced into microcosms of the following test stage "Mating".

Test stage "Mating"

Twenty microcosms (n = 10 for each treatment) should be prepared according to the instructions given in the second row of Table 1 (*L. terrestris*) or 2 (*A. caliginosa*) and following the procedure described for the preceding test stage.

The 20 test individuals of each treatment (GM and non-GM) should be assigned to 10 pairs per treatment, and then introduced into one microcosm each. The pairwise allocation shall, thereby, allow the identification of individuals at the end of this test stage. Weights of test individuals, usually, differ sufficiently, so that this could be done by means of biomass differences. If this is not the case, earthworms could additionally be tagged, for instance by use of viable implant elastomer as described by Butt & Lowe (2007).

Subsequent to the addition and rewetting of food material, microcosms need to be labelled and randomly placed in a climate chamber at permanent darkness and 15°C for two weeks. Aeration and remoistening shall be conducted as described before.

At completion of this period, earthworms should be removed from microcosms. Both individuals of one pair must be identified by either biomass differences or, if applicable, by tagging technique (e.g. Butt & Lowe, 2007). Biomass and physiological condition of each worm need to be recorded. The moisture content of the substrates from both treatments should be determined according to the instructions given before (see test stage "Single 1"). During this procedure, particular caution is necessary at this stage because cocoons present in the substrate must not be destroyed.

As a last step, earthworms are to be inserted into microcosms of the test stage “Single 2.1” (see below). The cocoons should be extracted from the substrate of each microcosm by use of a wet sieve method as described in “Test phase II”.

Test stage “Single 2.1 – 2.4”

Each test individual should be introduced into one of forty microcosms (n = 20 for each treatment), prepared according to the indications given in the third row (Test stage “Single 2.1”) of Table 1 (*L. terrestris*) or Table 2 (*A. caliginosa*). After adding and remoistening of food material, microcosms should be incubated at 15°C and permanent darkness for three weeks.

At the end of this period earthworms should be removed, watered, weight and their state of health determined as already described. The moisture content of substrate from both treatments shall be analysed (see above) and the cocoons should be extracted as described in “Test phase II”.

This test stage must be repeated four times (Test stages “Single 2.1” – “Single 2.4”), to ensure a maximum yield of cocoons. Upon completion of test stage “Single 2.4”, the microcosm experiments can be stopped, because no more cocoons are expected.

At this point, survival rates of test individuals can be determined for both treatments (**Endpoint: “Survival of initial generation”**).

Finally, the final biomass of test specimens should be determined. Based on initial and final biomass the weight change of each adult earthworm throughout the experimental runtime, as a measure of experimental and nutritional conditions, should be calculated (**Endpoint: “Change of biomass”**) (Fründ et al., 2010).

Test phase II – Cocoon production and hatchability

A wet sieving method is proposed to be used for the extraction of cocoons (Figs. 4a and b) from the test substrate. For that purpose, the entire soil material from one microcosm shall be placed on a test sieve (2 mm mesh size, suitable dimensions: e.g. 30 x 6.5 cm, diameter x height) which should then be transferred into a plastic tub. By means of a common water tube and low water pressure, the tub should be filled with water until the bottom of the sieve is covered, while destroying big soil aggregates by rinsing. Subsequently, the sieve can

be moved in the water until all aggregates are completely dissolved. This method ensures that the cocoons remain undamaged. Alternatively, a cylindrical sieve placed on a horizontal shaker (12 min at 100 rpm (Emmerling et al., 2012)) can be used as well.

Upon completion of the extraction procedure, the number of cocoons for each pair/individual must be recorded, and the amount per individual and month determined (**Endpoint: "Cocoon production"**).

This cocoon extraction process should be repeated five times, in total, as, beginning with the "Mating stage", it is to be conducted subsequent to each following test stage.

