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Standardised methods for the GMO monitoring of butterflies and moths: the whys and hows

Andreas Lang[1], Bernhard Theißen[2], Matthias Dolek[3]

1 *Umweltgeowissenschaften, Universität Basel, Bernoullistrasse 30, CH-4056 Basel, Switzerland* **2** *Research Institute for Ecosystem Analysis and Assessment e.V. - gaiac, Kackertstr. 10, 52072 Aachen, Germany* **3** *Büro für Ökologische Forschung und Planung, Obere Dorfstr. 16, D-82237 Wörthsee, Germany*

Corresponding author: *Andreas Lang* (lang@biologie.de, andreas.lang@unibas.ch)

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Abstract

Butterflies and moths (Lepidoptera) are related to many biotic and abiotic characteristics of the environment, and are widely accepted as relevant protection goals. Adverse effects on butterflies and moths through genetically modified (GM) crops have been demonstrated, by both insect-resistant and herbicide-tolerant events. Thus, Lepidoptera are considered suitable bio-indicators for monitoring the potential adverse effects due to the cultivation of GM crops, and guidelines were developed under the umbrella of the Association of German Engineers VDI (Verein Deutscher Ingenieure), entitled "Monitoring the effects of genetically modified organisms (GMO) – Standardised monitoring of butterflies and moths (Lepidoptera): transect method, light trap and larval survey". Here, the background and rationale of the VDI guidelines are presented, including a summary of the methods described in the guidelines. Special emphasis is given to the discussion of underlying reasons for the selection and adjustment of the applied methodology with respect to the GMO monitoring of day-active Lepidoptera, of night-active moths and of the recording of lepidopteran larvae, as well as to sample design and strategy. Further aspects possibly interfering with monitoring quality are treated such as landscape patterns, low species number and abundance in agro-ecosystems, or high year-to-year fluctuations of populations of Lepidoptera. Though specifically designed for GM crops, the VDI guidelines may also serve as a template to monitor the effects of a wider range of adverse factors on Lepidoptera in agriculture.

Keywords

Lepidoptera, non-target organisms, indicators, genetically modified plants, monitoring methods, transect count, light trapping, larval survey, beating sample, standardization, cost-efficiency

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Introduction

In the European community, the Directive 2001/18/EC on the Deliberate Release into the Environment of Genetically Modified Organisms (GMO) stipulates a monitoring plan in order to trace and identify any harmful effects on human health or the environment of GMO after they have been placed on the market (EC 2001). Guidelines with regard to the requirements for monitoring design, sampling methods and analysis techniques are outlined in further documents of the European Community (EC 2002, EFSA 2011). Under the umbrella of the Association of German Engineers VDI (Verein Deutscher Ingenieure; Züghart et al. 2013, this issue) guidelines for the GMO monitoring of Lepidoptera were developed, entitled VDI Guidelines 4330, Part 13, "Monitoring the effects of genetically modified organisms (GMO) – Standardised monitoring of butterflies and moths (Lepidoptera): transect method, light trap and larval survey" (VDI 2010).

Butterflies and moths were the first biological indicators considered for the purpose of GMO monitoring within the VDI guidelines series. In general, a bio-indicator can be defined as "a species or group of species that readily reflects the abiotic or biotic state of an environment, represents the impact of environmental change on a habitat, community, or ecosystem, or is indicative of the diversity of a subset of taxa, or of the wholesale diversity, within an area" (McGeoch 1998). Fleishman and Murphy (2009) define indicators as "scientifically reliable, cost-effective measures of the status or trend of an environmental phenomenon that is scientifically or logistically challenging to measure directly". Lepidoptera (butterflies and moths) are significantly related to various abiotic and biotic characteristics of the environment, e.g. to temperature, humidity, (micro)climate, soil nutrients, vegetation, habitat structure, or landscape patterns (Oostermeijer and vanSwaay 1998, Collinge et al. 2003, Aviron et al. 2007a, Settele et al. 2009a). Therefore, butterflies and moths have often been suggested and applied as suitable indicators for the monitoring of environmental quality and changes (e.g. Brunzel and Plachter 1999, Virtanen and Neuvonen 1999, Nowicki et al. 2008, Pascher et al. 2009), most recently as one of the major indicators to monitor and assess biodiversity change in Europe (EEA 2007, 2010; but see Fleishman and Murphy 2009 for a critical evaluation). For example, the monitoring data of Lepidoptera have been successfully used to detect declines of species and species richness (Maes and Van Dyck 2001, Conrad et al. 2004, Wenzel et al. 2006, Nilsson et al. 2008), to assess the effects of agri-environmental schemes (Aviron et al. 2007b, Roth et al. 2008, Merckx et al. 2009a), to monitor the impact of land use change (Ricketts et al. 2001, Feber et al. 2007, Merckx et al. 2009b, Stefanescu et al. 2009, van Dyck et al. 2009), to record direct effects of management measurements in arable land (Field et al. 2005, 2007, Dover et al. 2010), to indicate adverse effects of pesticide use (Johnson et al. 1995, Longley and Sotherton 1997, Severns 2002, Russell and Schultz 2010), or to assess the effects of climate change (Settele et al. 2008, VanSwaay et al. 2008a, Pearman et al. 2011).

