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Anuran amphibians as indicators of changes in aquatic and terrestrial ecosystems following GM crop cultivation: a monitoring guideline

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Abstract

Amphibians are a suitable indicator group for monitoring possible negative direct or indirect effects of GMO cultivation at the individual and population level. Direct effects could occur in aquatic ecosystems via uptake of GM pollen or GM detritus by anuran larvae. However, indirect negative effects caused by changes in cultivation practices (changes in pesticide use, for instance) are more likely. The VDI Guideline 4333 aims to ensure comprehensive monitoring of the different life-stages of anuran species that are common in agricultural landscapes of Austria, Germany and Switzerland. The guideline includes a novel approach to tadpole monitoring. To assess immediate effects, tadpole, metamorph and adult deformation rates are compared with naturally occurring deformation rates. Adult population size, adult body condition and juvenile emergence are monitored over multiple years to assess long-term effects of GM crop cultivation on population viability. At each study site, monitoring has to be carried out at multiple amphibian breeding sites which differ in their exposure to GM crop cultivation. All monitoring data have to be stored in a central database for future meta-analyses. This will ultimately allow for generalized statements about the impact of GM crop cultivation on amphibians. Although specifically designed for GM crops, VDI Guideline 4333 may also serve as a model for studying the effects of a wider range of stressors on amphibian populations in agriculture and forestry.

Keywords
GMO, monitoring guideline, agriculture, pesticide, deformation, population size, tadpole, recruitment, adult

Introduction

EU Directive 2001/18/EC on the deliberate release of genetically modified organisms (GMO) into the environment requires the assessment of environmental impacts of GMO including direct, indirect, immediate and delayed effects on the environment, further specified in Council Decision 2002/811/EC. In the case of transgenic crops resistant to herbicides, this means that besides evaluating the environmental impacts of the genetically modified plant itself, the environmental impacts of specific herbicide programmes and altered agricultural practices associated with this crop have to be assessed. GM crops may affect not only terrestrial, but also adjacent aquatic ecosystems such as ponds, ditches or run-off water bodies (Mann et al. 2009).

Amphibians are considered a particularly sensitive indicator group for environmental stress in aquatic and terrestrial ecosystems (e.g. Blaustein and Johnson 2003; Blaustein and Kats 2003). Owing to their complex life cycle, the most sensitive stages of embryonic and larval development are usually spent in aquatic habitats, while most juveniles and adults live in terrestrial habitats. Furthermore, as amphibians depend on water for breeding, adults of many species, especially in temperate regions, show extensive annual migrations between winter, breeding, and summer habitats.

As tadpoles of frogs and toads (anurans) are predominantly herbivores, they should be more sensitive to the entry of GMO products into aquatic systems than carnivorous newt and salamander larvae. Food uptake of GMO pollen, GMO detritus, or GMO-contaminated sediment could directly affect larval health, growth and development. However, indirect effects are expected to have much greater impacts, since GMO cultivation is likely to change the way farmers use pesticides, both in terms of cultivation practices and pesticide application regimes and doses. This may lead to significant effects, such as deformities and increased mortality as observed in anuran larvae (Mann et al. 2009; Paganelli et al. 2010; Relyea 2005b). While interactions between toxic effects and other ecological factors can amplify lethal effects of pesticides (Relyea and Mills 2001), even sublethal effects on larval growth and development can impair amphibian populations permanently (Jones et al. 2010; Relyea 2005a; Relyea 2005b). In agricultural systems, amphibians may be particularly susceptible to the toxic effects of pesticides because their breeding sites are often shallow ponds or temporary pools that may accumulate pollutants without substantial dilution (Howe et al. 2004; Mann et al. 2003). Furthermore, depending on application times and frequencies, lethal damage of amphibians during migrations in their terrestrial habitat is to be expected (Brühl et al. 2011).

