
**Project Number 289706**

**COLLABORATIVE PROJECT**

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

Start date of the project: 01/12/2011 Duration: 54 months

# D5.2 Set of standardised ERA protocols for Non-Target Organisms

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| **Dissemination Level** | | |
| **PU** | Public | **X** |
| **PP** | Restricted to other programme participants (including the Commission Services) | |
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# SUMMARY

AMIGA WP5, Deliverable 5.2 presents the design and evaluation of protocols to assess the possible impact of growing GM-maize and GM-potato crops in Europe on the abundance of Non Target Organisms in the context of Environmental Risk Assessment. The protocols have been designed based on the pertinent scientific and regulatory literature and the experience of the consortium partners. For field monitoring, two main methods have been employed, pitfall trapping during one week per month and direct visual observations once or twice a month. The application of these protocols has produced a wealth of standardised data over years and biogeographic zones, allowing statistical comparisons. Data have been aggregated to four functional groups (herbivores, parasitoids, predators and detritivores) and to at least family level. For some of the most abundant taxa, higher taxonomic resolution (genus or species level) has been achieved. Secondly, protocols for selection of focal species occurring in the maize and potato crops and for subsequent life table and behavioural experiments in greenhouse and climate chambers have been developed for early tiers in ERA, specifically considering the need of producing *in planta* generated data for non target organisms. In designing such protocols, practicality for applicants requesting admission of GM-crops has been taken into account with respect to taxonomic expertise, amount of labour required and associated costs.

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## General introduction

The EFSA Guidance on environmental risk assessment (ERA) of genetically modified (GM) plants (EFSA, 2010) gives broad guidance on the design and analysis of field experiments, adjusted and implemented case by case. EFSA adopts a 6-step process for ERA (Directive 2001/18/EC) which includes (1) problem formulation including hazard identification; (2) hazard characterisation; (3) exposure characterisation; (4) risk characterisation; (5) risk management strategies; and (6) an overall risk evaluation.

The EFSA GMO Panel considers seven specific areas of concern that are assessed when applicants submit ERA dossiers: 1) persistence and invasiveness of the GM plant , or its compatible relatives, including plant-to-plant gene transfer; (2) plant-to-micro-organism gene transfer; (3) interaction of the GM plant with target organisms and (4) interaction of the GM plant with non-target organisms (NTOs), including criteria for selection of appropriate species and relevant functional groups for risk assessment; (5) impact of the specific cultivation, management and harvesting techniques; including consideration of the production systems and the receiving environment(s); (6) effects on biogeochemical processes; and (7) effects on human and animal health.

In AMIGA WP5 (D5.2) we focus on ERA protocols for NTOs, specifically using case studies on maize and potato agroecosystems that have also been studied in WPs 5, 6 and 8, with associated statistical design and analysis of NTO data obtained in glasshouse, controlled environments and field monitoring studies; we here refer to D9.4/9.5 for detailed guidelines on design and analysis of NTO-field studies.

Each ERA begins with problem formulation, in which the most important environmental questions that merit detailed risk characterisation are identified. Problem formulation helps to make the risk assessment process transparent, by explicitly stating the assumptions underlying the risk assessment. At the end, the overall risk evaluation should result in informed qualitative and, if possible, quantitative advice to risk managers, outlining the nature and magnitude of uncertainties associated with the identified risks. The implications of the risk assessment for risk management measures should also be assessed (EFSA, 2010). The EFSA approach considers ‘intended’ (based on the expressed GM traits) and any ‘unintended’ effects due to the genetic modification process rather than the newly expressed GM trait(s), using a weight of evidence approach.

- **Intended**: Effects directly linked to the objective(s) of the genetic modification(s);
- **Unintended:** Effects not directly linked to the objective(s) of the genetic modification(s). On the basis of current knowledge of the inserted trait(s) and their metabolic connections, these can be either expected or unexpected.

Uncertainties arising from data gaps or unknown interactions (e.g. in stacked events) are also addressed by EFSA in their ERA guidance documents.

The AMIGA research project aims at providing more detailed ERA guidance for NTO studies in the form of protocols for design, sampling and analysis. This ERA protocol report links closely to (i) the Statistical Guidance report (D9.4) which provides statistical elements for

such protocols and also to (ii) Guidance on the newly developed AMIGA Power Analysis Tool (D9.5). Standardised ERA experimental designs, sampling methods for non-target organisms (NTOs), statistical tools for determining replication to achieve the required power for detecting different effect sizes (e.g. population numbers of focal NTOs) complement the general EFSA guidance but add further detailed information on optimisation and adaptation for different receiving environments, in particular considering different European biogeographic zones. Moreover, some of the protocols illustrated, specifically take into consideration the suggestions of EFSA GD to produce *in planta* data for estimating possible effects of GM plants on non-target organisms.
This document does not discuss the need of each of the studies during the ERA process, as this decision is a duty of applicants and risk assessors following the relevant EFSA Guidance documents. For discussions concerning the suggested use of each AMIGA produced tool for risk assessment and post market environmental monitoring, please refer to Deliverable 1.14.

## AMIGA ERA PROTOCOLS

# 1. Protocols for field experiments for NTOs

## 1.1. Monitoring occurrence of soil-dwelling NTO arthropods

### 1.1.1 Introduction

Surface-active arthropods are important components of the invertebrate biodiversity in most terrestrial habitats in the temperate region, including agro-ecosystems. The overwhelming majority of these are secondary consumers, and often prey on other organisms, such as pest insects on crops. They are also species-rich, so they are frequently used as indicators of biodiversity changes. The most frequently used method to collect and monitor them is by the use of traps, usually unbaited pitfall traps. The practice of using pitfall traps is well established (Southwood and Henderson, 2009) and the limitations of pitfall trapping are documented in the ecological literature. The main limitations are that (1) this method measures relative rather than absolute density and (2) it only indirectly supplies information on arthropod activity. The AMIGA project decided to use pitfall trapping as a major method of monitoring the possible long-term effects of the GM crop under field conditions. The modifications proposed here are meant to alleviate the known limitations of the methods and increasing the number of catches in order to allow a number of catches which is more likely to achieve a reasonable power for the statistical analyses.

### 1.1.2 Pitfall trapping: operation and sampling design

**Pitfall trap material**

4 plastic 500 ml cups, 10 cm diam.
Trapping fluid: ethylene glycol, 70% and a drop of odourless detergent.
20 cm x 20 cm metal pitfall trap rain covers
barrier; a 1m long, ca. 10 cm high (above ground part) straight barrier (plastic or metal)

**How to operate pitfalls in the field**

1. Double cups are used to stabilise the holes in the soil. At each pitfall location, dig a hole and insert a plastic cup. Insert a second plastic cup into the dug cup (see Fig.1). Make sure that the lip of the upper cup is level with the surrounding ground surface (this practically means the rim of the outer cup will be a little below surface). Also control that there are no gaps between the rim and the soil (control and correct this on every sampling occasion).
2. Put 100 ml of the trapping fluid into the cup. Add just one drop of detergent (if not already mixed with trapping fluid beforehand). Make sure you take sufficient trapping material, trapping fluid with you.
3. Place rain cover on the top of the trap. Push down the cover so that the distance between the roof of the rain cover and ground surface should be about 2 cm in order to prevent mice, frogs or other animals from entering the pitfall trap.