The features contributing to the value of Lepidoptera as environmental indicators further include the good knowledge on their faunistics, ecology and conservation biology (e.g., Settele et al. 2009b), relatively easy identification of species and the presence of field guides (e.g. Skinner 1998, Settele et al. 2009c, Bachellard et al. 2007), and the wider public acceptance of Lepidoptera as valuable, environmental protection goals (Brunzel and Plachter 1999). There exist well-developed and scientifically sound monitoring methods for the recording of butterflies and moths, and their larvae, i.e., transect counts for day active-butterflies, light trapping for night-active moths, and searching protocols for larvae (Pollard and Yates 1993, Southwood and Henderson 2000, Hermann 2007). Consequently, many routine monitoring schemes for Lepidoptera are already implemented and running (e.g., Pollard and Yates 1993, Weber et al. 2004, VanSwaay et al. 2008b, Schmeller et al. 2009, Kühn et al. 2010). In addition, Lepidoptera fulfil important ecological key roles as herbivores, pollinators and prey organisms in many terrestrial ecosystems, and, depending on the specific circumstances, can be representative for general biodiversity, thus potentially indicating changes in other animal groups and plants (Thomas 2005, Thomas et al. 2004) or habitats (van Swaay et al. 2010).

Most important, adverse effects of genetically modified (GM) plants on Lepidoptera have already been reported, which strongly supports their quality and significance for an appropriate GMO monitoring (Graef et al. 2005). Currently, the major events of GM plants developed and being cropped worldwide are insect-resistant and herbicide-tolerant (Kvakkestad 2009). Pollen of insect-resistent *Bt* (*Bacillus thuringiensis*) maize toxic to pest Lepidoptera may be drifted by wind onto host plants of non-target lepidopteran larvae growing nearby (Pleasants et al. 2001, Lang et al. 2004). Non-target lepidopteran larvae may be affected adversely by consuming this pollen attached to their host plants (e.g., Dolezel et al. 2005, Lang and Vojtech 2006, Lang and Otto 2010). The combination of transgenic, herbicide-tolerant crops together with the application of broad-spectrum herbicides, such as glyphosate or glufosinate-ammonium, is likely to change the herbicide regime, which can reduce the weed community within fields and in field margins, in turn affecting larval and adult butterflies associated with such food plants (e.g., Haughton et al. 2003, Roy et al. 2003). Direct toxic effects of the complementary broad-spectrum herbicides on non-target Lepidoptera have received less attention, but have been reported for glufosinate (El-Ghar 1994, Kutlesa and Caveney 2001). Potentially, cultivation of the above transgenic events put at risk non-target butterflies and moths occurring in agro-ecosystems as well as protected species living in habitats near the GM fields (Traxler et al. 2005, Hofmann and Schlechtriemen 2009).

The VDI guidelines describe the monitoring of Lepidoptera (butterflies, moths) as a whole, for adults as well as for larvae. This ensures that all potential effects of GM plants on Lepidoptera are covered adequately and increases the detection probability of such effects, thus meeting the required criteria for a GMO monitoring formulated by the EC (2001, 2002). Here, we present an overview over the VDI guidelines (VDI 2010), with special reference to the suitability of Lepidoptera as indicators, the rationale underlying the choice of the described monitoring methods and approaches as well as given problems, open questions and further recommendations.

The whys: the rationale behind the VDI guidelines

Sampling design and strategy

With regard to a GMO monitoring, various critical aspects are associated with farming practices and the possible distribution of GM and GM-free fields and areas. A major issue is that fields of GM crops and non-GM crops will vary in quantity and location from year to year, depending on implementation and use of transgenic crops, on cultivation practices, and crop rotation. It is important to note that sampling design and strategies can depend on the necessity to reveal cause-effect relationships of possible adverse GMO effects. This is accounted for by EC regulations and recommendations by separating the monitoring into g*eneral surveillance* and *case-specific monitoring* (EC 2001, 2002, EFSA 2011; see also Züghart et al. 2013, this issue). Case-specific monitoring should, when included in the monitoring plan, focus on potential adverse effects of GMOs that have been identified in the previous environmental risk assessment (ERA). Thus, a case-specific monitoring plan would serve to confirm or reject the assumptions of the ERA, and case-specific monitoring should address specific hypotheses associated with identified potential effects of the GM crop (EC 2002). In contrast, general surveillance should focus on unanticipated and unforeseen as well as on possible delayed and long-term effects that were not predicted in the risk assessment, and if unexpected changes in the environment have been observed, further risk assessment may need to be considered to establish whether they have arisen as a consequence of GMO cultivation (EC 2002). Often, a clear distinction between case-specific monitoring and general surveillance will not be possible or reasonable, and a combination of both approaches will make more sense, e.g. when focusing on protection goals (Raps 2007).