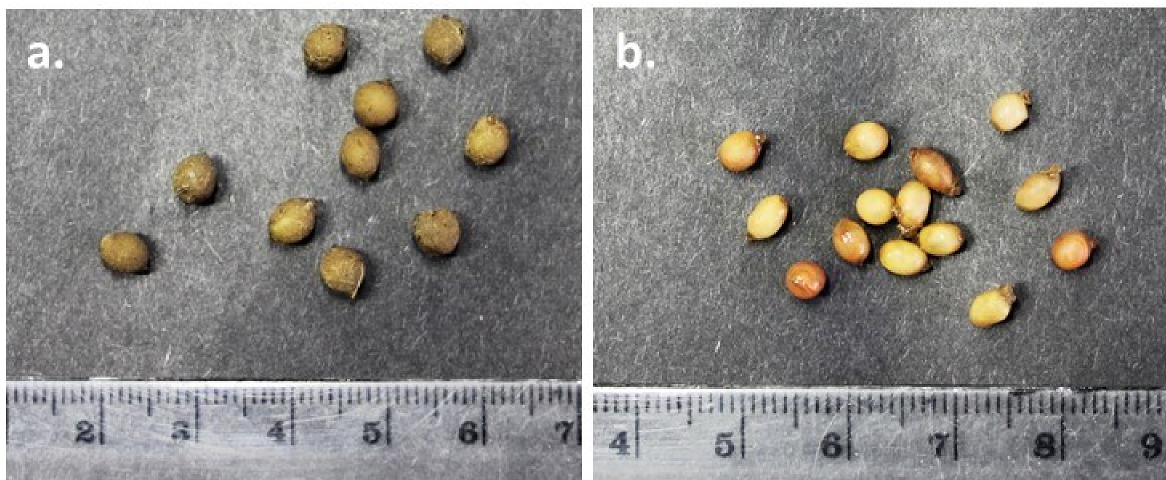

Fig 4: Cocoons of the focal test species *L. terrestris* (a.) and *A. caliginosa* (b.).

For cocoon incubation, the cocoons of one pair (test stage "Mating") or individual (test stages "Single 2.1-2.4") extracted at the same date should be transferred on one wet filter paper placed in a small petri dish immediately after sieving (Butt, 1991). It is recommended to place this small dish in a larger one to prevent escaping of earthworms and dehydration of cocoons (see above). Dishes should be marked according to treatment and respective pairs/individuals and then be placed in a climate chamber at 15°C and permanent darkness.

The petri dishes must be incubated for a total of 230 days for the species *L. terrestris* (mean incubation period: 135.3 ± 13.1 d; maximum incubation period: 231 d; minimum incubation period: 60 d until hatching) and of 110 days for the species *A. caliginosa* (mean incubation period: 51.4 ± 1.3 d; maximum incubation period: 110 d; minimum incubation period: 17 d until hatching). They should be controlled once a week for moisture content and bacterial contamination. Filter papers should be remoistened with distilled water and replaced if needed (see above).

The cocoons should be examined on a daily basis, starting, at the latest, with day 50 of *L. terrestris*, or day 15 of *A. caliginosa* cocoon incubation.

As earthworm cocoons are known to occasionally contain more than one individual (Butt, 1993), the number of hatchlings (Figs. 5a and b) emerging from each cocoon has to be recorded. Hatchlings need to be removed from the petri dishes (see test phase III) as soon as possible to avoid dehydration or bacterial infection. If ascertainable, empty cocoons should be removed from filter papers as well.

Unhatched cocoons can be discarded after 110 (*A. caliginosa*) or 230 (*L. terrestris*) days of incubation, as no more hatchlings are expected.

Upon completion of this test phase, the percentage of hatched cocoons (**Endpoint: "Cocoon hatchability"**) should be determined for both treatments (GM and non-GM).

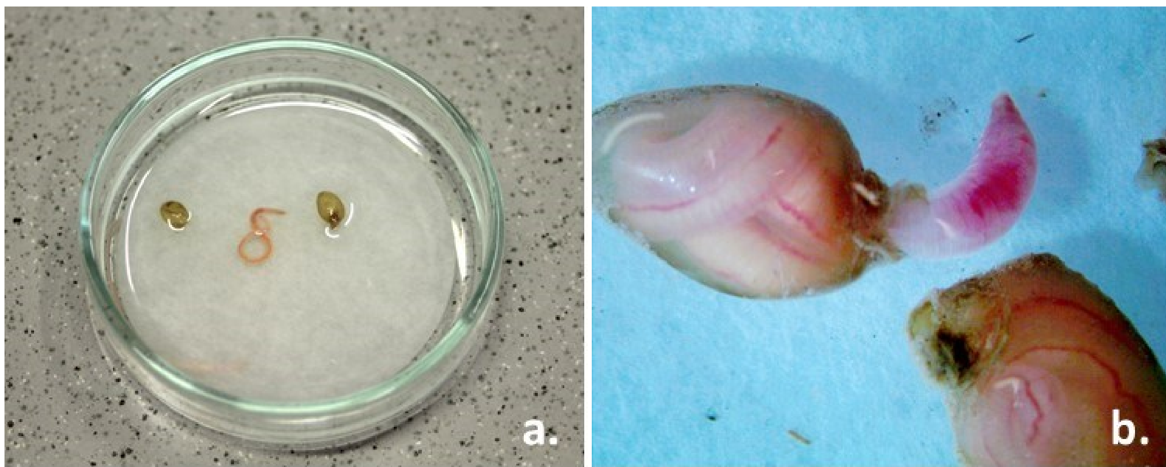

Fig 5: Hatchlings of *L. terrestris* (a.) and *A. caliginosa* (b.)

