Report as of FY2006 for 2006IN189B: "Environmental Risk Assessment of Soybean Rust Fungicides Use in Indiana"

Publications

- Conference Proceedings:
  - Cerbin S, Biallkowski W, Ochoa-Acuña H. 2006. Effect of Propiconazole and Strobilurin Fungicides alone or in combination on two algal species: Selenastrum capricornutum and Chlorella vulgaris. Society for Environmental Toxicology and Chemistry North America 27th Annual Meeting, Montreal, Quebec, Canada.
  - Ochoa-Acuña H, Bialkowski W, Cerbin S, Hahn L, Engel B. 2006. Probabilistic Evaluation of Potential Environmental Effects Caused by Soybean Fungicides use in Indiana. Society for Environmental Toxicology and Chemistry North America 27th Annual Meeting, Montreal, Quebec, Canada.
- unclassified:
  - Bialkowski W, Cerbin S, Ochoa-Acuña H. 2006. Acute and Chronic Toxicities of Parathion and Propiconazole Mixtures on the Aquatic Invertebrate, Daphnia magna. Society for Environmental Toxicology and Chemistry North America 27th Annual Meeting, Montreal, Quebec, Canada.
  - Hahn L, Zhai T, Lim K, Ochoa-Acuña H, and Engel B. 2006. Potential Water Quality Impacts of Asian Soybean Rust Fungicide Applications in Indiana. Society for Environmental Toxicology and Chemistry North America 27th Annual Meeting, Montreal, Quebec, Canada.

Report Follows

Title: Environmental Risk Assessment of Soybean Rust Fungicides Use in Indiana

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Problem

We evaluated the environmental safety of fungicides to be used for combating Asian soybean rust in Indiana. Soybean rust is a highly contagious, fungal crop disease recently detected in several US states. A recent study conducted by the USDA (Livingstone et al. 2004) estimates that conditions are favorable for the development of soybean rust during most years for the Midwest. The introduction of this pest has prompted the EPA to approve emergency registration exceptions for use on soybeans for several fungicides in the U.S. To date, 14 active ingredients have been approved or are under consideration for use against soybean rust, of which only three (chlorothalonil, azoxystrobin, and pyraclostrobin) have been granted full registration for use on soybeans.

Fungicide losses into surface water may become significant as the management recommendation is to use fungicides at their maximum allowable rate to prevent development of resistant fungal strains. According to the USDA study mentioned above, 8.6 million acres are regularly planted with soybeans in Lake States (Michigan, Minnesota and Wisconsin), whereas the acreage normally planted in Indiana exceeds 5 million acres. Because of the large acreage devoted to produce soybeans, control of soybean rust will likely result in large amounts of new fungicides entering the state's environment. We have already predicted the potential concentration in field runoff of these fungicides for Indiana based on their recommended application patterns. However, we are unable to provide an accurate estimate of the environmental risks associated with these concentrations given the scarcity of effects data for aquatic organisms. Given this lack of adequate data, a major effort of this project was to test the acute and chronic toxicity of these chemicals on algae and zooplankton.

Published information allows to predict that the toxicity of these fungicides is likely to be significant for both human and ecological receptors. The majority of these products are conazoles, or azole antifungal agents. Some conazoles induce tumors in rats, and others induce adverse developmental and reproductive effects. The EPA considers tetraconazole and cyproconazole to be possible human carcinogens and has derived oral cancer slope factors for them. Although the ecological effects database for these fungicides is very limited, the data available indicate that several are extremely toxic to aquatic biota, including fish, invertebrates, and algae. In addition, several studies have documented significant potentiation of adverse effects of pyrethroid and organophosphorous insecticides by previous exposure to conazole fungicides in rats, birds, fish, and invertebrates (Pilling et al. 1995, Ronis and Badger 1995, Johnston et al. 1996, Levine and Oris 1999).

Research Objectives: The main objective of this study was to evaluate the potential environmental impact of soybean rust fungicides to inform the development of best management practices for controlling this disease. This investigation focused on the following specific objectives:

- ☞ Objective 1: Determine the toxicity of fungicides to unicellular algae (*Selenastrum capricornotum*).
- ☞ Objective 2: Determine the acute toxicity of fungicides to representative zooplankton (*Daphnia magna*).
- ☞ Objective 3: Determine the potential synergistic action of conazole fungicides and organophosphorous insecticides

Methodology:

a. Toxicity testing of algae:

Six concentrations of each fungicide along with a control group were tested simultaneously. All experiments were run using four replicates. Tests were conducted on 30 mL flasks or using a new methodology based on 96-well microtiter plates, with each well containing 300 µL of test solution. Tests solutions were prepared using standard algal growth medium, prepared per U.S EPA specifications (U.S. EPA 2002).