Anurans are not only sensitive bioindicators for environmental change, they are also well suited for monitoring programmes due to:

- the substantial state of knowledge concerning the biology, ecology and conservation status of the different species,

- their predictability at the breeding site, and
- the fact that breeding, larval development and migrations occur in spring and summer, the season of pesticide applications.

Here, we summarize the possible negative impact of direct and indirect effects of GM crop cultivation on amphibians. We describe the study design of VDI Guideline 4333 that allows assessment of these effects using a standard protocol. This guideline deals only with anuran amphibians, as they are easier and more reliably to survey than salamanders and newts. Besides, anuran larvae should be more sensitive to direct effects of GMO products due to their herbivorous feeding behaviour.

Direct potential effects of GMOs on anuran amphibians

Direct effects of GMO products on amphibians have not yet been studied. However, effects of GM crops could result from the accumulation of detritus or pollen in the aquatic environment. Many tadpoles are non-discriminatory feeders and ingest high amounts of detritus and pollen (e.g. Wagner 1986). Although toxins in GM crops (e.g. *Bt* toxin) should not have an impact on tadpoles, such effects cannot be ruled out. Additionally, future GM crops may contain toxins that may have direct negative effects on amphibians.

Indirect potential effects of GMOs on anuran amphibians

Today, nearly all GM crops have a genetically engineered resistance to non-selective herbicides (ISAAA: Global Status of Commercialized Biotech/GM Crops: 2009. The first fourteen years, 1996 to 2009. http://www.isaaa.org/resources/publications/briefs/41/executivesummary/default.asp), mostly based on the active ingredient glyphosate (Duke and Powles 2008). The use of herbicides associated with the cultivation of GM crops may therefore indirectly affect amphibians.

Amphibians may be exposed to these pesticides in many ways. Frogs and toads may become contaminated; for instance, migrating terrestrial amphibians (adults, juveniles or metamorphs) can be over-sprayed or can get in contact with pesticides adhering to soil or plant material (Brühl et al. 2011). Pesticides may also contaminate amphibians via the food chain, when amphibians prey on insects that have come into contact with pesticides (McComb et al. 2008). Anuran eggs and larvae can be affected through the accumulation of pesticides or their degradation products in aquatic ecosystems via wind drift, drainage or run-off. In agricultural systems, amphibians may be particularly susceptible to the toxic effects of pesticides because their breeding sites are often shallow ponds or temporary pools that may contain higher concentrations than larger water bodies (Mann et al. 2003; Howe et al. 2004).

Within recent decades, the number of herbicide-resistant weeds has increased dramatically due to unsustainable weed management with the almost exclusive use of only one active ingredient (Heap 2011: The International Survey of Herbicide-Resistant Weeds. http://www.weedscience.org/ChronIncrease.gif). This is particularly true for herbicide-resistant GM crop cultivations (Powles 2008). Weed resistance leads to higher doses and additional applications of herbicides (Schütte and Mertens 2010). Furthermore, non-selective herbicides can be applied later in the year than selective herbicides (BVL 2010). Hence, the exposure risk for different amphibian species and different life-stages may shift. Applications later in the year may include an increased spraying height that can lead to higher aerial pesticide drift. The likelihood of heavy rainfall events in early summer is increasing in Western Europe due to climate change. Increased drift, heavier rainfall and greater erosion may cause adjacent aquatic habitats to become more heavily contaminated through the use of non-selective herbicides (SRU 2008).

Pesticides may lead to a reduction in insect abundance, and subsequently to reduced food availability for amphibians. Direct contact of amphibians with pesticides is known to have lethal and sublethal effects. These include increased mortality due to acute toxicity (Howe et al. 1998, Relyea 2005b) or teratogenic (Paganelli et al. 2010) and genotoxic effects (Clements et al. 1997).