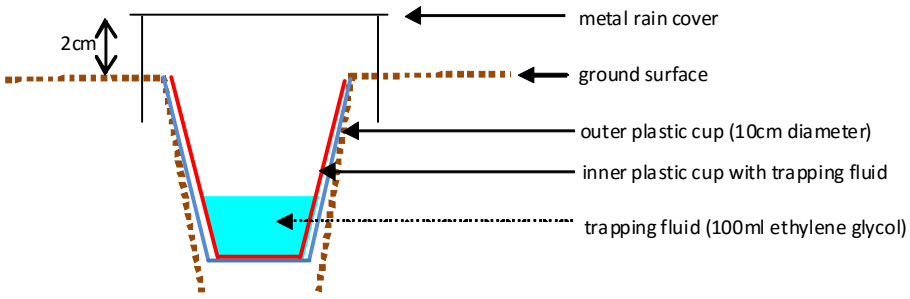


**Figure 1**. Pitfall trap cross-section.

**Sampling design**

Place two traps in the centre of each plot in the way described above. The two traps must be 1m apart and be connected with the barrier. The two traps are placed in different rows of the maize, so that the connecting barrier cuts one maize/potato row diagonally, touching each trap in the middle (see Fig. 2).

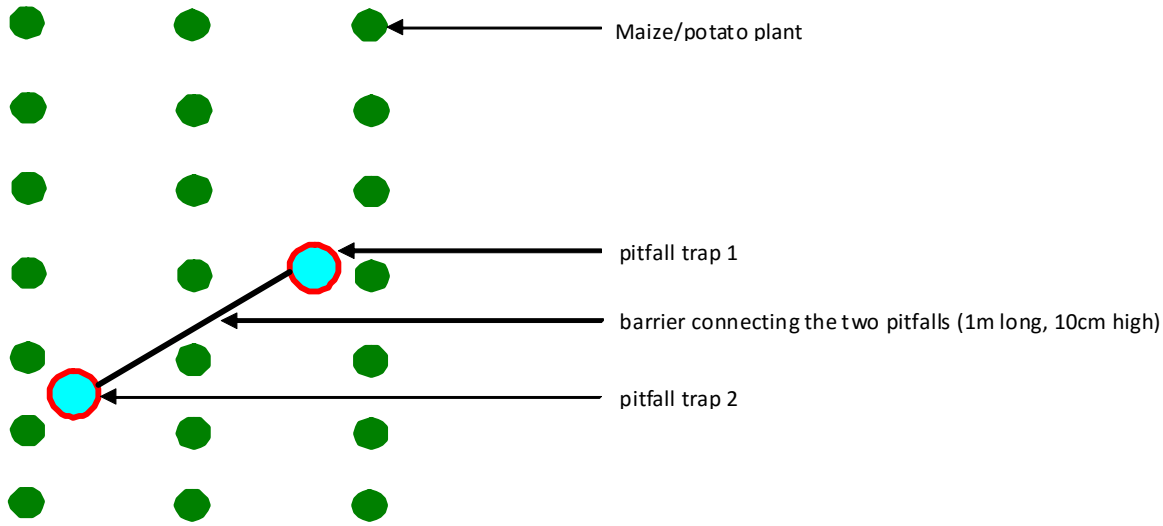


**Figure 2. Pitfall traps placed in the field.**

Pitfall trap cover:
Take a 20 cm x 20 cm galvanized metal sheet. Cut into this sheet (with scissors or saw) a straight line, running parallel with the edge at ca. 1 cm from the edge. Cut it only to 10 cm. Do the same on the other side, but start from the opposite corner. Fold down the two loose halves, and create a tip on each by making an additional, diagonal cut at the end of these narrower "legs". With some prudence, the cover can now be pushed into the soil.

### 1.1.3 Sampling period

The sampling period starts in Spring (e.g. April) immediately after the maize has been sown or potato has been planted, and ends shortly before harvest (e.g. September/October for maize and July-August for potato, depending on the region and the cultivar). Each month during the growing season, the pitfall traps are run for one week (7 days), commencing (if possible) in the first week of the respective month. In total, this results in 6-7 sampling dates during the season.

Make sure there is on-site recording of the main weather parameters: temperature (at soil level), rainfall, sunshine hours. If possible, measure temperature by using data loggers deployed directly in the plot/s. Use the attached site description sheet to describe the location (see 8. Background information).

### 1.1.4 How to collect samples from the field

Catch of the two traps can be combined (the two traps are not considered independent – they are too close on a 10 m x 10 m plot).

After 7 days of operation, collect the trap catches. Take a few extra cups (in case some of them are damaged), some fresh ethylene glycol (already diluted), squeeze bottle with 70%

ethanol, pre-printed labels, a hand trawl (almost certainly you will have to do some readjustment of the trap surroundings) and another (large) collecting bottle for trapping fluid that is too dirty, diluted or smelly. Check the required field equipment before going into the field (see attached “List of field equipment”).

There are two options to collect the catch from pitfall traps:
1. Use nappy liners. You can buy them in supermarkets, shops selling baby care products, etc.
2. If nappy liners are too big, cut them in half. You need a funnel, an empty container, and elastic bands.

**Procedure:** Take the inner trap with the catch and fluid. Line the funnel with the nappy liner unfolded, as if you were making a filter paper funnel. Place the lined funnel into the empty bottle. Swirl the catch around to mix it, and pour the content into the funnel. It is important not to pour slowly, because things will then settle and remain in the bottom of the trap. If that happens, pour the liquid back from the lower bottle, and repeat the procedure.
The liquid will go through the nappy liner fairly quickly, leaving the catch in the nappy liner “funnel”. Drop a label into it, and take it out, and close it by using an elastic band to secure the top, and put the catch into another bottle with ethyl alcohol. The catch may have some soil – that is not a problem for now. The liquid will collect in the lower bottle. If it is clean, you can re-use it, maybe by adding a little fresh solution. If it is too dirty, smelly or diluted, pour it into the waste collection vessel. Do NOT pour it on the ground – ethylene glycol is very poisonous (for this reason, it is forbidden to use it in several countries). Re-set the trap and close it by pushing the cover so that it is level with the ground. Make sure arthropods cannot crawl in – you may even use your hand trawl to close the edges by heaping some soil on the edges of the trap cover.

2. Use a sieve/tea strainer. Instead of the nappy liners, take a small tea strainer (metal, not plastic, less than 8 cm diam), an appropriate number of containers (a 200 ml container is usually sufficient), funnel, squeeze bottle with alcohol, extra alcohol, and the other items as above.
Procedure: lift the trap out of the second one. Put the funnel into a bottle . Swirl around the content and pour through the sieve into the funnel. The catch is now in the sieve. Take the funnel and the sieve, position the funnel into the container where you want to store the catch. Turn the content of the sieve (the catch) into the funnel. It is best to do this quickly, with a sudden rap against the funnel side, so that most of the catch is not only in the funnel, but near the lower opening. Place the sieve, upturned, into the funnel. Using the squeeze bottle, wash the catch into the container below with the alcohol. Skirt through the upturned sieve, making a circular movement around the perimeter of the funnel. This will dislodge small insects that often stick to the wire and to the sides of the funnel. Use alcohol sparingly - this needs some practice- otherwise you would fill up the lower container, and some of your catch may still remain stuck to the sides of the funnel. Drop the label into the lower container, close. Remember to close the trap by pushing the cover down so that it touches the surface, and move onto the next trap.

