

# High concentrations of protein test substances may have non-toxic effects on *Daphnia magna*: Implications for regulatory study designs and ecological risk assessments for GM crops

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**Abbreviations:** BSA, bovine serum albumin; ERA, ecological risk assessment; NTO, non-target organism

Laboratory testing for possible adverse effects of insecticidal proteins on non-target organisms (NTOs) is an important part of many ecological risk assessments for regulatory decision-making about the cultivation of insect-resistant genetically modified (IRGM) crops. To increase confidence in the risk assessments, regulatory guidelines for effects testing specify that representative surrogate species for NTOs are exposed to concentrations of insecticidal proteins that are in excess of worst-case predicted exposures in the field. High concentrations in effects tests are achieved by using protein test substances produced in microbes, such as *Escherichia coli*. In a study that exposed *Daphnia magna* to a single high concentration of a microbial test substance containing Vip3Aa20, the insecticidal protein in MIR162 maize, small reductions in growth were observed. These effects were surprising as many other studies strongly suggest that the activity of Vip3Aa20 is limited to Lepidoptera. A plausible explanation for the effect on growth is that high concentrations of test substance have a non-toxic effect on *Daphnia*, perhaps by reducing its feeding rate. A follow-up study tested that hypothesis by exposing *D. magna* to several concentrations of Vip3Aa20, and a high concentration of a non-toxic protein, bovine serum albumin (BSA). Vip3Aa20 and BSA had sporadic effects on the reproduction and growth of *D. magna*. The pattern of the effects suggests that they result from non-toxic effects of high concentrations of protein, and not from toxicity. The implications of these results for regulatory NTO effects testing and ERA of IRGM crops are discussed.

## Introduction

Ecological risk assessments (ERAs) are important components of regulatory decision-making about the cultivation of insect-resistant genetically modified (IRGM) crops. ERAs estimate the probability and seriousness of any harmful effects to non-target organisms (NTOs) that may result from exposure to the insecticidal protein during or following cultivation of the GM crop.

In many countries, regulatory ERAs for IRGM crops follow a tiered approach.<sup>1,2</sup> ERAs begin with laboratory effects (hazard) tests that expose representative surrogate species to concentrations or doses of the insecticidal active ingredient – almost always a protein – in excess of predicted worst-case exposures of NTOs in the field. If no adverse effects are detected, in other words, there is no hazard, minimal ecological risk can be concluded

without need for further consideration of exposure or effects. Adverse effects in such tests, however, do not necessarily indicate that adverse effects are likely in the field. Further analysis of the potential effects or exposure, or both, may show that the likelihood of ecological harm is negligible.

Many elements of the experimental design of effects tests for regulatory ERAs are closely specified by regulatory authorities. Usually, studies must conform to internationally accepted guidelines, such as those set by the Organisation for Economic Cooperation and Development (OECD). Guidelines stipulate study-design features such as the test organism, sample size, duration, endpoints, temperature, light, humidity, and concentration of test substance to which the organism is exposed.<sup>3</sup> Standardization helps to ensure the reliability and reproducibility of the studies, and provides decision-makers with readily interpretable data.<sup>4</sup>

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Guidelines for regulatory ecotoxicology studies often specify a minimum amount of active ingredient per unit weight of the organism, or a minimum multiple of a conservative estimate of the environmental concentration of the substance under the proposed pattern of use. Large amounts of test substance allow for scientific and policy uncertainty; for example, variation in the sensitivity of species to the active ingredient, or uncertainty about how to balance risks and potential benefits from using products containing the active ingredient.<sup>5,6</sup>

When using large amounts of test substance to help decision makers handle uncertainty, there must be confidence that adverse effects observed in a study accurately indicate the hazard of the active ingredient in the field. Adverse effects could indicate, for example, toxicity of impurities in the test substance, or that physical properties of high concentrations of the test substance adversely affect the test organism. Hence, the hazards of large amounts of a test substance in a laboratory study may be different from the hazards of its active ingredient in the field.

