Faculty of Health Sciences UiT

Ecotoxicological assessment of Roundup-ready soybean agriculture investigated in a *D. magna* model

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Thomas, - gode venn,

samvittighetsfulle veileder

og dansepartner.... - Takk!

"Writing a phd-thesis is like building a house
you know you're not going to live in"

- Svein-Anders Noer Lie

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The text is written by Marek Cuhra.

A controversial development in industrial agriculture

Traditional cultivation of landscape with deliberate intent to facilitate, improve and increase production of plant material for human consumption and farmed animal feed, has evolved into complex agro-industrial activities involving technological and agrochemical means. It involves use of machinery, chemicals, greenhouses and irrigation to alter the microclimate, supplementing nutrients and water and eradicating pests. Natural restrictions imposed by biological and climatological conditions are thereby to some extent manipulated and overruled.

Taking this development a step further, modification of cultivars by forced insertion of transgenes from other organisms has gradually been accepted in parts of the world as a legitimate and necessary step in the ongoing development of agriculture. This genetic manipulation has been presented as potentially beneficial for farmers, consumers and society in general, since it is intended to reduce production costs and create higher yields. Genetic manipulation has also been heralded as allowing for more environmentally benign agricultural practices, notably reduced use of pesticides and need for tillage (believed to be an important factor for soil conservation in areas subject to high rates of erosion).

Plant varieties modified to tolerate herbicide application were amongst the first commercially available cultivars to have been genetically manipulated. These plants still form the majority of all genetically modified organisms (GMOs) produced worldwide. Herbicide tolerance allows for reduced farming expenses, use of herbicidal chemicals substituting for generally more costly manual and mechanical eradicative techniques. Use of agrochemicals will affect both the quality of agricultural produce as well as the surrounding environment, which is measurable as chemical residue levels in agricultural commodities and impact on non-target organisms.

GMOs have been shown to facilitate certain aspects of plant cultivation and bring down direct production costs. This not withstanding, use of GMOs for production of human food and farm-animal feed remains the object of much public scepticism and scientific debate. In this regard, it might appear reassuring that agencies such as the US Food and Drug Administration (US FDA) as well as the European Food Safety Authority (EFSA), have both stated that food produced from GMO plant material generally should be recognized as safe. Furthermore, both are claimed to have concluded that due to the precision of transgenic techniques, GMO plants have the potential to produce safer foods than conventional (unmodified) varieties. However, this notion is contested. Numerous research findings document that the manipulation methods are imprecise and unpredictable and that the cultivation presents risk factors and adverse environmental impacts at production sites, in addition to undesirable effects on the quality of produce.

The inherent complexity of assessing risks posed by genetically modified (transgenic) cultivars has been addressed by means of traditional scientific approaches, including visual characterization, compositional analysis of constituents and animal feeding tests. These are deemed sufficient for quality-assurance of substances destined for human consumption or animal feed.

As in numerous other instances of innovation, the professionals charged with testing and assessing are not of one mind. Scientists evaluating transgenic cultivar produce hold widely differing opinions on issues such as need for precaution, relevance of surveillance and essence of analysis. This is presented as scientifically founded statements on issues such as determination of necessary length of exposure in feeding tests, discussions on relevance of new constituents such as novel proteins synthesized by inserted transgenes, labeling of consumer products and scope of environmental monitoring plans.

A review of scientific literature and regulatory documentation provides us with an understanding of societal developments over the last decades that have led to the present polarized situation in worldwide cultivation and consumption of genetically modified material. An overview of this evolution should start with analysis of regulatory processes leading to the 1992 US FDA policy brief on novel foods from biotech plants. This is vital to understanding the scientific and political exchanges leading to the present deregulation of transgenic produce in the United States as well as other countries in North and South America, including Canada, Argentina, Brazil and Uruguay. This policy of deregulation was opposed, based on claims of perceived risk, by the majority of EU constituent nations and--notably--Norway.

The scientific and political discussion on biotech food safety thus initially has been characterized as a trans-Atlantic rift, with other regions and individual countries joining in as further evidence accrued. More recently, these positions have changed somewhat. The formerly active scepticism regarding transgenic plants in Europe has largely been subdued into passive acceptance as gradually biotech cultivars are approved by the EFSA for import and cultivation.

By contrast, in the United States the discussion on safety of food from transgenic plants has re-erupted, generally focusing on mandatory labeling of foods containing transgenic ingredients. With both sides backed by their respective representatives in Congress and Senate, it led to recent referenda in several states. In most of these instances thus far, a narrow margin of voters rejected the proposals for labeling.

The safety issues concerning transgenic plants are still contested. Objective scientific evidence confirming their safety remains lacking.

In fact the overwhelming majority of data confirming safety of transgenic produce has been furnished by the biotech industry itself. Critics have pointed out that such industry tests are often methodologically flawed, among other things of insufficient duration, and thereby not useful in modelling the life-long exposure situation of consumers.

Independent research testing of transgenic produce has not been welcomed by biotech industry, which has refused to supply material for such testing. To date, few universities or research programs see the importance of independent testing of transgenic foods substances. Few independent researchers manage to obtain material for testing or adequate funding. For those who do, the reception of findings disfavourable to biotech products is generally hostile.

This has created a situation in which research failing to document potentially adverse effects of transgenic produce--even where exhibiting obvious methodical flaws--has largely been accepted as evidence of safety, whereas research indicating the contrary tended to be ignored or systematically discredited.

Contents

Abbreviations and key concepts

ADI	=	Acceptable daily intake (dosage related to bodyweight)
CAS#	=	Chemical abstract service of American Chemical Society
EC_{50}(48)	=	Concentration producing 50% effect (immobility) in test-animal in 48-hrs exposure
FOIA	=	US freedom of information act
GMO	=	Genetically modified organism
GT	=	Glyphosate tolerant
GTS	=	Glyphosate tolerant soy
HT	=	Herbicide tolerant
LC_{50}(48)	=	Concentration producing 50% lethal effect in test-animal in 48-hrs exposure
LOEC	=	Lowest concentration of observed effect
MRID	=	Master record identification number
MRL	=	Maximal residue level (limit)
MSDS	=	Material safety data sheet
NOEC	=	Concentration of no observed effect (see LOEC)
US EPA	=	Environmental Protection Agency of the United States
US FDA	=	Food and Drug Administration of the United States
USDA	=	United States Department of Agriculture

Table 1. Abbreviations and key concepts used in introduction, research papers and discussion.

Summary of key findings

Despite two decades of research into the quality and biosafety of genetically modified glyphosate-tolerant plant varieties, these issues are still contested by contradictory research findings. In a similar development, glyphosate herbicides such as the commercial product *Roundup* are more controversial now than ever before, - despite four decades of research into toxicological and ecotoxicological effects of these chemicals.

An unresolved issue seems to have been largely ignored by scientists as well as regulators: glyphosate tolerant crops are constituents of an industrial production system with specific agricultural practices and supplementary agrochemicals as interwoven additional elements. Thus the transgenic material produced should not be seen as an isolated product of a specific modified genotype but rather as a product of a tailored agriculture system.