Environmental effects may manifest differently in dependence of the spatial scale observed. Some adverse effects may only be detectable on a larger landscape scale and be masked on smaller local plots, e.g., small and chronic effects on meta-populations. For example, adverse pesticide effects on ground beetles (Coleoptera: Carabidae) were detected only on larger sampling plots but not on smaller ones (Duffield and Aebischer 1994), and this scale effect varies among different arthropod taxa to a different degree (Prasifka et al. 2005).

The interfering influence of agricultural practices and scale effects can be best encountered by applying two different monitoring approaches at the same time: small mobile monitoring sites (local monitoring), and larger stationary monitoring sites (landscape monitoring) (VDI 2010). The local monitoring is to be installed in and along GMO fields, and the locations change every year following GMO cropping (see above). A paired field approach, GMO field versus GMO-free field, enables a treatment comparison and potential effects can be attributed better to a specific location. This approach of paired fields in close proximity reduces the influence of environmental heterogeneity on data variance, and a paired approach provides a higher statistical power. However, care has to be taken that data dependence such as spatial autocorrelation does not decrease the power of the statistical test. On a landscape scale, sampling locations are fixed and stationary, and sampling should cover a wider area, e.g. longer routes for transects counts or more light traps. The comparison of sample grid squares or regions differing in the proportion of GM crop cultivation could enable a treatment comparison (Bühler 2006, 2007, Bühler et al. 2008).

Monitoring day-active Lepidoptera

Different approaches exist to record and monitor day-active Lepidoptera including complete inventories of a given sample area, standardised line-transect counts, point counts, distance-sampling, or mark-release-recapture (e.g., Hermann 1992, Pollard and Yates 1993, Mühlhofer 1999, Sutherland 2006, Nowicki et al. 2008). In the VDI guidelines (VDI 2010), the commonly applied transect-count approach is described in a standardised way (Pollard and Yates 1993, VanSwaay et al. 2008b). Line-transect counts are well suited to large homogeneous habitats, are highly adaptable, cheap and quite efficient in terms of recorded quantity and quality of data in relation to sampling effort, can be used to survey individuals, species or groups of species, and various indices can be calculated from the data (Pollard and Yates 1993, Sutherland 2006, Nowicki et al. 2008). Recently, transect counts are widely applied for the monitoring of species of the Habitats' Directive (EC 1992), based on species-specific guidelines (see e.g. in Leopold and Fartmann 2005). However, it is important to note that transect counts generate indices of relative abundance but not precise estimates of population densities, though transect count data can be correlated with population size, sometimes to a high degree (Haddad et al. 2008, Nowicki et al. 2008). Among others, differing detection probability by species, observer, region, year and season can bias the data recorded (e.g., Kery and Plattner 2007). Differing detection probabilities should be no major problem when a paired sample approach is applied (see below), in all other cases it should be taken into account as far as possible (Kery and Plattner 2007). Rough estimates of detection probabilities can be achieved without marking individuals e.g. by distance sampling (see below); some freeware programs such as MARK or PRESENCE might also be helpful in this respect. However, mark-release-recapture (MRR) methods are most often used to calculate detection probabilities for species as well as estimates of population sizes and survival probabilities (e.g., Haddad et al. 2008). Yet, MRR methods are quite laborious, may not be adequate for highly mobile species with low recapture rates, and are potentially harming the marked individuals or changing their behaviour (Sutherland 2006). The high temporal and financial investment involved for MRR is the main obstacle for their routine use in long-term monitoring programmes. However, it may be appropriate to utilise MRR for special needs such as calculation of species detectability or for monitoring rare and endangered species, and a simplified MRR approach reducing the cost of common MRR surveys by about two-thirds have been suggested by Nowicki et al. (2005). Another rarely applied method is distance sampling combined with line-transect counts, where the distances of the observed butterflies to the transect are recorded. Distance sampling provides estimates of density and detectability, but is a bit more laborious than simple transect counts, and the assumptions for applying this method may not always be met (Sutherland 2006, Nowicki et al. 2008). Due to the above described characteristics, the common transect count method appeared as the most appropriate approach with regard to invested effort and recorded results.