Here we report 2 regulatory studies of the effects on *Daphnia magna* (Cladocera: Daphniidae) of a test substance containing the insecticidal protein Vip3Aa20. The studies were conducted as part of ecological risk assessments for the cultivation of transgenic MIR162 maize,<sup>7</sup> which produces Vip3Aa20 to control various lepidopterous pests. The studies illustrate potential problems in interpreting effects in studies that use large amounts of test substance.

In the first study, individual *D. magna* (“daphnids”) were exposed to 752.6 µg Vip3Aa20/L (= 870 µg/L of test substance) for 10 d according to OECD Guideline 202. This concentration represented 10X the worst-case concentration of Vip3Aa20 in water following the cultivation of MIR162 maize calculated using United States Environmental Protection Agency’s Generic Estimated Environmental Concentration (GENEEC) model.<sup>7</sup> GENEEC is used in screening ecological risk assessments for synthetic pesticides and uses conservative assumptions to calculate exposure estimates. The model is even more conservative for estimating exposure to proteins in transgenic crops;<sup>8</sup> for example, proteins are unlikely to run-off from soil, and they are likely to become inactive in plant tissue before the material has aged sufficiently to be palatable to aquatic organisms.<sup>9</sup>

Survival, growth and reproduction of the daphnids exposed to Vip3Aa20 were compared with those of animals exposed to water only. Unexpectedly, small, but statistically significant, reductions in length and body weight were observed in the Vip3Aa20 treatment. A summary of this study has been published previously,<sup>7</sup> but we present here detailed experimental methods and data.

To investigate further the effect of Vip3Aa20, a second study exposed daphnids to 5 concentrations of Vip3Aa20 of between 188.1 µg/L and 3010.2 µg/L (up to 3480 µg/L of test substance) according to OECD Guideline 211. Again, survival, growth and reproduction of the animals were compared with those of a control group exposed to water only; however, in this study, exposure was for 21 d. This and other differences in the design of the studies resulted from their fulfilling different regulatory requirements.

An additional control group exposing daphnids to 3480 µg/L of bovine serum albumin (BSA) was included to test the hypothesis that the effect of Vip3Aa20 on the growth of *D. magna* in the

first study resulted from the effects of high concentrations of protein, and not from the specific toxicity of Vip3Aa20. The second study provided some corroboration of the hypothesis. Recommendations for designing protein toxicity studies using *D. magna* are made in light of these results.

## Results

### Water-quality parameters

The results of water-quality measurements for both studies are summarised in Table 1. They show that water quality was similar between the studies. They also show that the test and control substances did not affect water quality. The conditions remained within acceptable limits for the survival, growth and reproduction of *D. magna*.

### Biological observations: Study 1

Observations of *D. magna* survival, reproduction and growth from the first study are summarised in Table 2.

### Survival

Survival was 100% in the treatment and control groups. In the treatment group, all daphnids began reproducing on Day 7,