Tadpoles often show pesticide-induced deformations in body and tail shape (e.g. Cooke 1981, Lajmanovich et al. 2003). Some pesticides are also endocrine disruptors that potentially target the thyroid axis (Howe et al. 2004), leading to an imbalance in the sex ratio of the population (Hayes et al. 2006). They are also known to have an immunosuppressive effect (Rohr et al. 2008). In addition, pesticides may interact with other stressors such as predators (Relyea and Mills 2001, Boone and James 2003). All this applies to many pesticides, but some non-selective herbicides seem to be amongst the most dangerous for amphibians (see Williams and Semlitsch 2010).

VDI Guideline 4333: an anuran monitoring scheme to assess possible adverse effects of GMO cultivation on aquatic and terrestrial environments

The "VDI Society Technologies of Life Sciences" prepared a guideline on how to assess the potential negative effects of GM crop cultivation on amphibians, namely anurans, occurring in Austria, Germany and Switzerland. It is published as VDI Guideline 4333 "Monitoring der Wirkungen des Anbaus von gentechnisch veränderten Organismen (GVO) – Standardisierte Erfassung von Amphibien/Monitoring the effects of genetically modified organisms (GMOs) – Standardized monitoring of amphibians". Here we summarize the essential elements of this guideline; for details we refer the reader to the guideline itself (VDI 2013; www.vdi.de/4333).

VDI Guideline 4333 describes a study design that can be used to assess (i) immediate negative impacts, and (ii) long-term effects on anuran populations. To assess immediate effects, tadpole, metamorphic and adult deformation rates need to be recorded. Increases in these rates are common responses to herbicides (e.g. Cooke 1981, Lajmanovich et al. 2003, Paganelli et al. 2010). Herbicides can directly but also indirectly cause deformities by an enhanced susceptibility to trematode infection (Kiesecker 2002, Rohr et al. 2008). However, deformations, even when occurring at high rates, may not substantially affect population viability if density dependence is high (e.g. Forbes and Calow 2002; Veith and Viertel 1993). Thus, to assess long-term effects, the guideline stipulates studying changes in population size in order to track possible population declines.

Standard methods for monitoring adult populations of amphibians are well established (e.g. Dodd 2010; Heyer et al. 1994). However, tadpole monitoring is a rather new approach. Therefore, the VDI Guideline 4333 aims to set standards for assessing the immediate negative impacts of GM crop cultivation and pesticides on aquatic habitats using anuran larvae.

A first crucial step in monitoring is to define the study area and duration of the monitoring programme. Due to natural fluctuations in amphibian population parameters, potential negative impacts on amphibian populations in habitats influenced by GM crop cultivation should be controlled by comparison with unaffected habitats. However, since it is next to impossible to identify potentially unaffected sites *a priori*, VDI guideline 4333 defines a set of water bodies with different magnitudes of exposure, which are to be monitored per GMO cultivation site. In addition to the three most exposed breeding habitats, three additional, less exposed breeding habitats within a radius of up to 1000 m from the margins of the GMO field will be selected for monitoring (Fig. 1A). The expected magnitude of exposure is quantified via GIS on the basis of the distance between the water body and the GMO field, the prevailing relief (topography) and the water flow regime. This spatial set-up allows an assessment of possible negative effects along a gradient of exposure intensity. For each of these six monitoring sites, the expected magnitude of exposure will be quantified via GIS analysis: buffers with different radii are drawn around each site, and within the buffers the proportion of land used for GMO cultivation as well as the ratios of general land use (arable land, grassland, woodland, residential area) are determined (Fig. 1B).

To account for population trends, the monitoring has to be conducted during the period of GMO cultivation and should be continued for at least two times the generation time of a species after cultivation has ceased. Since generation time varies across species and may also be habitat-specific, the guideline suggests this extended period to last for ten years.