The objective of the research presented here has been to investigate quality and safety aspects of one such specific agriculture production system, that of the glyphosate-tolerant soybean. This globally dominant crop has been in open cultivation since 1995 and is still predominantly based on hybrid varieties of the GTS-40-3-2 transgenic event soybean (Roundup-ready soybean) in combination with glyphosate herbicide co-technology.

The published results were obtained via a review of published evidence four quite large laboratory experiments: a) an ecotoxicological study of a commercial Roundup herbicide and the glyphosate active ingredient, b) a study of biochemical composition in which genetically modif andied glyphosate-tolerant Roundup-ready soybean, conventional soybean and organic soybean are analyzed and compared, and c) two fully controlled laboratory feeding studies with soybean-meal, using water-flea *Daphnia magna* in life-long exposure as an animal model.

<u>Roundup and glyphosate:</u> The herbicides were tested for acute and chronic toxicity in a *D. magna* animal model. It was found that the ecotoxicity is more potent than what was previously assumed in regulatory assessments based on information provided by chemical industry. The tests in this recognized aquatic invertebrate indicator species

have yielded evidence of the general effects these chemicals might have in aquatic ecosystems that receive effluents from farmland. Glyphosate is found be distinctly more toxic than indicated in previously published studies. Analysis of vital parameters from *D. magna* in short-term 48-hour exposure demonstrate that acute-toxicity of glyphosate and Roundup herbicide formulation are two orders of magnitude higher than expected from previously published findings from industry testing. Also, life-long exposure to low-level concentration of either glyphosate or Roundup herbicide formulation demonstrates higher toxicity than expected from scientific literature. Notably, it was found that low concentrations of Roundup induce reproductive failure in *D. magna* (**paper I**).

Through a request for information-disclosure under the United States Freedom of Information Act, copies of the relevant industry tests from the archives of United States Environmental Protection Agency (US EPA) were obtained. The material revealed evidence of systematic flaws and erroneous interpretation of results. These irregularities partially explain the discrepancy between the findings on glyphosate ecotoxicity presented in **paper I** and the established assumptions in regulatory assessments and some scientific literature.

<u>Composition of soybean:</u> Laboratory analysis of selected minerals, nutrients and pesticide residues was performed in transgenic GTS-40-3-2 soy, conventional soy and organic soy obtained directly from farm-fields in Iowa, USA. The results show systematic differences which characterize these products. Substantial ppm-levels of glyphosate residues were found to be systematically present in all tested samples of Roundup-ready soybean. In seven out of ten samples these residue concentrations were unexpectedly high. Such residues were found in neither conventional soybean nor in organic soybean, although ppb- and ppt-levels of other pesticide residues were present (**paper II**).

<u>Feeding studies:</u> Vital parameters of *Daphnia magna* fed raw or heat-treated meal from either transgenic GTS-40-3-2 soybean, conventional soybean or organic soybean were analyzed. Results demonstrate significant differences in test-animal vital parameters, reflecting significant qualitative differences of feed. The first life-long feeding-study with soybean-feed in *D. magna* demonstrated that overall animal performance

measured as growth and reproduction was significantly affected by soy-meal diets. The best performance was found in animals fed diets with organic soy-meal. By comparison, animals fed transgenic soybean-meal showed significantly reduced growth and reproduction. The feeding studies documented that animals fed GTS-40-3-2 soybean showed fitness parameters inferior to animals fed conventional soybean (**paper III**).

<u>Animal performance is affected by glyphosate residues:</u> A subsequent life-long feeding-study using 8 distinct soybean-meal-diets from GTS-40-3-2 Roundup-ready soybean demonstrated that mortality, growth and reproductive maturity of *D. magna* are negatively correlated with levels of glyphosate residues (**paper IV**).

<u>Review of evidence:</u> Genetically modified glyphosate-tolerant crops have been subjected to numerous analyses and used as feed in animal feeding studies. Regulatory review of these studies in the United States of America and the European Union has concluded that they sufficiently document that such crops are essentially equivalent to unmodified varieties. However, the review indicates systematic omission of information on herbicide residue levels in the quality testing and risk assessment of genetically modified glyphosate-tolerant crops. This finding is an important background for understanding the protracted controversy on safety testing and indicates that the current regulatory requirements are insufficient to ensure identification of undesirable substance (**paper V**).

<u>Conclusions from the research:</u> The findings in **paper I** and the scrutiny of evidence from archives justifies a revision of industrial tests concerning glyphosate ecotoxicity in aquatic invertebrates. The general indications from the specific findings justify further measures for scrutiny of fundamental documentation on ecological effects, toxicology and health. These findings of faulty industry studies for assessment of glyphosate ecotoxicity are mirrored by the findings of **paper V**, which demonstrates similar grave shortcomings in industry studies assessing quality and safety of glyphosate-tolerant crops.

The research presented in **papers II, II and IV** contributes to a body of evidence indicating that directly and indirectly measurable material differences between transgenic and conventional cultivars are significant qualitative aspects, which challenge the concept of substantial equivalence. The traditional methodology for testing whether transgenic plants are substantially equivalent to their unmodified origins should be adjusted based on these findings.

Exposure to low-levels of glyphosate or Roundup in the environment will impair *D. magna* growth and reproduction. This indicates that adverse effects seen in feeding-studies could be caused by toxic effects from glyphosate residues.

The research leads to reflections on issues such as methods employed by biotech industry in production and testing of transgenic varieties, regulatory authority oversight and wider socio-political aspects.

Primary research findings:

- Glyphosate and Roundup herbicide are more toxic to non-target organism *D. magna* than previously published evidence would suggest (**paper I**)
- Glyphosate tolerant soybean accumulates residues of glyphosate and aminomethylphosphonic acid (AMPA)(**paper II**)
- Compositional characteristics of biochemical and mineral constituents in soybean is affected by glyphosate residues (**paper II**)
- Feed quality of soybean meal from glyphosate tolerant soy is not equivalent to that of organic or conventional unmodified comparators (**papers III & IV**)
- Important vital parameters of *D. magna* fed GTS-40-3-2 glyphosate are negatively correlated with glyphosate-herbicide residue levels (**paper IV**)
- Analyses for relevant herbicides residues are systematically missing in industry tests of herbicide-tolerant transgenic varieties (**paper V**)

Table 2. Primary findings from the presented research papers

Background

Soybean

Soybean (*Glycine max*) is predominantly cultivated in North and South America in industrial-scale farming system monoculture, which is often found to dominate the landscape (Pengue, 2005). Total global production of soybean was estimated to be in the order of 287 million ton in the 2013/14 growing-seasons (USDA, 2014) and is predicted to expand. The majority of this substantial harvest continues to be from glyphosate-tolerant soybean (Antoniou *et al.*, 2012; Binimelis *et al.*, 2009; Bonny, 2011; ISAAA, 2014). This large biomass is primarily processed into low-cost ingredients for farm-animal feed but also in part for human food. The GTS-40-3-2 variety of soybean is a patented GMO product developed to withstand glyphosate herbicides in commercial formulations such as Roundup. The plant is commonly known to farmers, agronomists, regulators and other professionals by its trade-name; *Roundup-ready soybean*. Roundup-ready soy is widely adapted by farmers growing soybean in the USA and in several countries in South America. As documented by Sylvie Bonny (2011), there are several reasons explaining this success. Even though the Roundup-ready seed is typically more expensive than conventional seed and farmers opting to use it are bound by stewardship agreement contracts, the benefits appear to outweigh the costs. Amongst these benefits we find lowered overall costs of herbicides, an initial decrease in number of seasonal herbicide applications, potentially higher yields caused by better control of weeds, and potential decrease in manual labour spent on weeding and plowing. However, it is also indicated that increasing weed-resistance necessitates increase in application rates of glyphosate often combined with use of other herbicides (in pre-emergence or post-harvest application), thus reducing overall efficacy of the Roundup-ready system.