Sampling effort is mainly determined by the number of visits and the length of the transect, and both strongly influence monitoring results. For GMO monitoring we suggested eight visits regularly distributed over the season, in order to cover the seasonal flight periods of all species and generations (see below; VDI 2010). The number of visits is usually a critical factor in maximising species detection (Pellet 2008). Thus, a higher number of visits ensures a better detection of potential adverse GMO effects, because it is not known beforehand which species or which generation of a certain species may possibly be affected. It has been argued that less than eight visits could still produce reliable data for a butterfly monitoring, although this has to be balanced by a higher number of monitoring sites (e.g. several hundreds depending on estimated power, see Roy et al. 2007, Brereton et al. 2011). It also depends on the transect length: longer transects (e.g. 2.5km) may still produce a sufficient number of species and abundance observations with 4 – 5 visits per season if counts are done during the predominant flight periods of butterflies (Lang, Bühler and Dolek, unpublished data). However, shorter transects (e.g. 250m) are less species-rich and much more variable in the number of species and individuals being observed (Lang and Bühler 2012), and less than 7 – 10 visits per season will drop monitoring results of observed species number in short transects drastically (Lang, Bühler and Dolek, unpublished data). This is especially true in species-poor agricultural settings, while in species-richer habitats a lower number of surveys may have less effect. In the VDI guidelines (VDI 2010), paired transects along specific GM field and control fields are suggested, i.e. transects would often be of a shorter type requiring a higher number of visits.

Often, day-active moths are also recorded within the framework of butterfly monitoring schemes, which is not generally requested by the VDI guidelines, but only for Burnet Moths (Zygaenidae). In addition, the VDI guidelines include the monitoring of Crambid Snout Moths (Lepidoptera: Pyralidae, Crambinae). The Crambid Snout Moths (Crambinae) are an important subfamily of the Pyralidae of about 80 species occurring in Central Europe with a few abundant species that can regularly be expected at field margins (Küppers 2008, Slamka 2010). Crambid Snout Moths are a useful supplement for the transect counts of adult butterflies as they are wide-spread, common and abundant in agricultural land, easy to catch and the species are easily identified (Slamka 2010). Especially in intensively managed agricultural habitats, the Crambinae might be more prevalent than other Lepidoptera species, thus supporting the GMO monitoring in butterfly-species poor transects (Lang et al. 2011).

Monitoring night-active moths

General surveys of moth diversity are not as common as monitoring of day-active butterflies. Nevertheless, since several decades monitoring schemes exist for the purpose of pest control as several species are harmful to crops like apple (e.g. *Cydia pomonella*) and maize (*Ostrinia nubilalis*), or in forestry e.g. in fir forests (e.g. *Lymantria monacha*). Recently, the interest in conservation monitoring of moths increased, and a few national organizations implemented first monitoring networks (Groenendijk and van der Meulen 2004, Parsons 2004).

The most effective and widely used method to assess the moth fauna is light trapping (Southwood and Henderson 2000). It utilises the moths' phototropic behaviour by attracting them to an artificial light source. Alternatives are pheromone and bait trapping which make use of the mating and foraging behaviour. In the case of a comparative monitoring of one or more sites light trapping is the most effective method to assess a diverse and representative portion of the moth fauna. Like the above described transect counts, light trap catches generate relative abundance and not population densities. Species are attracted to light traps in a species-specific amount and different numbers of attracted individuals of different species do not necessarily reflect different population densities.

The general problem of light trapping is that the individuals are *attracted to* the trap – thus it is not directly possible to draw a conclusion from the location where the species have been trapped to the habitat they were attracted from (but see Wirooks 2005 for a detailed study of the spatial relationship of light catches and origin of specimens, which may help to address this problem). Unfortunately, attraction to light is dependent on many factors that have an influence on the activity and mobility of species, e.g. the actual and recently prevailing weather conditions (temperature, rainfall, wind) and the attractiveness of the light trap. It is important to account for these factors with regard to a correct interpretation of light trap results. In the VDI guidelines, the method is adjusted to a monitoring of definite plots. Basically, the attraction of moths is limited by reducing the illuminating power to the plot as far as possible. Using a common light trap of the Minnesota type – which is suggested here – gives a few options to limit the catchment area. First, the power of the light source and the spectrum of the light can be influential on the catch. We suggest UV-light (highly attractive to most species, Cleve 1954), with a low total emission of radiation (Fayle et al. 2007) and with a low power (8W). This combined approach attracts individuals within an area of ~50m around the trap under optimum conditions (no moonlight or other competitive light sources) (Wirooks 2005). In addition, the height of the trap is influential. The light source should be adjusted to reduce the horizontal dimension of the catchment area. We recommend installing it just above the plots' vegetation. In habitats/crops with low growing vegetation or when the vegetation is low due to the season, the light source should be adjusted as low as technically possible. For the comparison of the matched-pair design, we assume a similar height of the vegetation in both plots. If the plots' vegetation, however, differs more than one meter in height, catches should be interpreted with some caution.

In addition to reduce the catchment area, it is obligatory to be well informed about the potential larval food plants in the plots and adjacent habitats in order to conclude on possible indigenous occurrence of the specimens.

In central Europe, moths can be trapped throughout the year. Even in early spring or winter several species are on the wing. For the sake of reducing sampling effort, the monitoring of moths should start in the second half of April and end in late August including at least eight sampling occasions (VDI 2010).