**Table 1.** Water-quality parameters in studies of the effects of Vip3Aa20 on *Daphnia magna*

Nominal Concentration	Range		
	Dissolved Oxygen mg/L	Temperature °C	pH
Study 1			
Control			
New	8.7 – 9.6	19 – 21	8.1 – 8.3
Aged	8.1 – 10	20 – 21	8.1 – 8.6
752.6 µg Vip3Aa20/L			
New	8.1 – 10	19 – 21	8.1 – 8.3
Aged	8.8 – 9.6	20 – 21	8.1 – 8.6
Study 2			
Control			
New	9.0 – 9.7	19 – 21	8.3 – 8.4
Aged	8.1 – 10.1	19 – 21	8.1 – 8.5
3480.0 µg BSA/L			
New	9.0 – 10.0	19 – 21	8.3 – 8.4
Aged	8.4 – 10.0	19 – 21	8.1 – 8.5
188.1 µg Vip3Aa20/L			
New	9.0 – 9.90	20 – 21	8.4 – 8.4
Aged	8.0 – 10.0	20 – 21	8.1 – 8.5
376.3 µg Vip3Aa20/L			
New	9.0 – 10.0	19 – 21	8.2 – 8.4
Aged	8.1 – 10.0	19 – 21	8.3 – 8.5
752.6 µg Vip3Aa20/L			
New	8.9 – 9.9	19 – 21	8.2 – 8.4
Aged	8.0 – 10.1	19 – 21	8.2 – 8.6
1505.1 µg Vip3Aa20/L			
New	8.9 – 9.9	19 – 21	8.2 – 8.4
Aged	8.1 – 10.1	19 – 21	8.3 – 8.5
3010.2 µg Vip3Aa20/L			
New	8.0 – 9.8	19 – 21	8.2 – 8.4
Aged	7.9 – 10.1	19 – 21	8.2 – 8.5

**Table 2** Summary of the biological observations made in the 10-day study of the effects of Vip3Aa20 on *D. magna*

Treatment	N	% Survival	Mean day of first brood release $\pm$ SD	Mean offspring per daphnid $\pm$ SD	Mean body length (mm) $\pm$ SD	Mean body weight (mg) $\pm$ SD
Control	50	100	6.92 $\pm$ 0.49	13.5 $\pm$ 6.2	3.72 $\pm$ 0.10	0.57 $\pm$ 0.10
752.6 $\mu$ g Vip3Aa20/L	50	100	6.98 $\pm$ 0.14	17.2 $\pm$ 9.2*	3.46 $\pm$ 0.10***	0.46 $\pm$ 0.10***

\*Significantly different from control,  $p = 0.05 - 0.01$ ;

\*\*\*significantly different from control,  $p < 0.001$ .

apart from one which began on Day 6. In the control group, 7 daphnids began reproduction on Day 6, 41 on Day 7, 1 on Day 8 and 1 on Day 9. The mean time to reproduction was not significantly different between the treatment and control groups.

#### Reproduction

All daphnids in the study reproduced. The mean number of offspring per daphnid was 17.2 in the Vip3Aa20 treatment group and 13.5 in the control group. The difference between the treatment and control means was statistically significant ( $t_{[85]} = 2.36$ ;  $p = 0.021$ ). The summary of these results published elsewhere<sup>7</sup> reported that the difference was not statistically significant: this was because the hypothesis under test was that exposure to Vip3Aa20 would not reduce the number of offspring (i.e., a one-sided test), not a 2-sided test for no difference in the number of offspring.

#### Growth

The daphnids in the Vip3Aa20 treatment group were smaller than those in the control group. The mean length of daphnids in the treatment group was 3.46 mm and the mean of the control group was 3.72 mm. The difference between the means was statistically significant ( $t_{[98]} = 13.0$ ;  $P < 0.001$ ). In the treatment group, the mean dry weight was 0.46 mg and the mean of the control group was 0.57 mg. Again, the difference between the means was statistically significant ( $t_{[98]} = 5.50$ ;  $P < 0.001$ ).

#### Biological observations: Study 2

Observations of *D. magna* survival, reproduction and growth from the second study are summarised in Table 3.

#### Survival

Survival was high in the study, with only a single death in each of 3 treatments. These deaths were caused by handling errors and were not treatment related.

#### Reproduction

In all treatment groups, the timing of the first brood was earlier than in the control group. In general, increasing concentrations of Vip3Aa20 were associated with shorter time to reproduction (Spearman rank correlation coefficient =  $-0.829$ ;  $p = 0.025$ ). However, only the differences between 752.6 and 1505.1  $\mu$ g/L treatment groups and the control group were statistically significant ( $t_{[10]} = 3.01$ ;  $p = 0.015$  and  $t_{[10]} = 2.88$ ;  $p = 0.015$ , respectively). All daphnids in the study reproduced. The earliest onset was Day 6 (1, 2 and 4 individuals respectively in the 376.3, 752.6  $\mu$ g/L and 1505.1  $\mu$ g Vip3Aa20/L treatment groups), and the latest onset was Day 8 (many individuals in all groups).