Test phase III – Biomass, maturation and survival of offspring

Generally, care must be taken when handling hatchlings as injuries or tail shedding (autotomy), which might reduce chances for a successful development of offspring, should be avoided.

Hatchlings can be removed from filter papers by using an angular dissecting needle or plastic forceps. According to the treatment of adult individuals, as a first step, juveniles must to be watered in cold tap water to remove adherent material. After some minutes, they need to be dried on cellulose tissues, weighed (**Endpoint: "Biomass at hatching")** and their physiological status can be determined. Each hatchling should, then, be transferred into a

closable plastic vessel (e.g. 10 x 5 cm, diameter x height) containing about 200 g of the same substrate as used for reproduction. After hatchlings have entered the soil, the respective food materials can be added (see below). For maturation juveniles need to be incubated in a climate chamber at 15°C and permanent darkness.

Offspring should be fed biweekly with finely ground plant material. With increasing earthworm biomass, food supply must be enhanced from 0.2 g for newly hatched *L. terrestris* and 0.15 g for newly hatched *A. caliginosa* up to 1 g for subadult specimens of both species. Juveniles should be weighed and examined for their physiological status in 2 week intervals. Any remarkable or abnormal features (as dehydration, injuries, resting stages or dead) should be recorded.

To ensure optimal conditions allowing unlimited growth, the substrate should be replaced regularly (Lofs-Holmin, 1982), at the latest when casts cover half of the soil surface. The frequency, thereby, depends on the size of the worms (Lofs-Holmin, 1982) and can individually be estimated.

The first individuals are expected to reach maturity after 50 days when using *A. caliginosa* or after 90 days with *L. terrestris*. From this date onward, respective worms should weekly be examined for their developmental stage. Earthworms can be classified as mature if tubercula pubertatis and clitellum are well developed and visible. At this stage, the biomass of each specimen (**Endpoint: "Biomass at maturity"**) and the duration of its development from hatching until maturity (**Endpoint: "Maturation"**) can be determined.

This final test phase is completed when the last specimen reaches maturity. According to own culturing experiences, mean durations of maturation periods comprise about 200 days for *L. terrestris* and about 100 days for *A. caliginosa*.

Finally, the survival rate of juveniles (**Endpoint: "Survival of offspring"**) should be determined for both treatments (GM and non-GM).

Risk assessment - Measurable endpoints and limits of concern

The present test system aims at analysing potential long term GM effects on soil processes and soil fertility by means of performance traits (reproduction, development and survival) of the earthworm species *L. terrestris* and *A. caliginosa* as focal non-target decomposers. GM crop risk assessment, thereby, bases on eight measurable assessment endpoints as listed in Table 3.

Table 3: Measurable endpoints of performance traits of both earthworm generations considered in the test system and respective limits of concern used for GM crop risk assessment.

Generation	**Test phase**	**Measurable endpoint**	**Limits of concern GM vs. non-GM**
Initial generation	I	Survival [%]	50% decrease
	I	Change of biomass (final vs. initial) [mg]	30% decrease
	II	Cocoon production [n $ind.^{-1}$ $month^{-1}$]	50% decrease
Offspring	II	Cocoon hatchability [%]	50% decrease
	III	Biomass at hatching [mg]	30% decrease
	III	Biomass at maturity [mg]	30% decrease
	III	Maturation [d]	50% increase
	III	Survival [%]	50% decrease

As it is usually impossible to scientifically assess limits of safety with any degree of certainty, risk assessment is carried out on the basis of limits beyond which there is a shared concern that ecological safety is at stake (here with regard to earthworm driven soil processes which influence soil fertility and ecosystem services). These “limits of concern” are, according to the EFSA Guidance document (2010), defined as the minimum ecological effects that are deemed biologically relevant and of sufficient magnitude to cause harm. Thus, if important ecological processes lie outside these limits, management should act to reverse harmful changes to the system. The limits of concern, therefore, indicate respective threshold values and provide the basis for interpretation of results.

The limits of concern of assessment endpoints measured in the course of the present test system (Tab. 3) 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 (Fründ et a al., 2010). Given limits for decreases or increases are, thereby, not to be understood as ranges of absolute effects, but as differences between GM as compared to non-GM effects on respective performance traits. Thus, it should be noted that detected absolute GM-effects, even if significant, do not automatically reflect potential harmful impacts (see case study).

Upon completion of the test system, results for each assessment endpoint of the GM treatment should be compared to the respective ones of the non-GM control treatment. 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 would be expected. If single or various values lie outside the limits of concern, harmful effects of GM crops on the fitness of the used earthworm species are possible. In that case, further studies are needed.