Glyphosate

Just as soy has become world number-one cultivar, glyphosate is world number-one herbicide. Commercial initiative combined these two successes into genetically modified glyphosate-tolerant soy thus creating an agroecological system which presents important biosafety challenges.

It has even been claimed that transgenic GTS-40-3-2 glyphosate tolerant soybean initially was developed by chemical industry primarily as a strategy to increase consumption of glyphosate-herbicide Roundup in agriculture (Charles, 2001). Thus, to evaluate the complexity still enshrouding GTS-40-3-2 glyphosate tolerant soybean nearly two decades after it was released onto the market, it is arguably essential to include glyphosate in the analysis. Furthermore, it has been known for more than a decade that glyphosate-tolerant soy can accumulate glyphosate residues, depending on factors such as application intensity and timing (Duke *et al.*, 2003). The herbicidal properties of glyphosate (N-phosphonomethyl-glycine) inhibit biosynthesis of chorismate from shikimate (Amrhein *et al.*, 1980), thereby lethally disrupting photosynthesis and plant cell metabolism. Transgenic bypassing of this vital plant metabolic pathway which is specificaly targeted by glyphosate, allows for herbicide application onto growing crops. Glyphosate herbicide main ingredients are usually isopropylamine-salt (or trimesium salt) of N-phosphonomethyl-glycine. These glyphosates degrade into metabolites, of which Aminomethylphosphonic Acid (AMPA) is recognized as the most important.

Glyphosate-tolerance is still the main genetic modification in crops used in industrialized agriculture (Benbrook, 2012). Glyphosate herbicides such as Roundup have been on the market for four decades and their use is still increasing, making them the primary category of pesticides world-wide. Thus both by volume and by revenue glyphosate is the globally dominant active-ingredient for herbicides, with recent estimates of global annual production reaching 1.1 million tonnes (Székács & Darvas, 2012).

Glyphosate herbicides are controversial. Even amongst scientists these chemicals are perceived very differently. Some describe them as *the ideal herbicide* (Duke & Powles, 2008), with little or no impact on non-target species (Giesy *et al.* 2000) and presenting no danger to human health (Williams *et al.* 2000). Other researchers have shown these chemicals to have considerable negative effects on non-target organisms such as aquatic invertebrates (Folmar *et al.*, 1979) and amphibians (Relyea, 2005; Mann *et al.,* 2009).

In similar ways, scientific research into transgenic cultivars also reaches opposing conclusions regarding health, ecology and safety questions. Both proponents and opponents present ostensibly valid scientific evidence to support their views, serving only to complicate the controversy.

Important societal challenges related to glyphosate-tolerant soybean production include ecological damage through deforestation and degradation of natural habitats (Pengue, 2005) and glyphosate pollution of environment (Benbrook, 2012). The large-scale cultivation of glyphosate-tolerant soy has also been identified as a main cause for emergence and widespread occurrence of numerous glyphosate-resistant agricultural weeds (Duke & Powles, 2008). The weed-challenges tend to be met with alternative and more potent mixes of herbicides, whereby older and arguably more toxic herbicides (notably atrazine) are reintroduced. This phenomenon has shown positive correlation with increased occurrence of severe medical problems in farmers and farm village populations in GTS-40-3-2 production areas in Argentina (Vazquez & Nota, 2011).

Despite the challenges associated with both the continued use of glyphosate as the principal herbicide and the continued cultivation of glyphosate tolerant crops, there are few attractive chemical-biotechnological alternatives at present. Several crop varieties tolerant to herbicidal chemicals glufosinate-ammonium, dicamba and 2,4-D are currently either in development, awaiting approval or already on the market. But, it is still an unresolved issue whether these crop varieties and agrochemical systems (which are relying on "old" herbicide technology) are as efficient, cost-effective or as environmentally benign, as the existing glyphosate-tolerant varieties currently available. It should also be noted that despite the aforementioned challenges posed by glyphosate-tolerant GMOs, several large biotech firms are now releasing “second-generation” glyphosate-tolerant cultivars touted as being even more efficient. Some offer “stacked-traits” biotech-packages; stacked-traits cultivars are hybrid varieties with a number of transgenes expressing herbicide tolerance of several active ingredients or inducing additional production of insecticidal Bt-toxins. This combining of approved transgenes into single cultivars is marketed in guise of a partial solution to problems posed by resistant weed and insect varieties. Sceptics judge these responses to constitute little more than temporary technological fixes, which are not sustainable for either farmers or environment (Antoniou *et al.*, 2010; Antoniou *et al.*, 2012; Heinemann *et al.*, 2014). Developing a new herbicide and getting it approved for use is very costly. According to some estimates, the financial investments of industry can amount to US $180 million and the regulatory approval can take a decade (Smith *et al.*, 2008; Mcdougall, 2010). Furthermore, it is challenging for industry to meet societal demands in such developments; new compounds are expected to have high target specificity and low general toxicity (for the environment, users and eventual consumers of agricultural commodities). The biotech-agrochemical industry therefore adheres to two general strategies: it develops and registers new transgenic cultivars and chemical compounds for the market (ISAAA, 2014); and it uses existing chemical compounds in new ways, notably through introduction of transgenic varieties that tolerate higher doses of approved agrochemicals such as glyphosate (eg. Cao *et al.*, 2012; 2013). The role of glyphosate herbicides can therefore be expected to remain predominant in global industrial agriculture, especially in cultivation of glyphosate-tolerant varieties. As such, it is relevant to consider the possible benefits and challenges associated with continued or increased glyphosate use.