Mean time to first brood in the BSA treatment was also earlier than that in the control treatment and the difference was statistically significant ( $t_{[98]} = 4.24$ ;  $P < 0.001$ ). The difference between the mean time to reproduction in the 752.6  $\mu$ g Vip3Aa20/L treatment group and the BSA treatment group was not statistically significant ( $t_{[17]} = 0.37$ ;  $p = 0.718$ ), nor was the difference between the 1505.1  $\mu$ g Vip3Aa20/L and BSA groups ( $t_{[12]} = 0.61$ ;  $p = 0.558$ ).

The mean number of offspring per daphnid showed no trend with increasing concentration of Vip3Aa20 after 10 d or 21 d. After 10 days, there were statistically significantly more offspring in the 1505.1  $\mu$ g Vip3Aa20/L treatment group compared with the control group ( $t_{[18]} = 3.01$ ;  $p = 0.003$ ). After 21 days, there was a statistically significant reduction in the number of offspring in the 3010.2  $\mu$ g Vip3Aa20/L treatment group compared with

**Table 3.** Summary of the biological observations made in the 21-day study of the effects of Vip3Aa20 on *D. magna*

Treatment	N	% Survival	Mean day of first brood release $\pm$ SD	Mean offspring per daphnid $\pm$ SD 10 days	Mean offspring per daphnid $\pm$ SD 21 days	Mean body length (mm) $\pm$ SD	Mean body weight (mg) $\pm$ SD
Control	10	100	7.90 $\pm$ 0.31	22.5 $\pm$ 6.7	218 $\pm$ 32	3.31 $\pm$ 0.20	0.28 $\pm$ 0.13
188.1 $\mu$ g Vip3Aa20/L	10	90	7.70 $\pm$ 0.48	21.8 $\pm$ 11.2	238 $\pm$ 18	3.28 $\pm$ 0.13	0.21 $\pm$ 0.10
376.3 $\mu$ g Vip3Aa20/L	10	100	7.60 $\pm$ 0.70	19.3 $\pm$ 6.8	235 $\pm$ 35	3.36 $\pm$ 0.13	0.28 $\pm$ 0.11
752.6 $\mu$ g Vip3Aa20/L	10	90	7.10 $\pm$ 0.74*	25.8 $\pm$ 14.1	249 $\pm$ 52	3.44 $\pm$ 0.20	0.39 $\pm$ 0.10
1505.1 $\mu$ g Vip3Aa20/L	10	100	7.00 $\pm$ 0.94*	33.0 $\pm$ 8.6**	214 $\pm$ 37	3.55 $\pm$ 0.09**	0.34 $\pm$ 0.07
3010.2 $\mu$ g Vip3Aa20/L	10	90	7.40 $\pm$ 0.70	30.8 $\pm$ 10.3	178 $\pm$ 27**	3.14 $\pm$ 0.14	0.26 $\pm$ 0.06
3480.0 $\mu$ g BSA/L	10	100	7.20 $\pm$ 0.42***	23.4 $\pm$ 14.0	182 $\pm$ 35*	3.13 $\pm$ 0.24	0.16 $\pm$ 0.10*

\*Significantly different from control,  $p = 0.05 - 0.01$ ;

\*\*Significantly different from control,  $p < 0.001 - 0.001$ ;

\*\*\*Significantly different from control,  $p < 0.001$ .

the control group ( $t_{[18]} = 3.02$ ;  $p = 0.007$ ). None of the other Vip3Aa20 treatments had a statistically significant effect on the number of offspring.