4. Case study - maize and potato

4.1 Material and methods

In a case study, the test system was applied to analyse impacts of insect resistant genetically modified Bt corn MON 810 and a fungal (phytophtora) resistant cisgenic potato grown in three biogeographically regions (Atlantic, Boreal and Mediterranean) in 2012 and 2013. Maize plants were cultivated on experimental field sites near Madrid in central Spain (Institute: INIA; cultivar: DKC6451YG (GM), DKC6450 (near-isogenic non-GM)) and near Lund in southern Sweden (Institute: Lund University; cultivar: DKC4442YG (GM), DK440 (near-isogenic non-GM)). Potato plants were cultivated on an arable field near Carlow in southeast Ireland (Institute: Teagasc; cultivar: Desiree + Rpi Vnt1 (GM), Desiree (near-isogenic non-GM)). Plant material (maize leaves and potato tuber material) was harvested, air dried and processed as described in the test protocol.

According to the occurrence of both focal species (see deliverable 4.7) test runs with plant material from each biogeographical region were conducted with the species *A. caliginosa*. The species *L. terrestris* was used to analyse impacts of Bt maize from Sweden and Irish potato tuber material.

Earthworms used during the present study were either purchased from a commercial supplier (*L. terrestris* bought at www.superwurm.de) or field collected (*A. caliginosa*). *A. caliginosa* specimens were collected by hand-sorting from 7 arable field sites (two in southern Lower Saxony (Gehrden and Burgstemmen) and 5 in Thuringia (Erfurt, Niederdorla, Pahren, Dornburg and Hessberg/Veilsdorf) cultivated with maize or *Silphium perfoliatum*.

As test soil a Luvisol (fertile, widespread soil in temperate regions (IUSS Working Group WRB, 2007)) derived from loess as parent material with pH 7.2; C_{org}: 1.25 %; N_{total}: 0.13 % and a relative water holding capacity (WHC) of 58.7 % was used. The soil was taken from the Ap horizon of an agricultural field site located near Hildesheim, Northern Germany. The texture comprised 13.9 % clay; 80 % silt and 6.1 % sand resulting in a clayey silt. The soil was defaunated and sieved as described above and moistened to about 23 % w/w.

Plastic vessels of 1 L (*A. caliginosa*) and 1.5 L (*L. terrestris*) volume (dimensions: *L. terrestris*: 20 x 9.5 x 9 cm, lengths x width x height; *A. caliginosa*: 9.5 x 9.5 x 12.5 cm, lengths x width x height) were used as microcosms. For test phase I, microcosms were prepared according the indications given in Tables 4 (*L. terrestris*) and 5 (*A. caliginosa*). All test procedures were conducted as described in the protocol.

Table 4: Test stages, individuals per microcosms [n], amount of substrate (wet weight) [g], amount of food (either potato tuber or maize leaves) [g], time [d] and procedure after completion of respective test stages for the species *Lumbricus terrestris*.

Test stage	Individuals [n]	Substrate [g]	Food 1 Potato tuber [g]	Food 2 Maize leaves [g]	Time [d]	Procedure after completion
Single 1	1	700	3	2	10	Biomass determination
Mating	2	1000	6	5	14	Biomass determination Cocoon extraction
Single 2.1	1	700	3	2	21	Biomass determination Cocoon extraction
Single 2.2	1	700	3	2	21	Biomass determination Cocoon extraction
Single 2.3	1	700	3	2	21	Biomass determination Cocoon extraction
Single 2.4	1	700	3	2	21	Biomass determination Cocoon extraction

Table 5: Test stages, individuals per microcosms [n], amount of substrate (wet weight) [g], amount of food (either potato tuber or maize leaves) [g], time [d] and procedure after completion of respective test stages for the species *Aporrectodea caliginosa*.

Test stage	Individuals [n]	Substrate [g]	Food 1 Potato tuber [g]	Food 2 Maize leaves [g]	Time [d]	Procedure after completion
Single 1	1	400	2	1	10	Biomass determination
Mating	2	500	4	2	14	Biomass determination Cocoon extraction
Single 2.1	1	400	2	1	21	Biomass determination Cocoon extraction
Single 2.2	1	400	2	1	21	Biomass determination Cocoon extraction
Single 2.3	1	400	2	1	21	Biomass determination Cocoon extraction
Single 2.4	1	400	2	1	21	Biomass determination Cocoon extraction

Statistical analyses

All results are presented as means (± S.E.).

A Kolmogorov-Smirnov test was performed to analyse whether data were normally distributed. If data were normally distributed, an ANOVA was performed and a Bonferroni post-hoc test implemented for pairwise comparison. Non-normally distributed data were analysed using a non-parametric Kruskal-Wallis H-test.