Risk-assessment of transgenic cultivars

To assure accurate assessment of potential risk stemming from cultivation or consumption of transgenic cultivars, it must be assumed that relevant qualitative differences will be detectable through existing methods. This may seem a trivial point but has in fact led to immense scientific and regulatory debate involving societal, political and commercial interests. This should not be underestimated; detailed guidelines on analysis and risk-assessment of transgenic plants for cultivation and consumption have been elaborated by the European Food Safety Authority (EFSA, 2008; 2010; Waigmann *et al*., 2012), the United Nations Food and Agriculture Organisation (FAO, 2011) as well as the Organisation for Economic Cooperation and Development (OECD, 1993; 1998; 2001; 2002 and 2006). The common recommended approach is to compare new transgenic cultivars with conventional comparators (near isogenic unmodified plants) by evaluation of phenotypical traits and by analysis of molecular and biochemical constituents. Material from such transgenic plants is compared with that from conventional varieties to ensure equivalence of nutrients, minerals and other important content. As there will be natural variation in levels of such constituents, it is advised that both the transgenic variety and the comparator of choice be cultivated in adjacent, controlled environments. Complimentary sprays applicable to transgenic herbicide tolerant varieties as well as the complex genetic background of transgenic cultivars bearing stacked events complicate such benchmarking by making representative agricultural conditions more difficult to model as well as making representative comparators difficult to obtain. Thus, in assessing biosafety aspects of transgenic cultivar GTS-40-3-2 Roundup-ready soybean, I have made certain assumptions and adaptations in order to perform relevant analysis, ecological modelling and feeding studies. One such adaptation has been the decision to use a selection of subsamples to produce representative test-material for diets of different soy-types, this is presented as an alternative to using near-isogenic comparators.

It must be assumed that not only genetic background of the cultivar in question, but also abiotic factors such as soil quality, environment and cultivating regime, will play a significant role for composition of the resulting produce. Different varieties of the same species of plant will be more or less suited for specific environments and growth conditions, thus the natural ”normal” variation in nutrients and specific elements can be interpreted as significant qualitative differences in produce from the same plant variety. Compositional differences will also be seen in produce from plants grown in the same environment, if these plants happen to be significantly different genotypes. Any living organism will display phenotypic variability and flexibility, establishing visible or at least somehow measurable and quantifiable differences, even amongst clonal or otherwise near-isogenic individuals.

Since the transgenic varieties of maize and soy presently used in agriculture are primarily optimized hybrids (produced by a mixture of transgenic methods and traditional breeding techniques), it is very difficult to come up with a comparator having only the absence of the specific modification as the sole differing factor. Given these facts, EFSA has presented requirements for inclusion of several comparators when assessing a transgenic variety. Thus, it is not seen as valid practice to compare one specific transgenic variety to one specific unmodified variety, rather the quality of the specific transgenic cultivar should be compared to the variability of several representative non-transgenic cultivars.

Substantial equivalence

The principle of substantial equivalence is at the core of an immense and complex discussion amongst scientists and some explanation is justified; “substantial equivalence is a concept, developed by OECD in 1991, that maintains that a novel food, for example, one that derives from genetic modification or engineering, should be considered the same as and as safe as a conventional food if it demonstrates the same characteristics and composition as the conventional food" (Womach, 2005 p. 248). In 1997, the European Commission regulated its policy on novel foods (from transgenic plants) stating that food and feed from such plants are expected not to "present a danger for the consumer", or "mislead the consumer", or "differ from foods or food ingredients which they are intended to replace to such an extent that their normal consumption would be nutritionally disadvantageous for the consumer" (EC, 1997 p. 5). The regulation goes on to state that "[this policy...] shall apply to foods or food ingredients [...] which, on the basis of the scientific evidence available [...] are substantially equivalent to existing foods or food ingredients as regards their composition, nutritional value, metabolism, intended use and the level of undesirable substances contained therein" (EC, 1997 p. 5). This raises questions concerning the qualitative evaluation of substances which vary from benign to harmful. It could be argued that pesticide residues should simply be termed "undesirable". Such interpretation would define material such as GTS-40-3-2 as substantially different from conventional soybeans due to its inherent glyphosate residue levels.

Substantial equivalence is difficult to confirm as it would imply full analysis of all constituents in produce of a specific new plant proving same characteristics and composition as a conventional comparator. For practical reasons, such analysis is limited to a spectrum of biochemical and mineral constituents. Rhetorically it could be asked whether the finding of no significant difference in a rather limited analysis in itself confirms that the tested biological materials are equivalent? Additional constituents can arguably be considered important in specific cultivars on a case-by-case basis supporting a demand that tests be performed using supplemental methods or other approaches. One such argument has been presented as the notion that it is not enough to assess only the potential for added qualities stemming from the insert, but also to investigate possible effects of the genetic modification itself (Traavik, 1999; Traavik & Lim, 2007). Opening this discussion would be lengthy, as it is a fact that the modification process itself has been shown to have surprising results in the host genome, often with bits and pieces of the transgene scattered onto the receiving DNA. In effect this interpretation can be extracted even from the US FDA policy brief on biotech plants (US FDA, 1992). Thus, this concern is expressed not only by independent scientists. Even EFSA has acknowledged the substance of this issue through its recent adoption of guidelines on risk assessment of genetically modified (GM) plants, stating that molecular characterisation is a first step to assess unintended effects of the modification (e.g. to ensure that there is no loss of endogenous gene function at the insertion site[s])(EFSA, 2010).

Published evidence on compositional analyses of glyphosate-tolerant transgenic crops has been reviewed by biotech industry researchers (Harrigan *et al.*, 2010) as well as independent researchers (Antoniou *et al.*, 2012; Ricroch *et al.*, 2011). These research groups reached differing conclusions. Based on a total of 1,840 statistical comparisons of produce from genetically modified glyphosate tolerant cultivars compared with corresponding conventional controls, Harrigan *et al.* found that the majority of parameters (88.5%) showed no significant differences. They conclude that natural variation in crop composition are the cause of the remaining significant differences, thus proving the transgenic varieties to be within the scope of conventional crop quality variation. The review by Ricroch *et al.* supports the conclusions presented by Harrigan *et al.* and concludes that the variation of transgenic crops compositional characteristics are within the range of natural variation of unmodified comparators, but goes on to suggest that new profiling techniques using proteomics and similar molecular fingerprinting would be useful in future evaluations and safety assessment of transgenic varieties. Contrary to this, Antoniou *et al.* mention several important biosafety issues and aspects of crop quality currently undercommunicated in industry research, one being glyphosate-tolerant varieties' (notably the GTS-40-3-2 glyphosate tolerant soybean) physiological potential to absorb and accumulate herbicide residues, which can then be passed on to consumers.

Agriculture affects aquatic ecology

Agriculture affects environment not only by the fact that large areas are under cultivation and thus denuded of native biosystems, but also through the effects that agriculture activities have on surrounding habitats. Aquatic habitats are affected by remains of fertilizers and pesticides leaking from farmlands and also by remains of plant material left in the fields after harvest. Such biomass is blown into ditches by wind and carried away by rainwater runoff, often ending up in the habitats of aquatic animals (Rosi-Marshall *et al.*, 2007). Inconspicuous plankton organisms such as the common water-flea *Daphnia magna* perform important ecological functions in ponds and lakes globally, even in areas where agriculture is a dominant activity (Benzie, 2010; Wesenberg-Lund, 1926). *D. magna* therefore constitutes a relevant model organism to test certain environmental effects of agriculture. Increasing use of plant-based feed ingredients such as soybean-meal in feed formulations for aquaculture of fish and crustaceans highlights the relevance of this animal model representative of farmed organisms in aquaculture as well as indicator of ecological effects. It is also relevant to further discuss the principal aspects of laboratory-modelling industrial-scale feeding of various materials to species of animal in production. Farmers and feed-producing industry continuously introduce unconventional ingredients into the diets of farmed organisms which may raise discussions on concepts such as natural food and the possible intuitive preconceptions of food and feed composition. Established methods for toxicological testing of chemicals use different species of microorganisms, plants and animals as indicators of ecological effects on organism, biotope and ecosystem level. In similar ways, tests with rodents and other mammals, as well as human cell-lines, are used to anticipate specific effects on human health.