The mean number of offspring in the BSA treatment group was not significantly different from the control group at 10 days, but was significantly reduced compared with the control after 21 d ( $t_{[18]} = 2.40$ ;  $p = 0.027$ ). At 10 days, the difference in the number of offspring between the BSA treatment group and the 1505.1  $\mu\text{g}$  Vip3Aa20/L treatment group was not statistically significant ( $t_{[18]} = 1.85$ ;  $p = 0.081$ ). At 21 days, the difference in the number of offspring between the BSA treatment group and the 3010.2  $\mu\text{g}$  Vip3Aa20/L treatment group was also not statistically significant ( $t_{[17]} = 0.28$ ;  $p = 0.786$ ).

### Growth

Exposure to Vip3Aa20 was not associated with a statistically significant reduction in body length or weight, even at 3010.2  $\mu\text{g}$  Vip3Aa20/L, which is just under 4 times the concentration in the first study (752.6  $\mu\text{g/L}$ ). The only statistically significant effect of exposure to Vip3Aa20 on the size of daphnids was in the 1510.1  $\mu\text{g/L}$  group in which the daphnids were on average larger than in the control group ( $t_{[12]} = 3.46$ ;  $p = 0.005$ ). The daphnids exposed to 1510.1  $\mu\text{g}$  Vip3Aa20/L were also significantly larger than those exposed to BSA ( $t_{[11]} = 5.18$ ;  $P < 0.001$ ).

Exposure to BSA did not reduce the mean length of daphnids significantly, although it was associated with a significant reduction in mean weight compared with the control ( $t_{[18]} = 2.31$ ;  $p = 0.003$ ). On average, the daphnids exposed to BSA were lighter than those in all the Vip3Aa20 treatment groups, apart from those exposed to 188.1  $\mu\text{g}$  Vip3Aa20/L.

## Discussion

The small, but statistically significant, reduction in size of daphnids exposed to 752.6  $\mu\text{g}$  Vip3Aa20/L in the first study was surprising because previous studies suggested that toxicity of Vip3Aa20 was limited to certain Lepidoptera.<sup>7,10-14</sup> The alternative hypothesis to toxicity of Vip3Aa20 is that reduced size was due to another mechanism affecting the feeding or nutritional status of *Daphnia*. Suspended particles, for example, are known to reduce the filtering rate of *Daphnia* species leading to reduced body size,<sup>15-17</sup> and the high concentration of protein test substance may have had similar effects.

Observations of reproduction in the first study could be interpreted as toxicity. Reduced nutrition usually delays reproduction and reduces the number of offspring produced by *D. magna*<sup>18</sup> and other species of *Daphnia*.<sup>19-21</sup> Toxicity, on the other hand, might explain the increased number of offspring because at low concentrations certain toxins are known to increase reproduction in *D. magna*.<sup>22-24</sup> However, they reduce it at higher concentrations. This stimulatory effect of toxins at low concentrations is called hormesis.<sup>25</sup>

To test further the nutrition and toxicity hypotheses for the effect of Vip3Aa20 on *D. magna*, a second study was conducted. If the toxicity hypothesis is correct, a dose – response relationship

between Vip3Aa20 and changes in growth and reproduction of *D. magna* is expected. To test this hypothesis, daphnids were exposed to several concentrations of Vip3Aa20. To test the nutrition hypothesis, daphnids were exposed to a non-toxic protein, BSA; if this hypothesis is correct, effects on growth and reproduction are expected from non-specific effects of high amounts of protein regardless of its toxicity, perhaps acting by reducing feeding.

Reduction in growth of daphnids exposed to Vip3Aa20 was not observed in the second study. There was no significant decrease in mean weight or length at any of the treatment concentrations, including 752.6  $\mu\text{g}$  Vip3Aa20/L, the concentration in the first study. The mean body length of daphnids in the group exposed to 1505.1  $\mu\text{g}$  Vip3Aa20/L was significantly higher than the mean of the control; however, there was no significant difference in the mean weights.