4.2 Results and discussion

Test phase I - Survival and biomass of initial earthworm generations

Survival of initial test individuals differed between species with *A. caliginosa* representing 100% survival in all treatments under each food source (Tab. 7). *L. terrestris*, by contrast, showed lower survival rates as compared to *A. caliginosa* and higher survival rates when fed on potato tuber compared to maize leaf material (Tab. 6). According to several previous findings (Saxena & Stotzky, 2001; Zwahlen et al., 2003; Vercesi et al., 2006; Schrader et al., 2008), no impact of genetic modification of plant material on survival rates of initial earthworms could be detected.

The biomass of initial specimens generally increased in the course of the test system, with individuals fed on GM crop material reflecting a higher weight increase as compared to those fed on control plant material (Tabs. 6 and 7). *L. terrestris* fed on potato tuber represented the only exception, as biomasses of this species decreased during the course of the experiment (Tab. 6). However, this effect did not result from unsuitable laboratory conditions or lower food qualities, but was due to insufficient food supply, as necessary quantities of plant material were not available during this year (2012). Generally, no adverse effect of GM crop material on biomass and growth of both focal species could be detected. The results, therefore, affirm the findings of Vercesi et al. (2006), Schrader et al. (2008), Zeilinger et al. (2010) and Emmerling et al. (2012), but contradict those of Zwahlen et al. (2003), who found adult *L. terrestris* to grow slower when fed on Bt compared to non-Bt maize.

Test phase II - Cocoon production and hatchability

The cocoon production of test individuals ranged between 0.5 cocoons ind^{-1} m^{-1} (*L terrestris* fed on non-GM potato tuber) and 8.6 cocoons ind^{-1} m^{-1} (*A. caliginosa* fed on GM potato

tuber) and differed between crops and biogeographical regions depending on species (Tabs. 6 and 7). The only significant effect of genetic modification of food was detected for the species *L. terrestris* fed on maize leaves, which reflected an about 55% increased cocoon production (p = 0.035) when fed on GM compared to non-GM plant material (Tab. 6). According to the findings of Vercesi et al. (2006), the cocoon production of the species *A. caliginosa* did not reflect any impact of the GM treatment.

The cocoon hatchability ranged within the bounds of literature data (Emmerling et al., 2012; Vercesi et al.; 2006) and differed between species, with *A. caliginosa* showing a higher hatching success (60-85%) as compared to *L. terrestris* (53-58%). With the exception of the species *L. terrestris* fed on (insufficient amounts of) potato tuber, the hatchability was generally slightly higher under GM compared to non-GM conditions. However, this effect was not significant. Whereas the hatchability of *L. terrestris* did not differ depending on plant material (Tab. 6), hatching success of *A. caliginosa* increased from Swedish via Spanish maize to Irish potato used as food source (Tab. 7).

Table 6: GM effects on performance traits of the focal species *L. terrestris* fed with maize material from Sweden (Boreal region) or Spain (Mediterranean region) or potato tuber from Ireland (Atlantic region).

■ Non-GM ■ GM * p < 0,05	*Lumbricus terrestris*			
Initial generation	**Maize (Sweden)**		**Potato (Ireland)**	
I Survival [%]	65.0	85.0	100.0	95.0
I Change of biomass [mg]	+ 651.0 ± 134.7	+ 923.4 ± 126.5	- 989.5 ± 152.4	- 815.8 ± 167.9
II Cocoon production [n ind.$^{-1}$ m^{-1}]	4.6 ± 0.8	7.2 ± 0.7*	0.5 ± 0.1	0.6 ± 0.2
Offspring				
II Cocoon hatchability [%]	56.2	57.8	56.3	52.6
III Biomass at hatching [mg]	42.1 ± 0.4	39.9 ± 0.4*	52.5 ± 2.1	43.0 ± 2.5*
III Biomass at maturity [mg]	2618.2 ± 57.9	2650.4 ± 49.0	3120.6 ± 111.5	3384.9 ± 97.8
III Maturation [d]	213.1 ± 5.0	183.4 ± 2.8*	163.9 ± 10.1	191.6 ± 6.5*
III Survival [%]	100.0	97.8	88.9	100.0

I-III: Test phases

Table 7: GM effects on performance traits of the focal species *A. caliginosa* fed with maize material from Sweden (Boreal region) or Spain (Mediterranean region) or potato tuber from Ireland (Atlantic region).