To minimize the impact of monitoring on the respective amphibian populations and to increase comparability across monitoring studies, VDI Guideline 4333 recommends focusing on species occurring in the agrarian landscape that are widely distributed throughout Austria, Germany and Switzerland. It therefore distinguishes between 'obligate' and 'supplementary' species. Obligate species, namely the Common toad, *Bufo bufo,* and the Common frog, *Rana temporaria*, are widely distributed in Western Europe and, as indicated by their vernacular names, are common in most areas and sites. Where one or both of these occur in sufficient numbers, they must be included in the monitoring. However, since amphibian species differ in their susceptibility to pesticides (e.g. Cooke 1981, Kerby et al. 2010), it is advisable to study additional species wherever possible. Therefore, supplementary species should be added where they

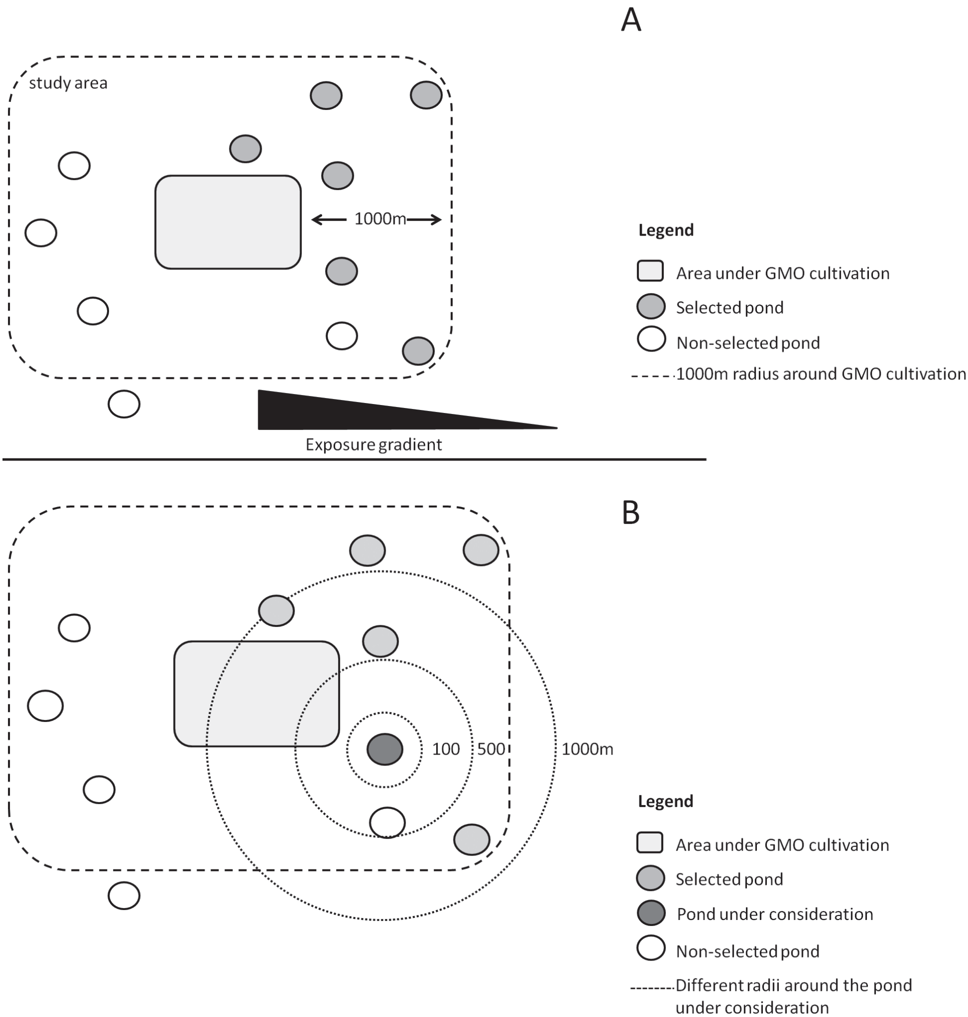

Figure 1. Spatial monitoring design of amphibian populations to quantify possible adverse effects of GMO cultivation. **A** Selection of an array of study ponds with different exposure probabilities **B** Quantifying the proportion of GMO cultivation within different radii around each study pond (in accordance with VDI guideline 4333).

are regionally or locally as common as or even more prevalent than obligate species. Supplementary species may be typical for substantial parts of the area of influence of the VDI guideline. They can therefore replace obligate species where one or both of them are not present in sufficient numbers.