Light trap surveys should wherever possible be performed from sunset to sunrise during dry, dark nights with little wind (anticipated wind speeds < 4 Bft), otherwise too few moths are active or attracted to the traps. It is essential to install death traps using e.g. chloroform as a killing agent, and provide structures to hide within the trap. This avoids external damage to the wings caused by flying individuals and wing patterns are essential for species identification. All specimens are identified and counted in the laboratory, which ensures a high quality and reliability of species identification, and specimens can be stored as evidence. Species that can only be distinguished by genital-morphology are assigned to a species complex, thus preventing misidentification by less experienced experts.

Monitoring lepidopteran larvae

So far, the monitoring of butterflies and moths has been strongly biased to adults (Nowicki et al. 2008), although the larvae of many native species can be recorded quite reliably (Hermann 1999). In the VDI guidelines, a list of 50 species is presented of which the larvae of a subset of at least five species should be monitored within a GMO monitoring (see below; VDI 2010). The species selection is based on a strong relation of the larvae to habitats predominant in the agricultural environment, such as fields, field margins, grassland and hedgerows, as well as on their detection and identification possibilities. The analysis and choice of species is based on experiences of the VDI working group and a literature survey (e.g., Ebert and Rennwald 1991, Ebert 1994-2003, Ebert 2005), additionally supported by the input of 27 lepidopteran experts, who answered to a questionnaire. Nevertheless, knowledge on species and larvae occurring in agro-ecosystems is limited but rapidly increasing, hence the presented species list should be checked and up-dated regularly, to allow for correction in species-specific monitoring methods, species determination, habitat use, conservation biology, or known impacts of GM plants.

Recently, the study and monitoring of larvae has received more attention (e.g., Hermann 2007). However, standardised and quantitative methods for a routine monitoring of larvae have not generally been developed, with only a few exceptions, mainly for species of the annexes of the Habitats' Directive (EC 1992) or other species of high conservation interest. Hence, the larval monitoring presented in VDI (2010) is breaking new ground to some extent. In our view, it is essential to start with the respective host plants rather than with a sole and limited focus on the lepidopteran species itself.

These host plants must be monitored for larvae by visual search according to a certain standardised protocol (see Annex in VDI 2010). Admittedly, the efficiency of a visual search approach depends on the current knowledge on larval biology and the (increasing) experience of the recording persons, which adds a certain amount of uncertainty and is difficult to assess in terms of standardisation. It is obvious that monitoring of larvae is more time consuming than monitoring adults per transect counts or light traps. Lang et al. (2011) tested the efficiency of the larval survey described in the VDI guidelines. They concluded that monitoring of larvae in agro-ecosystems is feasible and cost-efficient if the involved host plants and Lepidoptera species are common and abundant in arable land, and sufficient knowledge on the biology of the respective larvae exist. Thus, a monitoring of nettles (*Urtica*) was suggested (Lang et al. 2011), which are abundant plants in arable land (Gathmann et al. 2006), often grow in margins along fields, and host several larvae of common Lepidoptera species (Ebert 2005). In landscapes with a high proportion of bushes and hedgerows, beating samples on abundant shrub species that host common species can aid in collecting larvae in such habitats (IOBC/WPRS 1975). Generally, beating and visual search are two completely different sampling methods, which are applied in order to record different species groups. As a rule, visual search of host plants other than *Urtica* was reported to be laborious and to yield only low numbers of recorded larvae (Lang et al. 2011). Therefore, visual search of host plants other than nettles appears less suitable for a general routine monitoring (Lang et al. 2011). It may be used in a more case-specific and supplementary way, e.g., if certain species (groups) are to be monitored for protection purposes or if some species are known to occur abundantly (e.g., Lindzey and Connor 2011). However, this result might to a certain extent be driven by a lack of detailed knowledge on suitability of host plants, seasonal and diurnal timing, larval behaviour, and preferred plant parts. Often, this kind of knowledge is presented in handbooks, and is also included in the VDI guidelines to some extent (VDI 2010), but the development of larval studies of threatened species and especially species of the annexes of the Habitats` Directive shows that detailed studies of certain species can also generate new methodologies on larval monitoring (see examples in Dolek and Geyer 2005, Dolek 2006, Leopold and Fartmann 2005).

The hows: the approach of the VDI guidelines

As the European Community itself (EC 2001, 2002) demands a scientifically thorough monitoring plan in case of GMO releases, a comprehensive and multipart framework needs to be developed and established. The guidelines (VDI 2010) summarize methodologies on the monitoring of Lepidoptera from the choice of methods for surveying butterflies and moths in the field, the timing and frequency of fieldwork, over the appropriate design of study areas to a framework of data analysis and quality control. The suggested methodologies are based on well-established and widely applied procedures, but they are adapted to the specific requirements of GMO monitoring plans in agricultural landscapes. For fieldwork, the guidelines are structured in two pillars:

Pillar I: Survey of imagines. Species inventory and abundance of adult Lepidoptera are collected.

Pillar II: Survey of larvae. Abundance of larvae of selected species are recorded on host plants.

Both pillars supplement each other and have a different focus. In a monitoring plan, both must be applied.

How to survey butterflies and moths?