Non-GM (black values) / GM (red values); * $p < 0,05$

Aporrectodea caliginosa	Maize (Sweden) Non-GM	Maize (Sweden) GM	Maize (Spain) Non-GM	Maize (Spain) GM	Potato (Ireland) Non-GM	Potato (Ireland) GM
Initial generation						
I Survival [%]	100.0	100.0	100.0	100.0	100.0	100.0
I Change of biomass [mg]	+ 452.8 ± 87.0	+ 471.2 ± 82.3	+ 950.1 ± 82.1	+ 979.9 ± 72.7	+ 230.7 ± 108.5	+ 525.4 ± 120.2
II Cocoon production [n ind.$^{-1}$ m^{-1}]	2.8 ± 0.7	3.4 ± 0.8	5.9 ± 0.8	5.0 ± 0.8	8.6 ± 0.7	8.6 ± 0.5
Offspring						
II Cocoon hatchability [%]	60.2	69.6	67.8	71.8	72.3	84.8
III Biomass at hatching [mg]	14.5 ± 0.6	14.8 ± 0.5	16.4 ± 0.4	17.2 ± 0.4	15.7 ± 0.2	16.2 ± 0.2
III Biomass at maturity [mg]	761.8 ± 26.0	730.4 ± 23.3	682.4 ± 16.7	725.9 ± 22.5	593.5 ± 13.1	571.4 ± 9.8
III Maturation [d]	91.5 ± 2.3	89.6 ± 2.0	88.8 ± 1.6	92.7 ± 1.9	102.0 ± 2.4	93.7 ± 2.0*
III Survival [%]	100.0	100.0	98.6	98.3	96.9	96.6

I-III: Test phases

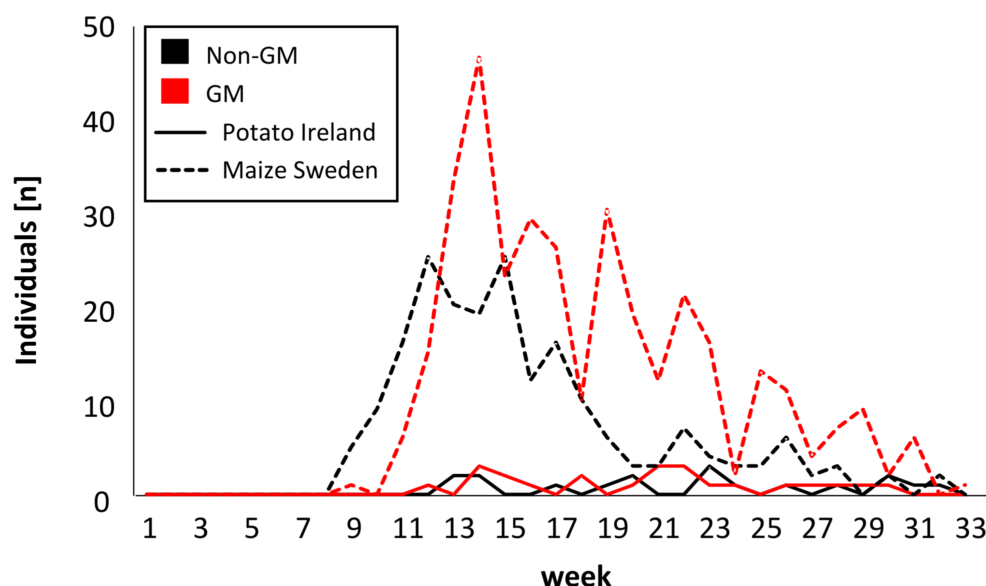

Figure 6: Hatching process of the species *L. terrestris* when fed with GM vs. non-GM material of maize leaves from Sweden and potato tuber from Ireland.