The mean weight of daphnids exposed to 3480  $\mu\text{g}$  BSA/L was significantly lower than that of the control group; however, there was no significant difference in mean lengths. This observation provides some corroboration of the hypothesis that if Vip3Aa20 does adversely affect the growth of daphnids in some circumstances, it does so through high concentrations of protein somehow reducing their nutritional status.

As in the first study, exposure to Vip3Aa20 was associated with the production of more offspring after 10 days, but only at 1505.1  $\mu\text{g}$  Vip3Aa20/L, which is higher than in the first study. After 21 days, only the 3010.2  $\mu\text{g}$  Vip3Aa20/L treatment was associated with an effect on the mean number of offspring, and in this case a reduction not an increase. A similar effect of reduced numbers of offspring was also seen in the BSA treatment.

Finally, 2 of the Vip3Aa20 treatments were associated with earlier reproduction. A similar effect was also seen in the BSA treatment.

Overall, the growth and reproduction endpoints in the 2 studies do not support the hypothesis of toxicity of Vip3Aa20 to *D. magna*. Unambiguous adverse effects were either not reproducible – in the case of reduced size – or were also seen in the BSA treatment – in the case of the mean number of offspring after 21 days. Similarly, earlier release of brood was not reproducible between the studies and was seen in the BSA treatment.

The only Vip3Aa20 effect that was seen in both studies and was not observed in the BSA treatment was the higher mean number of offspring after 10 days. It is speculative to infer that this effect is an indicator of toxicity. Based on life-history theory,<sup>26</sup> Bøhn et al.<sup>27</sup> suggested that greater investment in reproduction early in the life cycle of *D. magna* indicates toxicity rather than reduced nutritional quality. However, early reproduction was hypothesized as a response to conditions leading to reduced survival, which was not seen in either Vip3Aa20 study. One might also suggest that Vip3Aa20 is toxic at much higher concentrations than used in either study, and that the higher number of brood results from hormesis. Even if this hypothesis is plausible, it is not relevant to ERA for use of transgenic crops. Environmental exposures to Vip3Aa20 of over 3010  $\mu\text{g/L}$  resulting from the cultivation of a crop producing this protein are virtually inconceivable: this concentration is 40X the worst-case

predicted exposure from cultivating MIR162 maize calculated using the GENEEC model.

Ecotoxicology studies using *D. magna* tend to show small differences in response among studies, mainly because of variation in study conditions.<sup>28</sup> In the 2 studies reported here, there were no obvious differences in water quality between the studies (Table 1). However, clearly there must have been differences that led to some effects in one study not being observed in the other.

Given the sensitivity of growth and reproduction of *D. magna* to differences in diet quality, it is likely that subtle differences in starting conditions and interactions between the diet and test substance led to different results in the 2 studies, and to differences among treatments within studies. Furthermore, as the protein test substance was a potential source of nutrition, it was important to include a non-toxic protein control in the second study to help interpretation of the results. As the BSA treatment was associated with similar statistically significant differences from the control in some of the high concentration VipA20 treatments (earlier reproduction, reduced number of offspring after 21 d and reduced size), and also was not statistically significantly different from Vip3Aa20 treatments that were different from the control, inclusion of a protein control in future studies exposing *D. magna* to protein test substances seems warranted.

Taken together, the results of the 2 studies show that the Vip3Aa20 test substance has small, inconsistent, but statistically significant effects on growth and reproduction of *D. magna* at high protein concentrations. The results of the BSA treatment suggest a plausible origin of these effects is a non-toxic interaction between high concentrations of soluble protein and the diet leading to life-history variation in the daphnids in response to different nutritional status among treatments and between studies. These data provide further corroboration of the hypothesis that cultivation of MIR162 maize will not have harmful ecological effects.<sup>7</sup>