It will probably be easier to collect individual traps separately – but remember, the catch of the two traps connected by the fence should be combined. You can do this in the lab.

When opening the traps for the one-week catching period: take a few traps with you, in case some traps disappeared, or got damaged. Take a hand trowel, and some ethylene glycol solution as well. A sieve or nappy liner, an empty container and a container for used trapping fluid would also come in handy. Carefully lift the trap cover completely and check the inside of the trap. If necessary, remove any insect, soil, slugs, etc. from the trap. It may be easier to sieve the liquid through. Add some fresh liquid or change the trapping liquid if very dirty or smelly. Put back the cover and push it down to leave a ca. 2 cm gap. If you adjusted anything, record it. Move onto next trap.

Use the attached field plot protocol sheets to describe the plot (see 8. Background information).

### 1.1.5 Laboratory procedure: Sorting, identification and storage

*Sorting* – Take material from the field to the lab. Try to sort as soon as possible after collecting.
Sort all arthropods from contaminating material, label.
Pre-sort into different vials while discarding contaminating material (soil, debris): (1) adult beetles (counted); (2) spiders (counted); (3) rest.
Keep record of material stored (how many vials from which sampling, when sorted, by whom?)
*Identification* - (1) Identify Carabidae to species; (2) Identify spiders to families; (3) Identify rest to order and, when possible in view of expertise and resources available, to family.
For selected groups, higher level resolution (genus, species) has been achieved within AMIGA; see D5.1.
*Storage* - Store in 70% ethanol, cold room storage is preferred (if not possible, at room temperature, but preferably in darkness). Check every 6 months, top up with alcohol if necessary.
Keep reference material, discard bycatch.
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### 1.1.6 Labels to be used

Use pre-printed labels, using a laser printer and thick paper. If hand-written labels are used, write in lead pencil, not ink. Try not to use more than one label per vial.
The data have to indicate the place and time of collection, method, sample no., collector's name
Example:
AMIGA F'bjerg DK
GM maize plot G5
Pitfall, 2013 Jul 7-14
Coll. G Lövei

We suggest to use the year-month-day sequence as this makes it easy to sort samples by time in databases, folders and spreadsheets. Please note the date is a *period* not a single day.

Label use suggestion in the field: cut the labels into longitudinal strips; cut the labels also horizontally but do not cut them "free" – leave a small section uncut so that the labels are easily torn off the strip. You will save time not to have to search for the appropriate label. Do not allocate them into vials before going to the field – this just risks mixing-up. When you put the label into the vials in the field yourself, that is one final check that the label you are about to put into the vial is the correct one.

### 1.1.7. Datasheets

It is preferable to always have a hard copy of your data, stored as a safety measure. If you work on computer use paper and pen in the field – this will also serve as permanent record of your data.

When entering data into an Excel sheet, your first sheet should *always* be a metadata sheet. Write what is in the file, how were the data generated, what do the codes mean, who did the identification and data entering, etc. It is very important to have this background data with the data file.

Data recording sheet columns: Crop variety/line, country, location, treatment, plot, year, sampling period, species/category identity, number of individuals. See **Appendix 1** for a format suggestion.

This is the format most statistical programs require – i.e. all the information that is necessary to understand the response variable (the number of individuals) is on the same row.

When identifying the material, always make a written record, and archive this. This can be in free form, but has to have the necessary information so that any query emerging from the scrutiny of the electronic data sheets can be answered, or any suspected errors cross-checked. This means that all the information that is on the datasheet (see above) is also written in a notebook.

### 1.1.8. Background information

For reporting relevant variables, separate protocol sheets will be used, one for the location (the overall site), and extra sheets for each sampling occasion on the single plots.

The site description will include the following information (use the site description sheet provided in the **Appendix 2**). Use different site description sheets for different years.

1.) Name and location of the site
2.) Year
3.) Name of the recorder
4.) GPS coordinates of centre of the location

5.) Altitude (meters above sea level)
6.) Current crop
7.) Crop(s) of the previous year
8.) Information on field management practices: sowing/harvest dates, crop rotation, herbicide and insecticide application (date, brand, application rate), type and date of mechanical treatments (e.g., ploughing).
9.) Copy and print a Google Map (satellite view) of the location, marking the site.
10.) Provide information about field boundaries, also indicating them on the Google Map: presence/absence, size, habitat types, distance (estimated) to plots.
11.) A map of the plot arrangement with measurements (distance between plots), information on cover/surface between plots
12) Weather data. Make sure there is on-site recording of the main weather parameters: temperature (at soil level), rainfall, sunshine hours. If possible, measure temperature by using data loggers deployed directly in the plot/s.

The field plot protocols will include the following information (use the field plot protocol sheets provided in the Appendix). Use different sheets for different plots and years. Use the same sheet during all visits, adding the relevant new information. This will be the "visit sheet" of the plot.
1.) Location/site (does not change)
2.) Plot number (does not change)
*3.) Date (to be added every time)*
*4.) Name of recording person (to be added every time)*
5.) GPS coordinates of the plot (does not change)
6.) Soil type and soil texture (does not change)
*7.) BBCH growth stage of maize/potato in the plot* (Lancashire et al. 1991) *(to be added every time)*
8.) Provide an informal *estimate of the weed cover* in the respective maize plot, using the following classifications: **0** = none/low weed density, or weed control successful; **1** = medium weed density, or weed control only partly successful; **2** = high weed densities, or weed control not successful - *(to be added every time)*
9.) Record any other relevant information, e.g. extreme weather events, mass occurrence of maize pests and/or damage to maize plants, pitfall trap incidents (no trapping fluid, overflowing trapping fluids, dug-out traps, etc.). *(to be added every time)*
10. Record by-catch: what was caught (rough categories are sufficient like rodents, reptiles, amphibians), how many individuals?

**List of field equipment**
Check the required field equipment before going into the field by consulting the attached "List of field equipment" (**Appendix 3**).

## 1.2 Monitoring plant dwelling NTO arthropods

**Visual survey of live plants**

For maize, on 12 randomly selected plants per plot, select one leaf each, and count all insects found. Selected plants should be not growing at the field edge, but have at least 2 m in the case of the GM vs. non-GM plots, and 12 m distance to edge for the general surveillance. The selected leaf can be covered with plastic bag, and cut off, and checked in the laboratory. In that case, you can put the leaf into the freezer to kill insects, allowing a more precise counting. Select plants randomly, but take leaves regularly: 6 at low, and 6 at high positions. Collection every second week during the whole growing season, starting at 4-6 leaf stage of the maize plants. The lowest leaf is the first green, (largely) intact leaf. Low leaves include this one and the following 3 above the lowest. Likewise, the highest leaves include the first green, intact leaves from the top downwards.

During maize flowering, check the flowers for arthropods, identify and count them on the same 12 plants you use for leaf survey. When maize cobs appear, do the same – on one cob per plant.