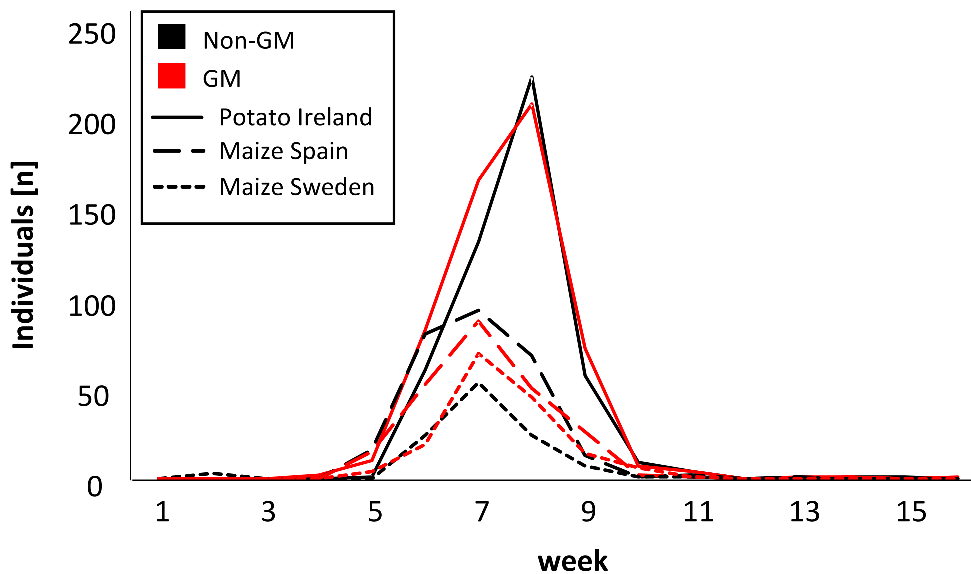

Figure 7: Hatching process of the species *A. caliginosa* when fed with GM vs. non-GM material of maize leaves from Sweden and Spain and potato tuber from Ireland.

Concerning the hatching progress of focal species, the results of the case study indicate a highly variable progress for the species *L. terrestris* with juveniles being hatched between 7 and 33 weeks after cocoon production (Fig. 6). Offspring of *A. caliginosa*, by contrast, generally hatched between week 5 and 10 after cocoon production, with highest hatching rates at week 7 (Fig. 7). No effect of genetic modification of food material on the hatching progress of both focal species could be detected.

Test phase III - Biomass, maturation and survival of offspring

The species *A. caliginosa* showed biomasses at hatching of between 14.5 and 17.2 mg independent of crop or genetic modification of plants. The biomass at maturity ranged between 571.4 and 761.8 mg with higher weights of individuals when fed on maize compared to potato tuber material ($p < 0.001$). Biomasses of *A. caliginosa* offspring affirm the findings of Vercesi et al. (2006) and Zeilinger et al. (2010) as they did not reflect any significant impact of GM compared to non-GM food sources ($p > 0.05$) (Tab. 7).

Hatchlings of the species *L. terrestris*, by contrast, showed significantly lower biomasses when the parental generation was fed with GM compared to non-GM plant material (Swedish maize leaves: $p < 0.001$; Irish potato tuber: $p = 0.008$). At maturity, in turn, biomasses of juveniles did not differ between GM and non-GM treatment ($p > 0.05$) (Tab. 6).

Concerning impacts of both crops themselves, *L. terrestris* offspring reflected higher biomasses when fed on potato compared to maize material (biomass at hatching: $p < 0.001$; biomass at maturity: $p = 0.001$) (Tab. 6).

The maturation period of test specimens clearly differed between focal species. While *L. terrestris* offspring reached maturity within 163 to 213 days (Tab. 6), *A. caliginosa* juveniles, according to the results of Vercesi et al. (2006), required a maximum period of 102 days to become mature (Tab. 7). Moreover, the maturation of earthworms reflected a species and crop specific GM effect. Whereas feeding GM compared to non-GM potato prolongated the maturation process of *L. terrestris* ($p = 0.048$), it reduced maturation periods of the species *A. caliginosa* ($p = 0.043$). Use of GM compared to non-GM maize, by contrast, shortened the maturation of *L. terrestris* ($p < 0.001$), but did not affect maturation processes of *A. caliginosa*.

As indicated by the survival rates of offspring, GM plant material did not increase the mortality of both species. The results of the present study, therefore, affirm the findings of Zwahlen et al. (2003). Moreover, the results indicate a suitable test design and appropriate laboratory conditions as survival rates of juveniles were at least 88% for the species *L. terrestris* (Tab. 6) and 96% for the species *A. caliginosa* (Tab. 7).

Risk assessment

The results of the case study indicate that the genetic modification of maize and potato induced changes in several performance traits of both focal species. Whereas GM maize led to (1) a higher cocoon production, (2) a lower biomass at hatching and (3) a shorter maturation of the species *L. terrestris*, GM potato induced (4) a decrease in the hatching biomass and (5) a prolongation of maturation of *L. terrestris* but (6) a shorter maturation period of *A. caliginosa*. Although these effects were significant, they did not allow to draw conclusions on potential risks of the GM crops to the focal non-target decomposer species.

To assess potential hazards caused by the considered GM maize and potato cultivars, GM effects have to be interpreted on the basis of the limits of concern. For that purpose, each detected GM-induced change was converted into percentage increase or decrease as compared to the respective control treatment (Tab. 8).

Tab. 8: Limits of concern and detected GM-induced percentage increases (+) or decreases (-) of performance traits of the species L. terrestris and A. caliginosa; food sources: maize leaves cultivated in Sweden and Spain; potato tuber cultivated in Ireland.