Within Pillar I the general survey of Lepidoptera is performed by two methods: Standardized transect counts during the day and light-traps during the night. Both methods are widely applied, but had to be adapted to suit the specific requirements of a monitoring scheme in an agricultural landscape.

To cope with the species-poor habitats, transect counts during the day include besides butterflies (Papilionidea et Hesperioidea) and burnet moths (Zygaenidae) additionally crambid snout moths (Crambinae). Some species of these crambid snout moths are widespread and abundant. They also occur on grassy field margins. Transects are split into sections of 50m length, if possible, each transect should be 20 sections long. However, transects of a length of 1km (20×50m) may not always be possible, and we consider a transect length of 250m the minimum length necessary in species-poor agricultural land. Appropriate weather conditions, speed of walking pace, general procedure, and additional parameters are defined in the guidelines.

Light traps with low illuminating power have to be used that do not attract individuals over longer distances in order to assess site-specific moth communities (see above). For the same reason, light traps must be located at least 50m from the edge of the habitat type studied. Field margins and hedgerows are usually narrow, linear landscape structures and, in this case, the location of light traps must be adapted adequately. In every case, light traps should not or as low as possible project vertically above the vegetation. More details of the application of the method are defined in the guidelines.

Within Pillar II certain species or species groups have to be chosen to assess the abundance of their caterpillars. On one hand, it is important that these species are abundant and widespread, and can be found by different field recorders in different regions, thus a statistical treatment among different sites and areas is feasible. On the other hand, regionally relevant species, e.g. rare or protected species, should be taken into account when necessary, too. This limits the choice in species-poor agricultural landscapes strongly. A list of suggested species is given in the guideline. Depending on the individual species, search must be adapted; two methodologies may be applied: visual search or beating method.

Visual search should be "success-oriented" (Hermann 1999), based on a sound knowledge of the behaviour of the caterpillars of the individual species. Only species that can be detected reliably should be considered. At least 50 suitable host plants or $20m^2$ of host plant area must be searched for each species and each site.

For species living on shrubs and trees the beating method is applied (Sutherland 2006). A beating tray is held underneath the branch, which is then beaten with a stick applying two short consecutive hits. These double hits are counted as a single strike. If possible, 100 double hits must be performed for each host plant type per site. More details are described in the guidelines.

When and how often go to the field?

Within Pillar I eight surveys have to be conducted between mid of April and end of August. This is a reduced approach compared to established monitoring schemes in order to minimize effort. The surveys have to be evenly distributed. The period from mid of April to end of August consists of nine half-month periods, the first or the last one may be omitted. If a prolonged period of bad weather prevents a survey in time it may be postponed to the following survey period.

Within Pillar II the timing for the counts of the larvae has to be adjusted to the species-specific activity periods. If the species produces a second generation, larvae have to be searched after both imaginal generations.

How to design study plots to gain optimal results?

In the guidelines, a paired field approach of GM fields and non-GM fields is applied to reduce secondary environmental influences. The two monitoring sites of one pair should be situated in a distance of 2–10km under the same natural conditions, to make them similar enough while avoiding an influence of GM on the non-GM monitoring site. All fieldwork within Pillar I and Pillar II is applied following this design.

As GM crops may be grown each year in different fields, while effects of GM may occur later or cumulative, two networks of mobile and stationary plots are described in the guideline. The mobile monitoring sites follow GM crops and are monitored during the year of GM crop use and, for field margins and hedgerows, the year following GMO cropping (see Figure 1). All field methods are applied in field margins and hedgerows, light traps additionally within the field, the latter only during the year of GM growth. The plot design of the paired, local approach must fulfil the following requirements: it must detect predefined effects with a specific probability, in-field and off-field effects, and regional and trans-regional effects. The monitoring plan has to include a prospective power analysis to determine the necessary sample size.

In addition, stationary plots must be established in the respective bio-geographic region, when GM crops are started to be grown in this region. Sampling sites and