Be very careful when approaching a plant – insects may see you and flee.

Cob – fold the leaves back carefully, to the top third-half, and check any insects inside. Refold the cob leaves after checking.

For potato, on 12 randomly selected plants per plot, select six leaves each (three from the bottom and three from the apical part), and count all insects found. Selected plants should be not growing at the field edge, but have at least 2 m in the case of the GM vs. non-GM plots, and 12 m distance to edge for the general surveillance. The selected leaf can be covered with plastic bag, and cut off, and checked in the laboratory. In that case, you can put the leaf into the freezer to kill insects, allowing a more precise counting.

Identification was conducted at family level or order level in view of the level of expertise and time avaialble in the project. For selected groups, higher level resolution was achieved e.g. for Carabidae  species level resolution was reached.

## 1.3 Monitoring flying arthropods

Sticky traps were operated during 2013 as a pilot study and the resource needs of processing the catch was analysed using the collected materials in Denmark. Several problems were unearthed:

1. The catch is not possible to “harvest” without a serious loss in quality. Due to wind, the traps are contaminated with dust, and later in the season, leaves brushing against

the surface damage the captured arthropods. Occasionally, birds attempt to take arthropods from the traps
2. When collected in the field, the traps are very fragile, which results in further damage to the trap catch
3. It is impossible to remove arthropods for the surface for close examination –and especially in the case of small insects, identification even to family is not possible
4. There is a large proportion of "passenger" or "tourists" captured i.e. elements of aero-plankton that have no trophic connection to the crop in question.
5. We tried to scan and automatically analyse the catch, trying to simply assess the size distribution of the catch using computer algorithms. These a) do not indicate difference between GM and non-GM plots and b) were judged irrelevant due to a large proportion of the above-mentioned "tourists".
6. The time necessary to obtain specimen in good conditions that allows a dependable taxonomic identification is very high compared to the informative value of the data

**Conclusion:** AMIGA data sets collected in 2013 with the sticky traps method were all affected by the same drawbacks described above. Due to these factors, we decided to abandon this method as a possible monitoring method and judge this as unsuitable method for routine ERA studies. The method could be useful for large-scale, landscape scale monitoring in case where a single type of GM crop is planted dominantly over a large area, where the percentage of "tourist" species are expected to be lower. However, in that case, the method needs further calibration.

## 1.4 Measuring activity of arthropod natural enemies under field conditions

### 1.4.1. Parasitism

At each sampling date, locate and check (possibly 5) naturally occurring aphid colonies per plot and record number (or percentages) of mummies present.

### 1.4.2. Predation

At each sampling date, locate and check (possibly 5) naturally occurring egg masses of e.g. Colorado potato beetle (in potato) or other coleopteran, lepidopteran or hemipteran egg masses (in potato or maize) per plot and record number (or percentage) of hatched, consumed and rotten eggs.

### 1.4.3. Predation pressure measurement

Additional quantitative methods to monitor NTO-field experiments are desirable. One such method was developed in the course of WP5. It makes use of 'dummy' prey, consisting of plastic, non-drying green plasticine 'caterpillars', glued to plant surface using instant glue. Leave dummy caterpillars out for 48 h, check and identify marks found. Glue 10 caterpillars per plot near ground level on maize stems, and 10 on same plant at breast height on leaf

midrib. Plants can be at regular distance from each other, leave 2 m edges. Operation – same as for pitfalls: one predation session per month.
Additional practical indications for using this method are given in the Appendix 3B.

For the AMIGA plots (10m x 10m), we placed 10 caterpillars: 5 near the edge (1 m from edge) and 5 in the centre, 2 m from each other, in a star-arrangement. On caterpillar in the centre, and 4 around this position, so that the four caterpillars mark the half position of a side of the square:

X

X X X

X

Collect the caterpillars into individual tubes – write the number on the outside of the tube, and keep them in a cool place. Examination can be made under a microscope or a hand-held magnifying glass.
This method allows the indirect measure of predation intensity and may include non-arthropod predators which are usually not considered with other sampling methods. Artificial caterpillars obviously can only mimic predator-prey interactions triggered by visual clues and are therefore complementary to other methods in which olfactory clue are essential.

## Adaptation of the protocols for surveys in commercial fields

In general, all the above indicated protocols are equally applicable to surveys in non-commercial fields foreseen in WP2, when this is deemed necessary in a post-market phase.
The minimum size of the commercial field should be selected according to the representative size for the area. Nevertheless, a **minimal size of 0.5 – 1 ha is necessary**. The number of fields to be sampled should be between 8 and 10 (to mirror the design of the NTO oriented field trials).
The number of pitfall traps should be adjusted to these surveys; a reduction of number of samples/surface by an order of magnitude can be adopted (e.g. **2 pitfall traps or 12 plants per 1000 m$^2$**).

# 2. Protocols for greenhouse and climate chamber experiments to assess effects on NTOs on trans- and cisgenic potato and transgenic maize

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### 2.1 Introduction

To support and further develop early-tier ERA-studies (cf. General introduction), greenhouse and climate chamber bioassays have been carried out for the same crops, maize and potato in controlled environments, i.e. greenhouses and growth chambers. For potato, non-target effects

of a late blight (*Phytophthora infestans*) resistant cisgenic and transgenic potatoes were assessed in relation to position and number of inserted resistance (R) genes. For maize greenhouse and laboratory bioassays have been performed on MON810 Bt-maize and its isoline.
AMIGA WP5 focussed on the interaction of the GM plant with non-target organisms (NTOs), including criteria for selection of relevant functional groups and focal species appropriate for experimentation (cf. Table 1). In WP5, NTO-species tested in greenhouse bioassays have been selected according to the EFSA Guidance Document (@EFSA, 2010).
We first report on the results of the NTO-species selection protocol for potato and subsequently describe two case studies on NTO-species on the second and third trophic levels for both maize and potato.

### 2.2 Selection of non-target species for *in-planta* testing

In any ecosystem, there is a potentially high number of NTO species that may be exposed to GM plants. Considering that not each of these species can be tested, a representative subset of NTO species should be selected for consideration in the risk assessment of each GM plant. The GMO Panel of the European Food Safety Authority (EFSA) proposed a risk assessment approach that utilizes some of the elements from a range of existing approaches (EFSA, 2010). The main criterion adopted in the Guidance Document, is the analysis of functional biodiversity. A particular emphasis is given to the consideration of the receiving environments for which the ERA is conducted. Therefore, the species selection process is aimed at the determination of "focal species" based on ecological criteria and practical considerations which lead to the final choice.
The scientific method calls for this selection process to be as standardized and transparent as possible. For this study, we selected a single species to be used based on a selection matrix for NTOs. This was based on the criteria indicated in the EFSA ERA Guidance Document (EFSA, 2010). The EFSA selection process includes four steps: (i) Identification of functional groups; (ii) Categorisation of NTO species; (iii) Ranking species based on the ecological criteria; and (iv) Final selection of focal species (Van Capelle et al., 2016).
Starting from the original table reported in the EFSA arthropod database (http://www.efsa.europa.eu/en/supporting/pub/956e; updated January 16, 2016), we constructed a selection matrix (Table 1).
The ranking exercise was undertaken by seven researchers at ENEA and Wageningen University, each of them gave an independent score and the average value was calculated. When standard deviation among scores was high, a joint discussion enabled to adjust the final score.
Based on final ranking, the two most suitable species on which to conduct risk assessment for the selected potato events would be the beetle *Leptinotarsa decemlineata* Say as a leaf feeder (preferable to the equally ranked lepidopteran *Phthorimaea operculella* Zeller for reasons of availability), and one of the three most common aphid species. The Green Peach aphid, *Myzus persicae* Sulzer (Hemiptera: Aphididae), was chosen for WP5 for several reasons: the simplicity in rearing this species in many laboratories and its unique reproductive biology, which allows for the measurement of survival and intrinsic rate of increase, which can be used

to estimate the population dynamics of this pest; and, it is listed as the second most collected species (first-most collected phloem feeder) in the EFSA arthropod database, giving it high relevance as a focal NTO.