These results have important implications for use of *D. magna* as a surrogate species in regulatory effects tests contributing to ERAs for the cultivation of transgenic crops. First, a more realistic model is needed to assess likely exposure of aquatic organisms in transgenic crops. While conservatism is useful in tiered testing,<sup>29</sup> overly conservative exposure estimates, along with high margins of exposure, could lead to non-toxic effects from high amounts of test substance. Secondly, when using protein test substances, a non-toxic protein control is useful to distinguish direct toxic effects from non-specific effects of proteins on the nutritional status of *D. magna*. Finally, conclusions about the cause of effects observed in *Daphnia* studies should be based on all the endpoints. Life-history theory may predict that toxicity leads to early reproduction; however, this is hypothesized to be a response to conditions leading to increased mortality. As there was no treatment-related mortality in either study, earlier reproduction is unlikely to be a sign of toxicity of Vip3Aa20 to *D. magna*.

## Materials and Methods

### Study 1

In this study, *D. magna* was exposed to 752.6 µg Vip3Aa20/L for 10 d according to OECD Guideline 202. The test substance

was an 86.5% pure preparation of Vip3Aa20 from *E. coli* transformed with the *vip3Aa20* gene. The concentration of Vip3Aa20 was chosen to represent 10X the worst-case concentration of Vip3Aa20 in water following the cultivation of MIR162 maize calculated using the GENEEC model.<sup>7</sup> The test substance was dissolved in purified water with alkalinity of 100 mg CaCO<sub>3</sub>/L and pH between 7.9 and 8.4.

The treatment group comprised 50 daphnids held individually in 100 mL beakers containing 50 mL of the Vip3A test solution. A control group of 50 daphnids were similarly exposed to 50 mL of the purified water used to prepare the Vip3Aa20 solution. The treatment and control solutions were replaced daily.

The beakers were held at 20 ± 2°C and were kept under a photoperiod of 16 hours of light and 8 hours of darkness. Daphnids were fed a suspension of unicellular green algae, yeast, cereal leaves and flaked fish food throughout the study.

The number of immobilised (assumed dead) daphnids was recorded daily. Offspring were counted and removed on the first release of brood in any beaker and daily for the remainder of the test. At the end of the test, the length and dry weight of all surviving parental daphnids were determined. The statistical significance of differences between the treatment and control groups was evaluated by 2-sample t-tests.

### Study 2

In this study, *D. magna* was exposed Vip3Aa20 for 21 d according to OECD Guideline 211. The test substance was the same as used in the first study. Several concentrations of Vip3Aa20 were used in order to test for a dose response: 188.1 µg/L, 376.3 µg/L, 752.6 µg/L, 1505.1 µg/L and 3010.2 µg/L. These represent 2.5X, 5X, 10X, 20X and 40X the worst-case concentration of Vip3Aa20 in water following the cultivation of MIR162 maize calculated using the GENEEC model. The test substance was dissolved in purified water with alkalinity of 186–194 mg CaCO<sub>3</sub>/L and pH between 8.1 and 8.6.

Each Vip3Aa20 treatment group comprised 10 daphnids held individually in 250 mL beakers containing 100 mL of test solution. A blank control group of 10 daphnids was similarly exposed to purified water only. To test for possible non-specific effects of high concentrations of protein, an additional control group was included comprising 10 daphnids individually exposed to 100 mL of 3480.0 µg BSA/L. This concentration is equivalent to the highest concentration of Vip3Aa20 test substance: 3480.0 µg /L of 86.5% pure test substance gives 3010.2 µg Vip3Aa20/L. All treatment and control solutions were replaced daily.

The beakers were held at 20 ± 1°C and were kept under a photoperiod of 16 hours of light and 8 hours of darkness. Daphnids were fed a suspension of unicellular green algae throughout the study. Biological observations were carried out in the first study, except that the study was ended after 21 d. Records of brood production were conducted daily, whereas size measurements could be taken only at the end of the study; therefore reproduction endpoints, but not size, can be compared directly between groups. The statistical significance of differences between groups was evaluated by 2-sample t-tests.

## Disclosure of Potential Conflicts of Interest

The authors are employees of Syngenta, which sells products containing Vip3Aa20.

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