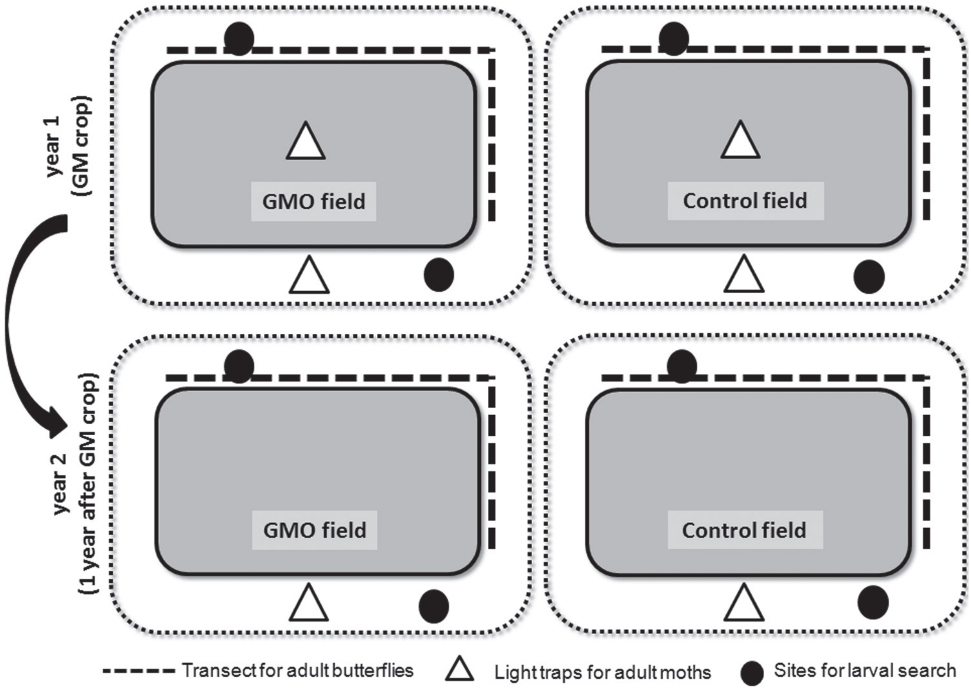

Figure 1. Mobile sites: schematic design of the paired field approach (GM fields and non-GM fields, with field margins) and the different samples to be taken during two years.

transects are fixed and will be regularly sampled during the established monitoring period. As currently the consent for GM crops is ten years in Europe, a monitoring period of at least eight to ten years is proposed (VDI 2010).

How to analyse data?

A complex framework, as it is the case in GMO monitoring, produces complex data sets. Therefore, consultations with professional statisticians are necessary during planning, implementation, and analysis of data. As a first approach, some basic procedures are suggested and described in the guidelines. Here, we only shortly summarise these procedures, for further particulars we refer to the VDI guidelines. In general, the analysis is based on a paired sampling comparing GMO monitoring sites with corresponding non-GMO sites. Due to the data structure of abundance counts a Poisson regression is favoured, but a standard regression may also be calculated after appropriate transformation of the data (generalised linear models). Additional co-variables have to be considered.

Besides these difference tests, equivalence tests may be applied. They examine whether an observed difference from a selected control can be regarded as equivalent.

The test is designed to find a significant equality as opposed to a significant difference, in other words null hypotheses and alternative hypotheses are turned around compared with difference tests. This approach is commonly used in the risk assessment of practical applications.

Population trends may be analysed using Mann-Kendall trend in the freeware program TRIM. This test can also be used to test differences between population trends in the GMO and non-GMO monitoring sites for the presence of a trend throughout the monitoring period, by determining annual differences in count data and testing such a difference of time series for a trend.

Finally, a statistical procedure is suggested, i.e. a randomisation method including a kernel density estimation, if only a small number of fields with GMO crops are cultivated and a paired comparison is not possible due to the small sample size.

How to assure quality of work and data?

A prospective power analysis is essential for a GMO monitoring plan, as it serves to estimate the sample size necessary to detect effects of a certain degree and/or to assess the likelihood of a given monitoring design to detect potential effects (Cohen 1988, Di Stefano 2001). A prospective power analysis requires prior knowledge of the data variance, which can be taken from preliminary trials, existing databases or from the literature (e.g., Lang 2004, Aviron et al. 2009, Lang and Bühler 2012). An 80% power is often used and considered adequate for experimental studies and GMO monitoring (Lang 2004, Perry et al. 2003). Depending on the specific circumstances, however, higher degrees of statistical power or lower effect sizes to be detected may be appropriate (Di Stefano 2003), e.g. for protected and vulnerable species.

The competence of the field observers is a fundamental requirement for assuring data quality. As an additional check, a certain amount of counts must be duplicated as double-blind trials. Plausibility testing of data and reports is a further useful quality control. A coordination centre for the collection and evaluation of data can also serve to assure and enhance the quality of a GMO monitoring plan.

General discussion and conclusions

The VDI guidelines (VDI 2010) provide the state-of-the art of a GMO monitoring of Lepidoptera as required by the European Community (EC 2001, 2002). They describe the best possible treatment of the demands of a lepidopteran GMO monitoring, accounting for the discussed difficulties, constraints and uncertainties as far as possible. The guidelines describe and ensure a suitable approach for a powerful and cost-efficient monitoring by:

(i) application of standardised sampling techniques,

(ii) diverse collection methods (transect counts, light traps and larval search) yielding a comprehensive and informative data set,
(iii) sufficient and efficient monitoring intensity (number of visits and samples, etc.),
(iv) paired sample approach for GMO and GMO-free sites reducing interfering environmental heterogeneity and increasing statistical power,
(v) accounting for different spatial scales of monitoring,
(vi) sample size or power estimation to plan for the appropriate numbers of samples to be taken,
(vii) various appropriate statistical methods to analyse the data set, and
(viii) giving instructions on data and organisational management.