A similar exercise was conducted considering the third trophic level, NTO-studies were focussed on natural enemies of the herbivores (see in Table 2 the example for predators in potato). In this case, we first scored the predatory guild at the family level and finally chose a species according to practicability, as suggested by the EFSA ERA GD.

We finally selected an aphid parasitoid species *(Aphidius colemani)* as natural enemy of *Myzus persicae* and a predatory beetle species, the coccinellid *Hippodamia variegata,* as natural enemy of aphids in maize and potato.

**Table 1**. Ranking of herbivore species to support selection of NTOs in potato based on the EFSA ERA Guidance Document (EFSA, 2010). The lower the score, the higher the relevance of species for focal species selection.

| Taxon | Guild | Exposure | Presence/ Occurrence | sensitivity | Linkage | Ecological sign. | Abundance | Susceptibility | Ranking |
|---|---|---|---|---|---|---|---|---|---|
| ***Leptinotarsa decemlineata*** | Leaf feeder | 1 | 1 | 5 | 2 | 5 | 2 | 5 | 3,00 |
| ***Phthorimaea operculella*** | Leaf miner | 2 | 1 | 5 | 2 | 5 | 1 | 5 | 3,00 |
| ***Myzus persicae*** | Sap feeder | 2 | 1 | 5 | 3 | 5 | 1 | 5 | 3,14 |
| ***Aphis nasturtii*** | Sap feeder | 2 | 1 | 5 | 3 | 5 | 1 | 5 | 3,14 |
| ***Macrosiphon euphorbiae*** | Sap feeder | 2 | 1 | 5 | 2 | 5 | 2 | 5 | 3,14 |
| **Aleurodidae** | Sap feeders | 2 | 1 | 5 | 4 | 5 | 1 | 5 | 3,29 |
| **Thripidae** | Sap feeders | 2 | 1 | 5 | 4 | 5 | 1 | 5 | 3,29 |
| **Alticinae** | Leaf feeder | 2 | 2 | 5 | 3 | 5 | 2 | 5 | 3,43 |
| **Acarina** | Cell content feeders | 2 | 1 | 5 | 5 | 5 | 1 | 5 | 3,43 |
| ***Tuta absoluta*** | Leaf miner | 2 | 2 | 5 | 4 | 5 | 2 | 5 | 3,57 |
| **Agromizidae** | Leaf miners | 2 | 1 | 5 | 5 | 5 | 2 | 5 | 3,57 |
| ***Lygus sp.*** | Sap feeders | 2 | 1 | 5 | 4 | 5 | 4 | 5 | 3,71 |
| **Cicadellidae** | Sap feeders | 2 | 1 | 5 | 5 | 5 | 3 | 5 | 3,71 |
| **Scarabeidae** | Root feeders | 1 | 2 | 5 | 5 | 4 | 5 | 5 | 3,86 |
| **Elateridae** | Root feeders | 2 | 3 | 5 | 4 | 5 | 3 | 5 | 3,86 |
| **Noctuidae** | Root-leaf feeders | 2 | 2 | 5 | 4 | 5 | 4 | 5 | 3,86 |
| **Pentatomidae** | Sap feeders | 2 | 2 | 5 | 5 | 5 | 4 | 5 | 4,00 |

**Table 2**. Ranking of predator species to support selection of NTOs in potato based on the EFSA ERA Guidance Document (EFSA, 2010). The lower the score, the higher the relevance of species for focal species selection.

| Taxon | Exposure | Presence/ Occurrence | sensitivity | Linkage | Ecological sign. | Abundance | Susceptibility | Ranking |
|---|---|---|---|---|---|---|---|---|
| ***Coccinellidae*** | 1 | 1 | 5 | 1 | 1 | 1 | 5 | 2,14 |
| ***Chrysopidae*** | 2 | 1 | 5 | 1 | 1 | 1 | 5 | 2,29 |
| ***Syrphidae*** | 1 | 1 | 5 | 1 | 1 | 2 | 5 | 2,29 |
| ***Miridae*** | 1 | 1 | 5 | 2 | 1 | 2 | 4 | 2,29 |
| ***Araneae*** | 3 | 2 | 5 | 1 | 1 | 2 | 3 | 2,43 |
| ***Anthocoridae*** | 1 | 1 | 5 | 2 | 1 | 3 | 4 | 2,43 |
| ***Carabidae*** | 3 | 2 | 5 | 1 | 1 | 4 | 3 | 2,71 |
| ***Nabidae*** | 2 | 1 | 5 | 2 | 2 | 4 | 4 | 2,86 |
| ***Lygeidae*** | 2 | 1 | 5 | 2 | 3 | 4 | 4 | 3,00 |
| ***Staphilinidae*** | 3 | 2 | 5 | 2 | 2 | 4 | 3 | 3,00 |
| ***Reduvidae*** | 3 | 1 | 5 | 2 | 2 | 5 | 4 | 3,14 |

The use of the selection matrix can also be applied to other functional groups. In AMIGA work package 4, a selection of earthworm focal species was done considering available literature on this group of non-target soil organisms. For details see Van Capelle et al., (2016).

### 2.3 Case study 1: Effects on NTOs on transgenic and near-isogenic maize

**Plant material and chemical analyses**

1) Plant morphometrical determinations (leaf number, culm diameter and plant height) at 10, 20, 30, 40 days after sowing (DAS);
2) For each cultivar, 3 bulk samples of leaf tissue are collected at three different growth stages (early, medium and late stage);
3) During the flowering period, 11 g of GM pollen grains and 15 g of NIL pollen grains are collected and stored at -80°C.
4) Quantification of secondary plant metabolites (bound and free polyphenols) in leaf tissues;
5) Determination of Cry1Ab protein concentration in pollen grains and leaf tissues of GM and NIL samples, by ELISA methodology.

**Aphid life history**

Climatic conditions: 21:15°C L:D, 70%RH, 16:8 L:D photoperiod.
Replication: Individual plants are the unit of replication. Minimally 10 plants per potato- or maize line. Replicates can be accumulated over two or more rounds in sequence. Rounds can be defined as blocks in the statistical analysis.
Insect inoculation: 3 newborn aphid nymphs per plant, each its own clipcage. Feeding history of the aphid colony has preferably taken place on the conventional comparator line / NIL.