Test-animal biology

Daphnia magna is an omnivorous planktonic filtrator known to feed on a variety of organic material, such as unicellular algae, bacteria, yeast cells and suspended particulate material of plant and animal origin, even benthic biomass. Under optimal conditions *D. magna* form large clonal populations by asexual parthenogenic reproduction. The species is a slow swimmer highly vulnerable to predation from fish, and is thus found primarily in smaller bodies of stagnant water. *D. magna* is abundant in Europe and is uncomplicated to rear in aquaria and under laboratory condition due to tolerance to changes in temperature, water-quality and the overall phenotypic plasticity and adaptability of the species. *D. magna* is arguably the best studied of the cladoceran species and subject of extensive research for more than two-and-a-half centuries since this species was described by Schäffer in 1755 (Fryer, 2008). Daphnid breeding in captivity is relatively undemanding and species of water-flies are used in recreational aquaristics as well as intensive cultivation of aquaculture biomass, often serving as feed for larger and commercially attractive species. Daphnid species *pulex* and *magna* are globally recognized as indicator organisms. Published evidence shows *D. magna* to be a useful indicator species for aquatic ecotoxicity testing of materials, substances and effluents, starting well before 1930 (Naumann, 1929; 1933; 1934a; 1934b). Daphnids have been used for centuries in biological research and for decades in specific toxicological testing. National and international institutions, industrial organisations, universities and laboratories have developed specific guidelines and protocols for use of daphnids in acute toxicity testing and reproductive studies (US-EPA, 1996; US-EPA, 2002; OECD, 2004; OECD, 2008). At present, laboratories deploy established standard guidelines for testing of acute toxicity (OECD, 2004; US-EPA, 1996) or testing for potentially more subtle chronic effects in long-term exposure reproduction studies (OECD, 2008; US-EPA, 1996).

D. magna also has a demonstrated history as test-species in evaluating and comparing transgenic plant material such as Bt-maize MON 810 (genetically modified with insert of toxin-producing gene from *Bacillus thuringiensis*) in aquatic suspension of powdered kernel (Bøhn *et al.*, 2008; 2010) and powdered leaves (Holderbaum *et al.*, submitted). Industry studies have tested other varieties of transgenic Bt-maize in *D. magna* ecotoxicological testing of whole pollen (Collins, 1994; Privalle, 1997) or merely the Bt-toxin itself in aquatic dilution (Raybould & Vlachos, 2011).

D. magna is very adaptable and its use in testing is not limited to that of hydrophilic substances or materials that can be suspended as particulate matter. Interestingly, in recent work testing biological effects of electromagnetic fields, aquaria holding *D. magna* were simply placed relative to physical location of a source of radiation, exposing replicate experimental units to gradients of the investigated factor (Krylov, 2008). In a recent testing of qualitative aspects of laboratory plastics, juvenile *D. magna* were reared in a variety of plastic environments, with distinct and measurable effects on such parameters as growth and reproduction (Cuhra, in prep.).

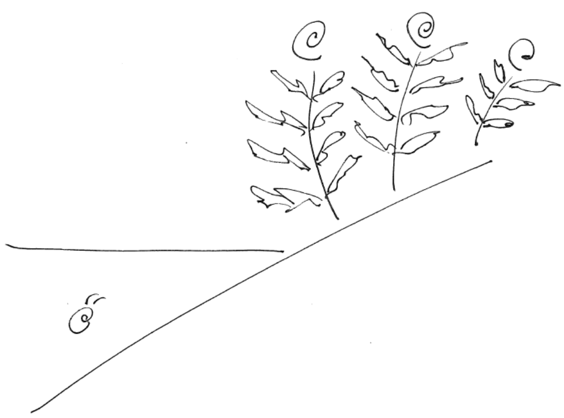
Related work

Important evidence concerning possible adverse effects of genetically modified plants emerged when it was demonstrated that transgenic MON-810 Bt-maize variety was not substantially equivalent to near-isogenic conventional maize cultivars grown in parallel plots at the same Philippines farmland (Bøhn *et al.*, 2008; 2010). Despite strong critique against the 2008 study (EFSA, 2009; Ricroch *et al.*, 2010, Romeis *et al*., 2013) it was considered important evidence of negative impact from MON-810 towards non-target organisms and used for reassessment of industry application for cultivation of MON-810 maize (Bøhn *et al.*, 2012). The study demonstrated the applicability of a well known traditional method for substance-testing as indicator of qualitative aspects of plant materials. Animals receiving feed produced from the transgenic variety had reduced reproduction and higher mortality than those fed conventional maize. This indicated that either the variety of Bt-toxin produced by the transgenic plant was having adverse effects on organisms which are not supposed to be affected, or some other compound or mechanism or possibly some unspecified consequence of the transgenic modification itself, was having significant negative impact on the *Daphnia*. The evidence indicated that potentially adverse ecological effects of MON-810 cultivation on non-target organisms could not be ruled out. The initial feeding studies in Bt-maize were performed with feed prepared from maize kernels, which is representative of the main produce entering human food chain directly or via animal feed. These experiments were supplemented by series of *D. magna* feeding studies using feed produced from lyophilized leaves from MON-810 maize and conventional parent-line comparators. These experiments are more representative in testing of ecological effects from monoculture waste biomass consisting of non-harvested material such as leaves, stems and roots. In a review of published evidence, EFSA (2009) concluded that the study by Bøhn *et al*. (2008) was flawed, mainly because a) maize flour is not part of the natural diet of *Daphnia* and the observed unusual delays in development therefore could be caused by nutritional deficiency, and b) internationally accepted guidelines for toxicity and reproduction testing in *Daphnia* were not followed. This critique however, is not a scientifically based rejection of the findings of effects on non-target organisms and must be discussed further.

It is also relevant in this context to mention the controversial work by Séralini *et al.* (2012), a life-long (two-year) study in which rats manifested adverse effects from Roundup herbicide formulation (in drinking-water) and from feeding glyphosate-tolerant NK-603 maize. It should be noted that the work by Séralini *et al.* immediately provoked a storm of critical questioning from biotech industry, researchers related to biotech industry as well as independent researchers. The EFSA reviewed the study and in 2013 concluded that it was methodologically flawed and that the results should be disregarded. Subsequently, in 2013 it was retracted by the editor of the journal that originally reviewed and published it. However, the following year the work was republished by a different journal (Séralini *et al.* 2014).