Several aspects may interfere with the information value of the monitoring and should be kept in mind. Landscape patterns can strongly influence butterfly and moths species number, and effects of farming practice may differ between different landscapes (e.g. Weibull et al. 2003, Rundlöff and Smith 2006, Merckx et al. 2009a). It is therefore important to analyse monitoring data in a stratified approach on a regional level, which also decreases the necessary sample size to detect a given effect (Lang and Bühler 2012). Other interfering variables in (arable) landscapes include habitat type and structure, especially in field boundaries, e.g. the occurrence of host plants for larvae and nectar plants for adults (Settele et al. 2009a). Ideally, such variables should be recorded additionally and introduced as co-variables in the analysis of monitoring results (e.g., through a vegetation survey or estimates of the density of nectar plants, see VDI 2010).

Although the inclusion of abundant Crambid Snout Moths occurring in grassy field margins helps to overcome the possible problem of low abundance and few species, the necessary sample size (according to the prospective power analysis) may be extremely high and impracticable when the abundance of single species are to be analysed (Lang 2004, Aviron et al. 2009). In such cases, the analysis of patch occupancy (presence-absence data) would be a recommendation requiring a lower sample size (Royle and Nichols 2003, McKenzie and Nichols 2004, Lang and Bühler 2012). Another approach is to pool species by assigning them to ecological traits, e.g. with respect to their mobility or larval food plant (Mattila et al. 2008, Lang and Bühler 2012). Nevertheless, effects on rare or cryptic species may still be difficult to detect (e.g. Kéry and Plattner 2007), and species-specific approaches must be applied additionally. Again, the development of monitoring methods for species of the Habitats' Directive provides a good example.

Butterfly populations show highly dynamic annual fluctuations due to environmental factors (Van Strien et al. 1997, Wilson and Roy 2009), which can prevent the uncovering of cause-effect relationships of trends with regard to a certain factor of interest, e.g. of a GMO effect (Fleishmann and Murphy 2009). In addition, highly mobile adult Lepidoptera can be recorded off the localities where the original causes for a change in their abundance occurred. Therefore, the monitoring of any trends of Lepidoptera populations requires longer-time series or pair-wise comparisons to separate natural between-year fluctuations from actual trends and underlying causes (Roy et al. 2001). In the VDI guidelines, such pair-wise comparisons are of extreme importance in order to be able to interpret possible effects and trends (Bühler 2006, 2007, Bühler et al. 2008). The recording of larvae allows for assigning effects locally, which may be supported by analysing only a certain subset of the observed adults such as stationary species (Lang and Bühler 2012).

Many environmental studies examine effects by using biodiversity indicators such as species richness and abundance, or various diversity indices (e.g., Weibull et al. 2003, Roth et al. 2008). These indicators are considered as easy to measure, as appropriate approximations to general biodiversity, and are thus popular and wide-used (Filippi-Codaccioni et al. 2010). Sometimes, information on species richness and on abundant, widely distributed species can provide information on occurrence of rare species (Thomas 2005, Thomas et al. 2004, Pearman and Weber 2007). However, the possible impact of a transgenic crop will most likely differ among the species, because the species are differing in their susceptibility to a given stressor (e.g. Peacock et al. 1998) or are exposed to a different degree to the stressor (e.g. Traxler et al. 2005). Using indicators or sum parameters such as species richness may mask adverse effects on certain (specialist or rare) species, thus possibly having poor ecological relevance and causing misleading conclusions (Filippi-Codaccioni et al. 2010, Rosin et al. 2012). Therefore, analysing a species assemblage with respect to certain classifications reflecting ecological or functional traits of the involved species is a recommended approach in comparison to solely considering abundance and species number (e.g. Aviron et al. 2007b, Lang and Bühler 2012). Often, sum indicators require lower sample sizes and are less laborious than the recording of single species (Lang and Bühler 2012), however, care should be taken not to miss possible effects on single vulnerable species of conservation concern (Filippi-Codaccioni et al. 2010, Rosin et al. 2012, see also above discussions). Theoretically, GMO cropping may have impacts on many aspects of butterfly life histories, on larval survival and overwintering mortality, on residence time, or movement in landscape, to which abundance and species richness indices may not be sensitive enough indicators. Also, a monitoring approach of a more experimental than observing kind could be feasible, e.g. experimentally exposing lepidopteran larvae in the field to different GM crop intensities. Apart from its given application value, the VDI guidelines constitute a good starting point and can stimulate more in depth research on the possible effects of GM crops and monitoring issues. The study on the recording of larvae along field margins by Lang et al. (2011) may serve as an example for such in-depth research inspired by the VDI guidelines.

It is important for monitoring programmes to be cost-effective, which is not equivalent to being cheap, but means generating data of high (or sufficient) quality with an acceptable and justified effort (Lovett et al. 2007). For this, an iterative quality control of the monitoring results is paramount, regularly checking the relation between invested efforts and value of generated data, and continuously adapting the monitoring programme to scientific progress and new knowledge (Lindenmayer and Likens 2010).

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