Observations:

- Daily monitoring until death, loss or reproduction in the second generation of aphids. Monitoring can be done starting 6 or 7 days after initial inoculation, to avoid unnecessary plant damage and to optimize workload. Once the time until first reproduction is known, aphids can be checked for daily fecundity for that length of time. This suffices for calculation of $R_m$.

- Two generations of aphids are monitored, the second generation on another plant of the same planting date, removing offspring as they are found; once the initial aphids have started reproducing, extra clip cages are attached to each plant to enclose each of the three newborn nymphs.

Measurement endpoints:

- Survival time
- Pre-reproductive period
- Intrinsic rate of increase

**Performance of the coccinellid aphid predator *Hippodamia variegata* feeding in *ad libitum* or food-limited conditions on maize aphids or pollen**

Climatic conditions: 25 ± 1°C, R.H. = 60-80%, = 16:8 L:D periods
Replication: Individual *H. variegata* adult females are the unit of replication. Minimally 10-15 ♀♀ per maize line. Replicates can be accumulated over two or more rounds in sequence. Rounds can be defined as blocks in the statistical analysis.

Bioassays are carried out in the laboratory to assess the demographic responses of the coccinellid *H. variegata*, feeding as larvae and adults on aphids (*Rhopalosiphum maidis*) reared on Bt maize and near-isogenic plants.
Potted corn plants are greenhouse-reared and when plants reach ≈1 m in height they are inoculated with *R. maidis*. For each treatment, 15 *H. variegata* adult females are individually maintained and daily monitored for oviposition and survival for 21 days, to collect necessary data for constructing the life tables. Each female coupled with a male is put in a Plexiglas cylindrical cage (Ø = 9 cm) that is covered with a screened lid. Each cage is lined on the inside with an air bubble plastic film to act as oviposition substrate. The adults are daily fed *ad libitum* with *R. maidis* infesting either Bt maize or near-isogenic plants. The coccinellids are allowed to feed also on honeydew since aphids are provided on pieces of leaf tissue. The number of eggs laid by each female, including the cannibalised ones, are recorded daily for 21 days. Any deceased males during the experimental period are replaced. The preoviposition period is calculated as the number of days between emergence and the first oviposition.
In order to determine preimaginal development time, preimaginal survival, and sex ratio, 10 newly emerged larvae are collected from different females of the two treatments. The operation is repeated every about 10 days throughout female lifespan until 50 larvae are collected for each treatment. The larvae are incubated at 25 ± 1°C, R.H.60-80%, L:D = 16:8 and placed individually in cylindrical containers (Ø = 4 cm) to avoid larval cannibalism. Larvae are fed *ad libitum* with *R. maidis* and examined every day. Only the individuals reaching adulthood are taken into consideration for determining development times. After emergence adults are sexed.
Demographic parameters, including net reproductive rate ($R_0$), intrinsic rate of increase ($r_m$), mean generation time ($T$), doubling time (DT), and finite rate of increase (λ) are calculated. Jackknife method is used to calculate the variability of parameters.
Life table data are also used to generate an age-classified Leslie projection matrix (as described in Stark *et al*. (2007) BioControl 52: 365-374) with the aim of modelling the impact that exposure to a prey reared on GM-plant would have on a population of coccinellids. The model consists of a matrix including survival probabilities ($P_i$) and fertilities ($F_i$) of a population. We multiplied this matrix by a starting population vector which contains information on the age distribution of the studied population. Population growth across time can be then found via repeated matrix multiplications.
Demography is used to evaluate the total effects, lethal and sublethal, of GM-plant-exposed population by means of a Life Table Response Experiments (LTREs) (as described in Caswell, 1996 Ecological Modelling 88: 73-82). Finally, an application of matrix models, the

Delay in Population Growth Index (as described in Wennergren and Stark, 2000 Ecol. Appl. 10: 295-302), a measure of population recovery, is calculated to compare the time required to a control population and GM-plant-exposed populations to reach a predetermined number of individuals. In this study population delay is determined by choosing a population size of 100,000 individuals.

**Performance of Coccinellids feeding in reduced-availability-of-prey conditions**
Protocol is as previously described but adult *H. variegata* females are provided with aphids once every three days, to expose the coccinellids to starvation stress.

**Performance of Coccinellids feeding on GM pollen**
Protocol is as previously described but adult *H. variegata* females are provided with pollen as food along with a low amount of aphids (for example *Myzus persicae*), to ensure oviposition.

**<u>Measurement endpoints for all coccinellid experiments</u>:**

- Offspring preimaginal development times, preimaginal survival, and sex ratio
- Adult fresh weight
- Fecundity, fertility, and preoviposition time
- Demographic parameters: net reproductive rate ($R_0$), intrinsic rate of increase ($r_m$), mean generation time ($T$), doubling time (DT), and finite rate of increase ($\lambda$)
- Delay in Population Growth Index
- Analysis of Life Table Response Experiments (LTREs)

### 2.4 Case study 2: Case study 2: Performance of the Green Peach aphid *Myzus persicae* and its parasitoid *Aphidius colemani* on potato resistant to P*hytophthora infestans*

**Aphid performance**
Intrinsic rate of increase and survival of aphids on non-transformed Désirée and six different GM Désirée events were quantified. Then, to test reproducibility, Wageningen University and ENEA performed similar experiments comparing specifically the cisgenic events A15-31 and A15-45 to the non-transformed Désirée. Lastly, we compared several conventional potato cultivars with these same measured endpoints.
Each experiment began with one day-old aphids, adults from the rearing were isolated on a potato leaf in a Petri-dish. Aphid nymphs were taken from the Petri dish after 24 h and placed singly in clip-cages (25 mm diameter; 10 mm high) on the abaxial surface of two (at ENEA) or three leaves (Wageningen University) on each plant. Ten (at Wageningen University) to fifteen (at ENEA) plant replicates of each event and the non-transformed Désirée cultivar were used, and randomly distributed in the climate room.

We monitored the performance of *M. persicae* for two generations. Aphids were checked every day for mortality and for offspring production; neonate nymphs were counted and removed daily. At Wageningen University, once the first generation produced its first nymphs, one of these was caged on another leaf of the same plant; at ENEA second generations were transferred to a new plant. The parameters collected were: pre-reproductive period and total fecundity, for calculation of intrinsic rate of increase ($R_m$), and aphid mortality of both generations. Intrinsic rate of increase was calculated as described in Wyatt and White (1977): $R_m = 0.74 (\ln Md) / d$, where Md is the effective fecundity and d the length of the pre-reproductive period.

The same methodology was applied to a second experiment in a greenhouse comparing the first generation of aphid life-history parameters on one cisgenic event (A15-31, highly resistant) and four conventional cultivars varying in their foliar resistance to *P. infestans*. Cultivar Bintje has a resistance rating of low to very low, cultivar Première and Désirée rate low to medium, and Sarpo Mira rates highly resistant to *P. infestans* (ECPD, 2015).

**Performance of the parasitoid *Aphidius colemani***

Climatic conditions: 21:15°C L:D, 70%RH, 16:8 L:D photoperiod.

Replication: Individual plants are the unit of replication. Minimally 10 plants per potato- or maize line. Replicates can be accumulated over two or more rounds in sequence. Rounds can be defined as blocks in the statistical analysis.