Regardless of whether the conclusions drawn by Séralini *et al.* were fully valid or not, it is important to acknowledge that the methodology in the mentioned study attempted to assess effects from; a) consumption of a glyphosate-tolerant transgenic variety in feed, b) ingestion of drinking-water containing the relevant herbicide, c) in life long exposure. This raises similar biosafety questions as the reflections leading to the work in GTS-40-3-2 soybean investigated here.

The relevance of the re-assessment of fundamental issues raised in the retracted study was further commented upon in an objection on final rule to the US EPA in 2013 (OPP Docket, 2013). The objection highlighted aspects of a US EPA internal process in 1981-83 on assessment of data from a two-year rat-feeding study submitted by industry. The rats were fed relatively high doses of glyphosate but the initial assessment of adenoma and carcinoma incidence was re-evaluated by US EPA and subsequently the conclusions were changed. The objection argued that there were findings in our studies presented here -- and the mentioned study by Séralini *et al.* -- which indicated that the US-EPA evaluation of the 1981-83 study was due for revision. However, this need for regulatory re-assessment still remains to be acknowledged.



Aim

Research question

Research question: The fundamental reflections leading to the research project can be condensed in the following research question: *Is GTS-40-3-2 genetically modified glyphosate-tolerant soy substantially equivalent to, or significantly different from, its unmodified counterpart?*

Scientific assumptions: In order to investigate the research question, the following fundamental assumption relating to relevance of scientific method is stated: *Substantial equivalence and/or significant differences are qualitative parameters which in GTS-40-3-2 genetically modified glyphosate-tolerant soy can be estimated by: 1) analysis of composition of soybean-meal, and 2) through life-long* feeding studies with soybean-meal in test-organism *Daphnia magna*.

Since GTS-40-3-2 soybean is designed to be cultivated in agriculture system dependent on glyphosate-herbicide application, agroecological aspects of these chemicals are included in the analysis and assessment.

Research hypothesis

Research hypothesis: Based on the assumption of method relevance the research question is investigated through the following hypothesis: *Composition characteristics and feed quality of meal from GTS-40-3-2 soybean will not differ significantly from meal from unmodified comparators.*

Research methodology: Following methods were used to investigate the research question; a) literature studies of relevant published evidence, b) laboratory testing of soybean material c) feeding studies with soybean material in an animal model, d) toxicological and ecotoxicological testing of glyphosate chemicals and commercial herbicide formulations, and e) revision of specific studies extracted from archives in the United States of America via a FOIA-request.

Form of presentation

The writing will present research conducted to answer the specific research question and will further reflect on the wider environmental and societal implications.

Thus the writing conforms to a traditional logical structure as presented below:

- an **introduction based on mandate and observations**
- leading to a relevant specific **research question**
- which is **tested by scientific methods**
- producing **results that are discussed**
- leading to **further reflections**
- opening a **wider environmental and societal context**

Materials and methods

Research materials

In 2009 I contacted biotech industry companies Monsanto and Dow AgroSciences to obtain plant material for testing. Monsanto replied to none of my calls, emails or regular letters. Dow AgroSciences sent me an email in response to one of my letters, politely declining to furnish material for my research. I thus had to obtain GTS40-3-2 glyphosate-tolerant soy materials by other means.

An aqueous solution of 40 % b.w. glyphosate in the form of N-(p hosphonomethyl) glycine-monoisopropylamine salt (glyphosate-IPA) was obtained from Sigma–Aldrich, St. Louis MO 63103 USA (Batch no 10519EJ). A typical commercial brand of Roundup formulation was bought from a US retailer a few months prior to the testing. (Lot I08080/FI/1/5), containing 18 % b.w. glyphosate, 0.73 %diquat-dibromide and, according to label, 81.27 % ''other ingredients''. The producer is not required to specify these other ingredients but they are generally thought to consist of mainly water, activator adjuvants and various surfactants. The brand name of this herbicide is Roundup Weed & Grass Killer Concentrate Plus (**paper I**).

Animal model

Experiments in the *D. magna* model are initiated by preparing individual beaker glasses, typically pre-fed (feeding studies) or prepared with exposure gradient toxins (toxicological studies). A healthy stock of mother animals 15 – 40 days old are kept in 4 - 8 large 4 liter holding-tanks (MF-beakers). The day before experiment is to be initiated, all juvenile animals are removed from MF-beakers by; a) transferring mothers to petri dishes and subsequently b) filtering the holding medium through 200μm plankton netting. Following this cleaning the mother animals are transferred back into the MF-beakers. No later than 24 hours following this procedure, new-born juveniles approximately 850μm in length are carefully collected from MF-beakers and transferred to a single large petri dish. Juveniles are allowed to mix freely for a few minutes and following this homogenization, individual experimental animals are taken with a glass pipette (figure 1) and allocated to individual 100ml beaker glasses. Each beaker glass contains one *D. magna* which is daily monitored and fed according to

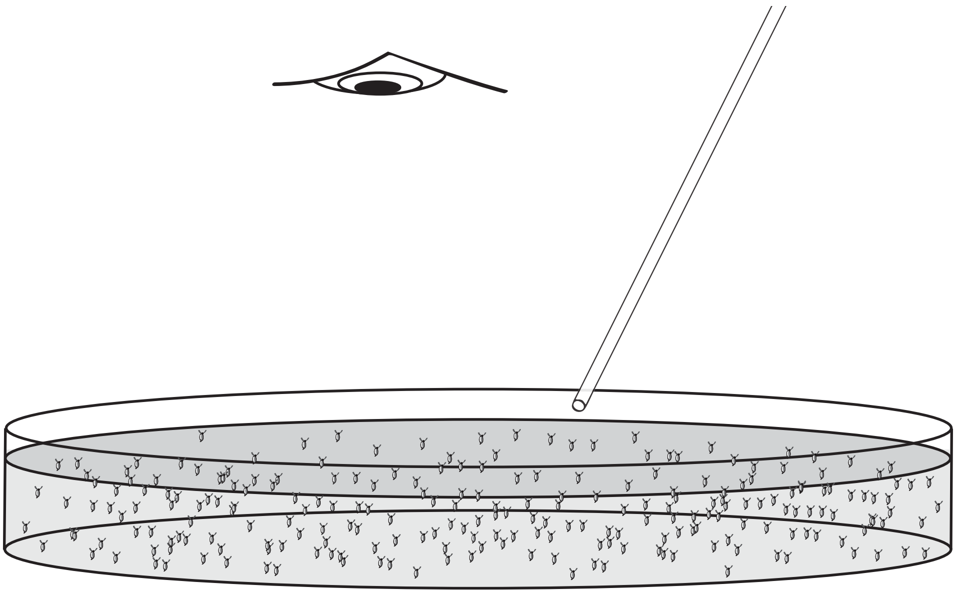


Figure 1. Petri dish with more than 300 juveniles born within 24 hours. The observer selects individual juveniles and allocates these to individual experimental units (glasses) according to randomized setup.