The monitoring guideline stipulates investigating the following life-stages:

(i) Reproducing adults: In an initial step, information about the reproductive population in a given year and its changes over time has to be recorded. All males and females migrating in spring to a breeding site are counted using drift fences with pit fall traps (e.g. Dodd 2010; Hachtel et al. 2009; Henle and Veith 2004). Besides identifying species and sex, all specimens are examined for abnormalities. Information on individual body condition, which is inferred from measurements of body length and body mass, may provide further insights into long-term effects of GM crop cultivation on population viability. The counted number of amphibians is only a reliable index of population size if detection probabilities are high (Bailey et al. 2004; Schmidt and Pellet 2009). Therefore, the drift fence must be operated very carefully to ensure that detection probabilities are high and show low variability.

(ii) Tadpoles: Deformations in body and tail shape or of extremities are naturally observed at a rate of less than 5% in most anuran larvae (Cooke 1981; Mann et al. 2009; Piha et al. 2006). Monitoring tadpoles during early and late development will therefore allow the detection of unusual increases of deformation rates.

(iii) Metamorphs: Metamorphs are counted using drift fences with pit fall traps. They are checked for abnormalities in the same way as adults. Comparison of quantitative data on juvenile emergence across ponds along the exposure gradient and across years provides the opportunity to estimate the effect of GMO cultivation on the annual recruitment of a population. In the case of an apparent mass mortality, VDI Guideline 4333 recommends preserving metamorphs for subsequent histological examination.

If negative effects are detected in tadpoles or metamorphs, detailed water and sediment analyses have to be carried out in the respective water body in subsequent years. These along with the accompanying monitoring data will show during the following years if negative effects are related to GM crop cultivation. Such supplementary analyses should focus on transgenes, GMO by-products and pesticides using available standard procedures (EC 2009; EC 2010; Züghart and Dörpinghaus 2004).

The results for each monitoring must be stored in a central database for meta-analyses. Ultimately, this will allow for generalized statements about the impact of GM crop cultivation on anurans across different species, habitats and exposure intensities.

Discussion

Agricultural practices are known to affect biodiversity (Ellis et al. 2010; Jenkins 2003). The use of GMOs in agriculture is likely to affect biodiversity as well. Here we describe how to assess the impact of GM crop cultivation on amphibian populations. Our assumption is that GM crops probably have no direct negative effects on amphibians, whereas indirect negative effects are to be expected. These may be caused by changes in pesticide use that are typically associated with the cultivation of GM crops.

Pesticides are well known to affect amphibians (Bridges 1997; Brunelli et al. 2010; Davidson 2004, Howe et al. 2004; Paganelli et al. 2010; Relyea 2005a; Relyea 2005b;

Relyea et al. 2005). Though effects on individuals do not necessarily impair population viability (Forbes and Calow 2002; Rohr et al. 2006; Schmidt 2004), an ultimate deterioration cannot be excluded. A comprehensive assessment of the impact of GM crop cultivation on amphibians that includes short and long-term effects is therefore necessary.

VDI Guideline 4333 is designed to assess the potential impact of GM crop cultivation on amphibian populations in an agricultural landscape. However, it can also serve as a general guideline that can be used to study the effects of a wide range of chemical stressors (pesticides, fertilizers, endocrine-disrupting chemicals, etc.) on amphibian populations in agriculture or forestry. The guideline describes how to quantify negative effects on amphibians during their aquatic and terrestrial life-stages. This comprehensive approach is novel as it includes the most sensitive stages of larval development in their aquatic environment.