Insect inoculation: Individual plants (3 weeks old) are enclosed in a glass container, its opening covered by textile netting and a rubber band or in a similar suitable container of cage. Twenty *M. persicae* 2$^{nd}$ to 4$^{th}$ instar nymphs were allowed to feed on the plant for 24 hours, after which a mated female of *A. colemani* (naïve, i.e. having no previous experience with plant or host) is introduced that can parasitize the aphids for 24 hours, and is then removed. The infested plants are checked daily in order to locate mummies. Each mummy found is placed in a Petri dish on humid filter paper. Petri dishes were checked every day for adult eclosion and moisture suplemented. Once the adults emerge, their sex is identified and their fresh weight is recorded.

Since mummies can be difficult to locate, and location can be inaccurate (found a day after it forms, or found at a different stage of formation), it might be better and easier to check the containers daily for emergence of adult parasitoids and remove parasitoids as they emerge inside the containers. This will still give us the measure of total development time (from parasitism until emergence) and parasitism rate.

**Measurement endpoints:**

- Parasitism rate
- Failure rate (parasitoids failing to eclose on a plant divided by total # mummies on the plant) (accuracy may be lost if not all mummies are found- and this is sometimes difficult)
- Total parasitoid development time (from parasitism date to adult emergence)

- Adult fresh weight

**Parasitoid behavior**

Odour preference behaviour of mated female aphid parasitoids is observed by allowing them to choose between two different odour sources consisting of plants and aphids in a Y-tube olfactometer (Figure 1) .

Climatic conditions: 20-24°C, 70% RH, 16:8 L:D photoperiod.

Replication: Plant-pairs are the unit of replication. Minimally 10 plant-pairs per potato- or maize lines. Replicates can be accumulated over two or more days in sequence or spaced with intervals of one or a few days. Days can be defined as blocks in the statistical analysis.

**Y-tube olfactometer assay**

Climatic conditions: 22 ± 2 °C.

The set up consists of two glass jars (30 liters), each connected to one of the two branches of a glass Y-tube olfactometer (Figure 3; diameter 3.5 cm, length of the stem 22 cm). Pressurized air is charcoal-filtered before passing through the two glass containers. Each of these contains one (or more; the same number of similarly sized plants per container) potato or maize plants, 3-4 weeks old, infested with 100 *M. persicae* nymphs (mixed instars) for 48 h before the Y-tube test. The airflow from the containers is kept at 2,3 L $min^{-1}$ through both branches of the Y-tube. Ca. 1 m above the setting two fluorescent tubes (32 W, Philips) are placed to obtain a homogeneous light distribution; the set-up is shielded at the back and the sides with white cloth or paper. In order to limit odors from soil and plastic, the pots are completely covered with aluminum foil.

For each choice test, e.g. comparator line vs. a transgenic line, minimally 10 plants are tested pairwise and 20 wasps are tested per plant pair; after testing 10 half of the parasitoids per plant pair, the jar position was changed to avoid positional bias. In total 200 wasps are studied per choice situation between plant comparator – trans/cisgenic line. Each wasp is released individually into the Y-tube and a choice was made if she crossed the far line close to the odour source. If she crossed the first line (2 cm after the split) then 15 minutes was allowed to make a choice. If she did not cross the first line before 10 minutes, “no choice” was assigned.

**Identification of plant volatiles**

During Y-tube bioassays, headspace trapping of plant volatile organic compounds and their chemical analysis was performed according to the methods described by Kos *et al.* (2013) Pest Management Science 69: 302 -311.

**Measurement endpoints:**

- Choice distribution over comparator and trans/cisgenic lines
- Time taken until a choice was made
- Volatile emission

**Wind tunnel assays**

The wind speed, monitored with a hot-wire anemometer (Alnor, model CGA-26), was adjusted to 20 + 1 cm/sec in all experiments. The exhausted air was vented outside the wind-tunnel rooms. The light intensity in the flight chambers was around 3600 lux at the take off point. A black and white striped pattern was placed beneath the flight chamber floors to aid visual orientation. Experiments were carried out at 20 + 1°C and 65 + 5% relative humidity.

Individual parasitoids were used once and released from an open-ended glass test tube held on a plastic base so that the take-off point was exactly in the middle of a cross section of the flight chamber. Between 40 and 80 parasitoid females were tested for each target by releasing them individually in the odor plume, 20 cm downwind from the target. Parasitoids were observed for a maximum time of 10 min, and flight behavior data were recorded and analyzed with the aid of event-recording software (The Observer, Noldus Information Technology, Wageningen, The Netherlands.) A flight response was considered as oriented towards the experimental target when the female flew directly upwind and landed on or not further than 5 cm away from the target. Experiments were conducted on several days, and targets were presented in a random order to reduce the effect of uncontrolled temporal variability of behavioral responses.

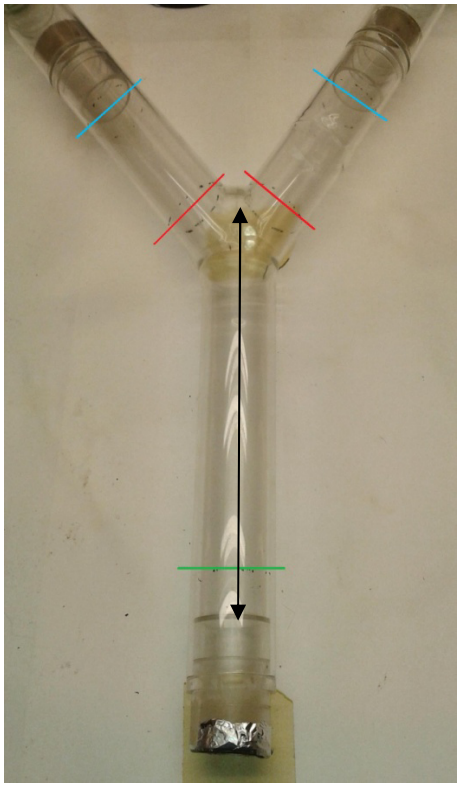

**Figure 3**: Y-tube olfactometer. Wasps are released on the green line. The red and the blue ones are the choice lines: respectively first choice and final choice. The double-headed arrow is 22 cm long.

## 3. References


Caswell (1996) Ecological Modelling 88: 73-82.
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Southwood, T. R. E., & Henderson, P. A. (2009). *Ecological Methods*. John Wiley & Sons.
Van Capelle *et al*. (2016) Science of the Total Environment 548–549: 360–369.
Wennergren and Stark (2000) Ecological Applications 10: 295-302.
Wyatt & White (1977) Journal of applied Ecology 14: 757-766.

## Appendixes

### Appendix 1A. Field plot protocol (pitfall traps)

| **Location:** | | | **Plot no.** | | **GPS co-ordinates of plot:** | |
|---|---|---|---|---|---|---|
| **Crop:** | | | **Soil type:** | | **Soil texture**: | |
| **Visit** | **Date** | **Recorder** | **BBCH stage[1]** | **Weed score[2]** | **By-catch[3]** | **Notes[4]** |
| 1 | | | | | | |
| 2 | | | | | | |
| 3 | | | | | | |
| 4 | | | | | | |
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| 6 | | | | | | |
| 7 | | | | | | |
| 8 | | | | | | |
| 9 | | | | | | |
| 10 | | | | | | |

[1] BBCH stage of crop according to Lancashire et al. (1991), Ann. Appl. Biol. 119, 561-.