operating procedures and specific regime of individual experimental setup (figures 2 and 4). Each beaker is an experimental unit, which is randomly allocated a position within the experiment. Individual experimental units are permanently allocated to defined treatment groups. Treatment groups contain 10, 15, 20 or 30 experimental units, depending on experimental setup. Each beaker has a unique identifier, either as a marking on the beaker itself, or as a marking at the allocated position. To enable randomization of positions, these are affixed to plastic trays each containing 6 to 7 treatment groups, with 5 experimental units in each group. According to number of individual treatment groups, chronic toxicity experiments employ 150-250 individual animals and feeding studies employ 100-300 animals. Such large quantities of experimental units are organized on up to 9 trays with 35 (30) beaker glasses (figure 4). Tray position (A, B, C) on individual tables, tray orientation and table position/orientation is changed according to outcomes of randomization software at defined intervals, e.g. days 2, 4, 6 etc. or days 3, 6, 9 etc. for the full 42-day duration of experiments. The tables have wheels, enabling rotation and relocation. In order to facilitate handling, daily feeding and collection of data, the individual trays with 35 (30) experimental units are transferred from tables in experimental area (figure 4) to a working area equipped with light-tables, stereo-loupe microscopes and highly specific

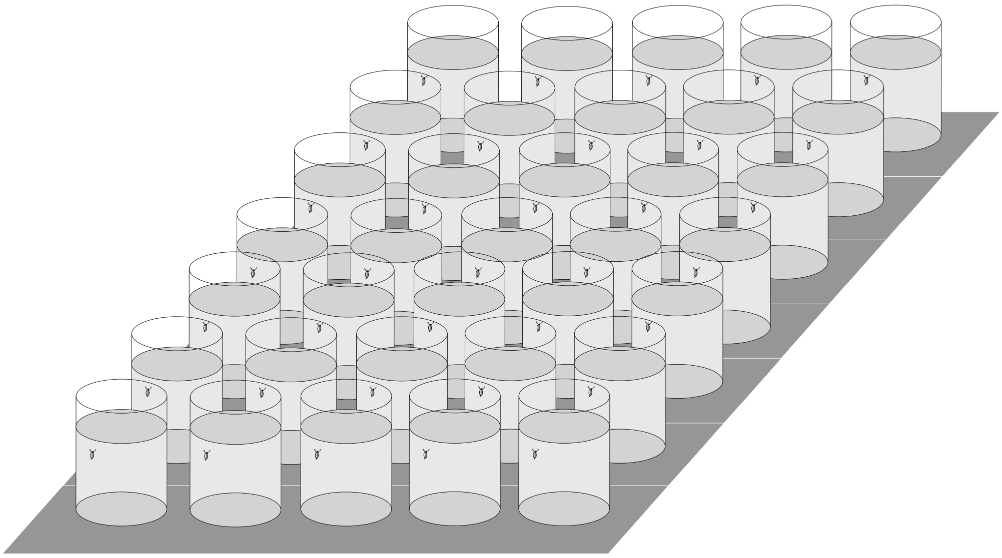


Figure 2. Tray with 7 rows, each consisting of 5 experimental units. Row position and tray position and orientation is randomized within experimental setup, and changed at regular intervals according to outcome of randomization software.

tools designed and adapted for efficient and non-disruptive handling and photography of individual animals. To describe these daily and weekly procedures in detail is not possible here, however it suffices to note that the specific daily routines involving animal handling, preparation of holding media, feeding of treatment-groups, washing glassware, harvesting information and registration of data typically demand the full attention of 1-2 primary scientists and 1-3 assistants on a daily basis (weekends included) for a 42-day period spanning the typical longevity of a *D. magna* individual. The operating costs for one such 42-day experiment are estimated at 0.25 M NOK, which is relatively low when considering the relatively high number of experimental units. Experiments in *D. magna* are established as an acknowledged standard in toxicological and ecotoxicological testing. Researchers employed by biotechnological industries Monsanto and Syngenta began using *D. magna* models to assess toxicological effects from genetically modified crops, initially as studies of pollen from Bt-varieties of corn (*Zea mays*) (Collins 1994; Privalle 1997). The model developed by GenØk (figure 3) and described here, was used for obtaing data for Bøhn *et al.,* 2008; 2010 and **papers III and IV**. Employed in traditional ecotoxicological testing of herbicides in acute exposure and chronic exposure, the GenØk model yielded the data reported in **paper I**.

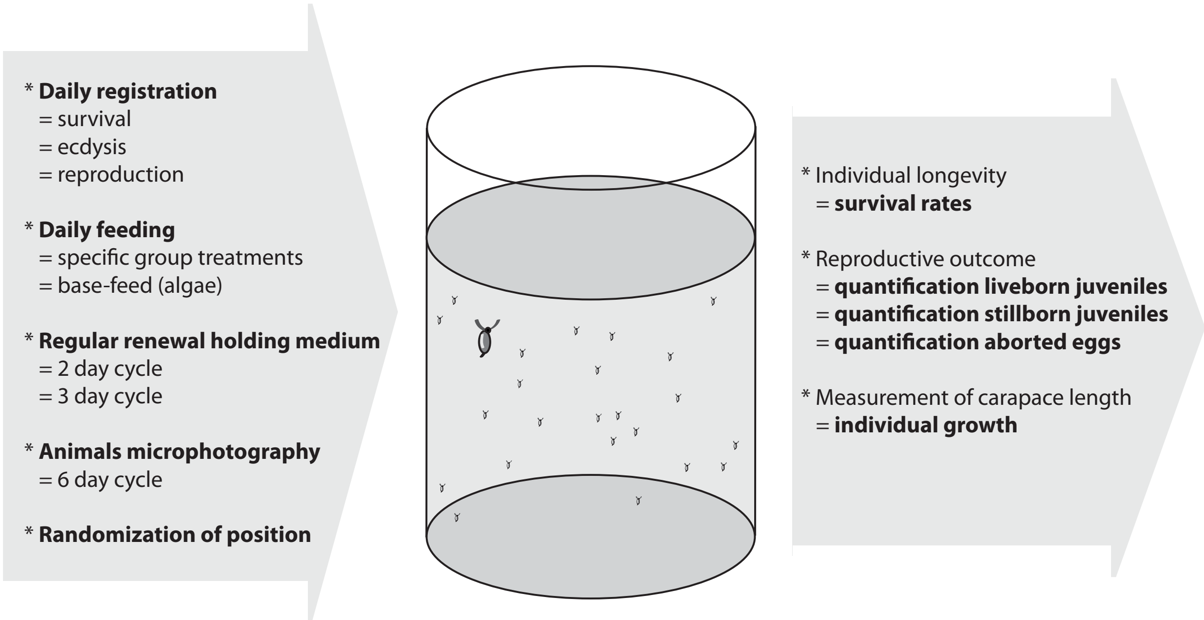


Figure 3. Schematic overview of daily routines and harvesting of endpoints from individual experimental units.