Quantifying the possible effects of GM crop cultivation on natural amphibian populations is challenging. A variety of effects may occur that have to be considered and measured in the field. To design an operational monitoring scheme, we decided to focus on four response variables that can easily be measured in the field: (i) rate of deformations in the larval, juvenile and adult life-stages, (ii) number of metamorphs, (iii) number of breeding adults, and (iv) body condition of breeding adults. We excluded further potential response variables (e.g. histological analyses of damaged tissues, skeletochronology to determine shifts in adult age structure due to massive die-offs during pre-reproductive stages, capture-mark-recapture estimates of survival and detection probabilities, structural changes in the agricultural landscape associated with GM crop cultivation) primarily because they would be too difficult to be quantified on a large-scale. Nevertheless, we are confident that the chosen response variables are adequate to detect potential effects of the cultivation of GM crops on individual and population levels.

Deformities of tadpoles and body condition of adults quantify effects on individuals, while adult population size and the number of metamorphs (emergence) measure effects at the population level. Individual-level effects may be viewed as precursors to future negative population effects. Reading (2007) showed that there was a positive correlation between adult survival and body condition. Emergence is a crucial component of recruitment and thus a major driving force of amphibian population dynamics (Hels and Nachman 2002; Lampo and De Leo 1998; Schmidt 2011). A decline in abundance is a population-level effect which indicates either that the population was unable to compensate a prior negative effect on individuals (e.g. increased mortality in the tadpole stage) or that post-exposure effects may have occurred (Forbes and Calow 2002; Rohr et al. 2006). Because some effects may only become apparent years after exposure, it is important to continue studying the possible consequences of GM crop cultivation after cultivation has ended.

To assess the impact of GM crop cultivation on amphibians, their populations must be studied at multiple ponds. As “control ponds” are hardly found at study sites, in VDI Guideline 4333 a GIS-based approach was taken to determine how much each pond may be affected by GM crops. Using circular buffers with varying radii around the ponds and determining the relative extent of GM crop within these buffers, ponds can be arranged along a gradient of exposure to GM crop cultivation. We expect that this approach along with a subsequent meta-analysis of all studied GMO sites will allow a fairly precise determination of how GM crop cultivation affects amphibian populations.

The approach described in VDI Guideline 4333 to quantifying the effects of cultivation of GM crops on amphibians is correlative. Therefore, it cannot prove that any observed alteration was caused by either direct or indirect effects of GM crop cultivation. Experiments that could prove causality at population level would in principle be possible (see Krebs et al. (1995) and Hudson et al. (1998) for case studies where population dynamics were experimentally manipulated). However, they would be extremely laborious and, at the very least, in most, if not all, cases unethical.

The proposed study design can be viewed as a pseudo-experiment, with each cultivation of a GM crop in the vicinity of amphibian breeding sites being a different and independent experimental treatment. If, in fact, effects are observed, it is then necessary to conduct experiments to establish cause-effect relationships. Deformities, tadpole survival or adult body condition may also be response variables in experimental studies. A combination of field studies, experiments and population modelling will certainly give the most compelling evidence as to whether the cultivation of GM crops negatively affects amphibian population dynamics. EU legislation requires that the potential effects of the cultivation of GM crops are assessed whenever and wherever GM crops are used. The data from all these studies need to be stored in a central database, so that it will be possible to conduct a meta-analysis at a later stage. Such a meta-analysis based on a large number of studies would gain a high statistical power and would therefore allow a much greater understanding of the effects of GM crop cultivation on amphibians.

Conclusions

Monitoring the effects of GM crops on biodiversity is an important and challenging task. Guidelines for standard monitoring, such as VDI Guideline 4333, are an important step towards this direction. Importantly, the methodology described in the guideline and in this article is general and could also be used to design an observational study to assess the effects of other anthropogenic stressors on amphibians.

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