[2] Estimate of weed cover: 0 = none/low weed density, or weed control successful; 1 = medium weed density, or weed control only partly successful; 2 = high weed density, or weed control not successful.

[3] What by-catch was caught, rough categories such as rodents, reptiles, amphibians, number of individuals.

[4] Any other relevant information, number consecutively and describe on back page, e.g. extreme weather events, mass occurrence of pests, damage to plants, pitfall trap incidents (no trapping fluid, overflowing trapping fluids, dug-out traps, etc.).

**Appendix 1B. Field plot protocol (plant dwelling arthropods on maize)**

| **Location/site:** | **Plot no.** | **GPS co-ordinates of plot:** |
|---|---|---|
| **Date:** | **Maize growth stage:** | **Recorder:** |
| **Notes:** | | |

| **Taxon/guild** | **Low1**[5] | **Low 2** | **Low 3** | **Low 4** | **Low 5** | **Low 6** | **Upp1**[6] | **Upp2** | **Upp3** | **Upp4** | **Upp5** | **Upp6** | **Flower**[7] | **Cob**[8] |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | | | | | | |
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| | | | | | | | | | | | | | | |

5 The first 4 intact leaves counted from the ground qualify as "Lower Leaves".

6 The first 4 intact leaves counted from the top qualify as "Upper Leaves".

7 Insert individual numbers of the 12 plants in this cell, separated by semicolons.

8 Insert individual numbers of the 12 plants in this cell, separated by semicolons.

**Appendix 1C. Field plot protocol (plant dwelling arthropods on potatoes)**

| **Location/site:** | **Plot no.** |
|---|---|
| **Date:** | **Plant growth stage:** |

| **Plant No.** | **Taxon/guild** | **Low1** | **Low 2** | **Low 3** | **Up1** | **Up2** | **Up3** | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | | | | |
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**Appendix 2: Site description**

<table>
<tr><td colspan="2">Name and location of plot:[9]</td><td>Year:</td></tr>
<tr><td colspan="3">Name of the recorder:</td></tr>
<tr><td rowspan="2">GPS co-ordinates (centre of location)[10]:</td><td colspan="2">Altitude (m a.s.l.):</td></tr>
<tr><td>Current crop:</td><td>Crop previous year:</td></tr>
<tr><td colspan="3">Field management, type and date[11]:<br><br>Field boundaries</td></tr>
</table>

[9] Make sure there is on-site recording of the main weather parameters: temperature (at soil level), rainfall, sunshine hours. If possible, measure temperature by using data loggers deployed directly in the plot/s.

[10] Copy and print a Google Map of the site (satellite view), marking the site. Draw a map of the plot arrangement with measurements (distance between plots), provide information on cover/surface between plots (e.g. grass, bare ground). Attach the map to the site description sheet.

[11] Description of any management measures carried out, e.g. sowing/harvest dates, crop density, application of pesticides (brand, date, application rates), type and date of mechanical treatments (e.g., ploughing), etcetera. If necessary, use a separate sheet and clip to site description sheet.

***Pitfall traps***

*Implementing the traps*

- 2 outer and 2 inner cups (pitfalls) per sampling plot
- 2 one meter long barriers per sampling plot
- Ethylene glycol (70% and with a drop of odourless detergent)
- 2 non-transparent rain covers per sampling plot
- Spade, small shovel
- A measuring meter
- Field plot protocol sheets
- Writing board
- Pencils
- Eraser
- Waterproof pen
- Camera
- GPS
- Data loggers (if any)
- Gloves
- Paper tissue
-

*Collecting the catch*

- Metal tea strainer/sieve (if you use sieves), or nappy liners, elastic band (if you use nappies)
- Sampling containers (to take the catch of the traps)
- Collecting bottle for dirty trapping fluid
- Extra 70% ethylene glycol in squeeze bottle (for rinsing the pitfalls)
- Squeeze bottle with alcohol
- Funnel
- Extra pitfall cups, covers (2 – 3) and barrier (to replace damaged ones)
- Pre-printed trap labels
- Field plot protocols
- Writing board
- Pencils
- Eraser
- Waterproof pen
- Scissors
- Forceps
- Camera
- GPS
- Dataloggers (if any)
- Gloves
- Paper tissue

**Plant dwelling arthropods**

- Field protocol sheets
- Writing board
- Sampling protocols, or plastic bags, scissors & waterproof pen (if leaves are cut and collected to laboratory)
- Pencils
- Eraser
- Pincers
- Sheet with definitions of BBCH growth stages
- Camera
- GPS
- Dataloggers (if any)
- Aspirator (to collect individual arthropods from leaf or stalk)

## Appendix 3B. Predation pressure measurement

### Materials required

Green plasticine
Modified garlic press
Small pieces of bamboo or reed stick
Instant glue (attack or similar) – we found a gel is better than fluid glue – the latter tends to run and spread. The aim is to use the smallest necessary amount of glue, and on a small spot only. This is usually enough to keep the caterpillar in position.

Take a small blob of plasticine that fits the garlic press. Massage it in your hands to warm it up – when warmer, it is softer, and easier to work with it. Once malleable, place it in the garlic press. Make sure the blob is solid and that there are no bubbles inside. With a gentle, steady pressure, expel the plasticine through the hole to produce a ”snake”. Make sure the surface where the ”snake” emerges is clean (use a clean sheet of A4 paper, for example).
Once cut, examine the snake for any marks – it should be completely smooth. If necessary, you can cut away parts of it, or roll it by hand, up-and-down, on the smooth paper sheet.
Once with smooth surface, use a sharp knife to cut the ”snake” into even pieces, ca. 2 cm long. Handle them very gently so as not to make marks on them. Always work on clean, non-dusty surfaces.
Take a piece of bamboo or reed, and using a small drop of glue, glue one caterpillar to the stick. Press only gently, not to distort the caterpillar. Put it in a small container so that it does not get ”marked” while you transport it to the field.
Once in the field location, you can place the stick, with the caterpillar on it, at a selected spot. Try to avoid direct sunlight – the plasticine tends to become very soft at ca. 35 C. You can place the caterpillar directly on the soil surface, or on the plant. If you want, you can also glue the caterpillars directly onto plants, but this requires very gentle handling. In this case, place a drop of glue onto the caterpillar, and gently push it against the surface where you want to fix it. Keep it pressed for a few seconds so that the glue sets.

Pilot study experiments indicate that if the distance between caterpillars is 1m (we call this grouped, 5 caterpillars were in one group) vs. 5 m, the overall % predation is not different. However, at the group level, the probability that at least one caterpillar is attacked is higher than for individually placed (5 m distance) caterpillars.

Do not leave the caterpillars out for too long – we usually do a 24 h exposure. Longer exposure may confound the results – dust can stick to the caterpillar, esp. if it is windy, etc.

Make sure you identify the place where the caterpillars were put. Marking is useful – but not directly near the caterpillar. It is very difficult to find them again, even after just 24h!