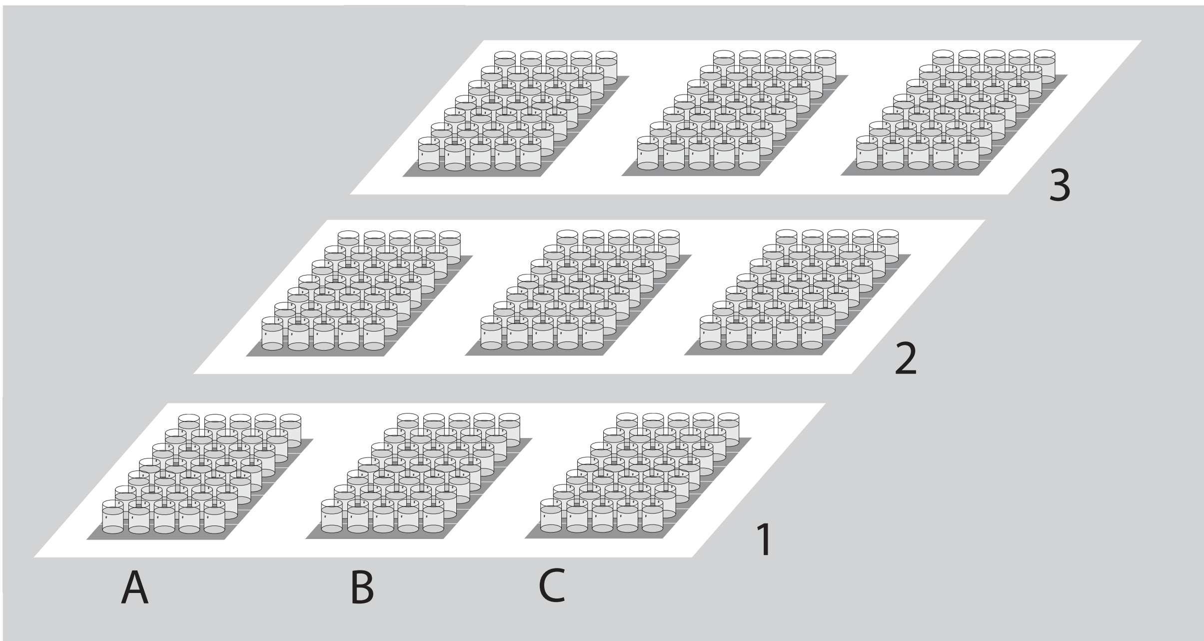


Figure 4. Full experimental setup consisting of 9 trays, each with 7 treatment groups. Individual treatments typically consist of 15-30 experimental units and thus will be distributed as 3-6 rows within the setup. Labels A, B and C denote positions on individual tables 1, 2 and 3.

Archive studies

In order to investigate the research question, it was found important to access relevant archives to evaluate original toxicological and ecotoxicological studies which form the basis of present regulation of the herbicides investigated through laboratory testing.

In November 2011 I submitted a request[1] for disclosure of information to the US EPA, pursuant to US Freedom of Information Act (FOIA). The targeted information was contained in 21 specific Master Record Identification Number (MRID) study reports. These reports were originally submitted by industry applicants as evidence of ecotoxicological effects of glyphosate, AMPA, herbicide formulation (Roundup, surfactants and inert ingredients). The MRID is the US-EPA file containing the expert evaluation of the specific test reported by the applicant[2]. My request was granted and I received the materials in early spring of 2012.

There are two ways that documentation is made available. The first is through the ongoing scanning of archives and publishing of pdf-files on searchable databases. This we may call “server-push”; it constitutes the bulk of the material. The second is by “client-pull”, through specific requests to government agencies, in this case the EPA and FDA. The 2011 EPA report to the US Department of Justice describes in some detail how the intentions of the FOIA are being implemented in the agency, with specific measurables on implementation, training, evaluation, dialogue with the public, proactive disclosure, use of social networking channels and reducing backlog (Jackson, 2011). Also, a US EPA/FOIA Chief Officer's report to the US attorney general (US EPA, 2009) states that proactive measures have been initiated regarding Office of Pesticide Program (OPP) documents, releasing a large number of scientific reviews as searchable documents in an Internet database called “The Electronic Reading Room”. It is estimated that approximately 13,000 OPP scientific reviews on 300 active ingredients are available through this EPA website. The purpose of this proactive disclosure is specifically stated as disclosing “science reviews and pesticide registration files [...] vital

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[1] Case HQ-FOI-00325-12 US EPA FOIA 2011.

[2] The Master Record Identification Number is defined as the unique cataloguing number assigned to an individual pesticide study at the time of its submission to the EPA.

to industry, interest groups, state, local and foreign governments, and many other stakeholders...” (US EPA, 2009).

The proactive disclosure is an ongoing process. Files from the EPA archives are continuously being scanned and made available to the general public. For the purpose of assessing biosafety of modern biotechnology in agriculture and studying effects of transgenic plants and associated herbicides, both EPA and Department of Agriculture (USDA) and Food and Drug Administration (FDA) documents are of importance. Coming into office, President Obama initiated a change in the policy of his predecessors: “In the two years since the President issued his FOIA Memorandum directing agencies to apply a presumption of openness to all decisions involving the FOIA, agencies across the government have taken steps to create a more open and accountable government" (FOIA, 2011).

The administrative procedures by which different public offices and parts of the US administration incorporate the intentions of the FOIA into their daily routines vary, but there is an underlying principle of openness which is valuable to science as well as to the interests of the general public. “The U.S. Freedom of Information Act (FOIA) is a law ensuring public access to U.S. government records. FOIA carries a presumption of disclosure; the burden is on the government - not the public - to substantiate why information may not be released. Upon written request, agencies of the United States government are required to disclose those records, unless they can be lawfully withheld from disclosure under one of nine specific exemptions in the FOIA. This right of access is ultimately enforceable in federal court” (FOIA, 2009).

Research manuscripts

List of papers

Paper I: Cuhra M, Traavik T, Bøhn T (2013)
Clone- and age-dependent toxicity of a glyphosate commercial formulation and its active ingredient in Daphnia magna.

Ecotoxicology, 22: 251-262.

Paper II: Bøhn T, Cuhra M, Traavik T, Sanden M, Fagan J, Primicerio R (2014)
Compositional differences in soybeans on the market: glyphosate accumulates in Roundup Ready GM soybeans.

Food Chemistry, 153: 207-215.

Paper III: Cuhra M, Traavik T, Bøhn T (2014)
Life cycle fitness differences in D. magna fed Roundup Ready soybean conventional soybean or organic soybean.

Aquaculture Nutrition 1365/2095. DOI 10.1111/anu.12199.

Paper IV: Cuhra M, Dando M, Traavik T, Primicerio R, Holderbaum, D, Bøhn T (2015)
Glyphosate-Residues in Roundup-Ready Soybean Impair Daphnia magna Life-Cycle.

Journal of Agricultural Chemistry and Environment, 4, 24-36.

Paper V: Cuhra M (2015)
Analysis of herbicide-residues are still missing in risk-assessment of glyphosate-tolerant GMO-cultivars.

Environmental Sciences Europe (manuscript accepted for publication)