		Limits of concern	*Lumbricus terrestris*		*Aporrectodea caliginosa*		
Initial generation			**Maize (Sweden)**	**Potato (Ireland)**	**Maize (Sweden)**	**Maize (Spain)**	**Potato (Ireland)**
I	Survival [%]	- 50%	+ 30.77%	- 5.00%	± 0.00%	± 0.00%	± 0.00%
I	Change of biomass [mg]	- 30%	+ 41.83%	+ 17.55%	+ 4.05%	+ 3.13%	+ 127.75%
II	Cocoon production [n $ind.^{-1}$ m^{-1}]	- 50%	+ 55.52%	+ 18.75%	+ 21.55%	- 14.78%	+ 0.32%
Offspring							
II	Cocoon hatchability [%]	- 50%	+ 2.95%	- 6.43%	+ 15.48%	+ 5.92%	+ 17.33%
III	Biomass at hatching [mg]	- 30%	- 5.30%	- 18.09%	+ 2.28%	+ 4.48%	+ 3.00%
III	Biomass at maturity [mg]	- 30%	+ 1.23%	+ 8.47%	- 4.12%	+ 6.37%	- 3.73%
III	Maturation [d]	+ 50%	- 13.94%	+ 16.88%	- 2.03%	+ 4.43%	- 8.18%
III	Survival [%]	- 50%	- 2.20%	+ 12.50%	± 0.00%	- 0.24%	- 0.28%

I-III: Test phases

When now, firstly, comparing the direction of change with the direction of limits of concern, it gets obvious that only three of the 6 detected significant GM-impacts have to be classified as negative: (2) GM maize-induced decrease of biomass at hatching of *L. terrestris*, (4) GM potato-induced decrease of biomass at hatching of *A. caliginosa*, and (5) GM potato-induced prolongation of maturation of *L. terrestris*. When then, additionally, comparing magnitudes of effects with threshold ranges given by limits of concern, it gets obvious that no single negative effect laid within the set limits (Tab. 8). For that reason, GM-induced changes of performance traits, detected in the present case study, are not of sufficient magnitude to cause harm.

With regard to earthworm driven processes and functions, cultivation of both crops analysed during the present study can, therefore, be considered as non-hazardous.

5. General discussion

Generally, the results of the case study reveal that laboratory conditions and experimental design used within the test system are appropriate and suitable for analyzing GM effects on performance traits (Pey et al., 2014) of the focal species *L. terrestris* and *A. caliginosa,* which have the potential to indicate possible long-term effects and changes in ecological functions. Due to the reproducibility of the experimental design, the determination of measurement endpoints and the setting of limits of concern, which allow a hazard characterization of each detected adverse effect (EFSA, 2010), the present test systems could be used for standard testing of GM effects of maize and potato from Atlantic, Boreal and Mediterranean regions.

Comparing the applicability of both selected focal species used in the test system, *A. caliginosa* might, if reasonable in the context of scope, be preferred as indicator organism due to shorter generation times and higher reproduction rates compared to *L. terrestris*.

As indicated by the different amounts of food used within the case study compared to those stated in the protocol, it gets obvious that some indications in the protocol are to be understood as guidelines. Some decisions, during testing, have to be made for each individual case according, for instance, to the possibilities offered by size and experimental design of GM crop cultivation areas. Such minor modifications of the test system are possible as long as the validity of experimental results is not reduced. This refers, primarily, to those indications for which, anyway, different amounts are recommended and described as optimum in literature studies. Individual adjustments of food supply (for *L. terrestris* optimal food supplies from 0.49 g ind^{-1} $month^{-1}$ (Zwahlen et al., 2003) up to > 20 g ind^{-1} $month^{-1}$ (Lowe & Butt, 2005) are described), for instance, are possible without any noticeable negative consequences. Instructions concerning obligatory basic requirements of earthworm species, like soil moisture or temperature, in turn, have to be followed precisely as respective tolerance ranges of earthworm species are often limited and deviations might lead to decreased reproduction or hatching success (e.g. Butt, 1993), resulting in a reduced experimental validity.

Beside potato and maize from the Atlantic, Boreal and Mediterranean region, there are certain other GM crops from several other biogeographical regions which, within Europe and

globally, play an important part (for instance soy or rice), but were not considered within the present test system up to now. In general it is conceivable and possible to analyze impacts of other GM crops by means of the present test system. However, in this context it has to be taken into account, that for each crop/region combination respective focal species must be selected, which, potentially require different conditions (for instance with regard to time scheduling; soil moisture; temperature; vessel sizes, food supply, pH or texture of substrate). Therefore, if risk assessment of other crops or from other biogeographical regions are intended, used species as well as experimental conditions and design have to be modified according to the respective requirements. The test system should then be evaluated again for its efficiency.

Altogether, the present test system provides a valuable new tool for GM crop risk assessment, which allows to assess impacts and potential hazards of GM crops on focal earthworm species as important non-target decomposers.

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