At the start of the test, each well was inoculated with 20 µL of an algal suspension containing approximately 3.7 x 10^5 cells/mL. Light was provided at a rate of 1500 lux 24 h/day. Plates were constantly shaken for the 3-day test period using an IKA MTS microplate orbital shaker (Wilmington, NC).

Algal concentrations were determined every 12 h using a Dynex Revelation MRX II automatic plate reader (Chantilly, VA) setting the wavelength to 680 nm. Previous studies have shown this wavelength provides the best fit between absorbance and count data of unicellular algae (Kasai et al. 1993, Ma et al. 2001). Calibration curves were checked for accuracy during each plate reading.

The percent growth inhibition was calculated by comparing the algal concentration in each treatment to the concentration of algae in the control groups after 72 h of exposure. Using these data, the fungicide concentration associated with a 50% decrease in algal biomass (i.e., the median inhibition concentration, or IC_{50}) was calculated.

b. Acute toxicity testing of zooplankton:

D. magna were exposed to the same six concentrations used in the algal tests. All experiments were run using four replicates. Tests were performed in 30-mL borosilicate glass beakers.

Tests were commenced by adding five 1-day old *D. magna* to each beaker. Beakers were checked twice daily for a period of four days. The number of surviving animals at different time intervals (24, 48, and 96 h) were used to calculate median lethal concentrations (LC_{50}) for each zooplankton and fungicide.

c. Synergistic action of propiconazole and parathion:

Animals that received pretreatments were first exposed in groups of 60 using 600-mL glass beakers containing 500 mL test solution. Following pretreatment, animals were separated in groups of five and transferred to 30 mL glass beakers for subsequent exposures to parathion.

All solutions were prepared ≤48 h prior to the initiation of testing. Technical grade propiconazole, parathion-methyl, and piperonyl butoxide were procured from Sigma Aldrich (St. Louis, MO, USA).

We selected the ergosterol biosynthesis inhibiting fungicide propiconazole because it is present in several soybean rust fungicide formulations and has been shown to act synergistically with organophosphorous insecticides in fish, birds, and mammals. It has been hypothesized thay conazole fungicides induce cytochrome P450-dependent monooxygenases that result in an increased oxidative activation of organophosphorous insecticides. However, there are no studies to date testing whether the same phenomena are observed in an invertebrate system, although in *Daphnia* dose-response experiments have demonstrated that the oxon metabolite is more toxic than the parent organophosphorous compound. We also used piperonyl butoxide in our experiments because it is known to act as a non-selective cytochrome inhibitor. If propiconazole enhances toxicity of parathion through activation of cytochrome P450-dependent monooxygenases, we expected to see a decrease in the joint toxicity when piperonyl butoxide is present.

This objective was carried out with a series of four separate experiments which constitute the MS thesis of Walter Bialkowski. Results from Objective 2 were used to ascertain the toxicity of chemicals in single exposures and to determine the concentration ranges to be used in the binary exposure experiments. Effect concentrations were established by exposing animals for 24 hours in 12.5, 25, 50, 100, 400 and 1600 µg/L propiconazole prior to treatment with parathion. An exposure duration threshold was determined by exposing animals to propiconazole for 2, 12, 24 and 48 hours prior to exposure with parathion. Another series of animals were exposed to parathion first, followed by exposure to propiconazole for the same time periods.

In experiment 1, *Daphnia* ≤24 hours old were placed in 600 mL beakers for 24 hour pretreatment exposures in one of four propiconazole concentrations: 0, 100, 400, and 1600 µg/L. Two replicates were included for each treatment. Following pretreatment, animals were allocated into 30 mL beakers containing 0, 2, 4, 8, and 16 µg/L. Six post-treatment replicates were used. In experiment 2, piperonyl butoxide was used to evaluate the influence of cytochrome enzymes in enhancing the toxicity of parathion. Pretreatment (24 h of propiconazole) and post treatment (72 h of parathion) exposures commenced as above. Pretreatments included 0, 100, and 400 µg/L propiconazole with or without 933 µg/L piperonyl butoxide. This concentration of piperonyl butoxide is one-tenth the experimental 48-h LC_{50}. In experiment 4, a 48 h immobilization test was performed using both contaminants simultaneously immobilization tests were performed by preparing solutions containing combinations (16) of 0, 50, 100, and 400 µg/L propiconazole and 0, 4, 8, and 16 µg/L parathion. In experiment 4, pretreatment durations were 8, 16, and 24 hours. Propiconazole concentrations were 0, 100, and 400 µg/L and included replicates with and without piperonyl butoxide (933 µg/L). The compliment of a total 96 hour exposure time consisted of the parathion post treatments (0, 8, and 16 µg/L). For example, animals receiving an 8 h pretreatment were transferred into appropriate parathion solutions for an additional 88 h (88 + 8 = 96).

Principal Findings

We found that several soybean rust fungicides can elicit significant toxicity to unicellular algae and invertebrates at concentrations expected to occur, at least during short (4-day) periods in edge-of-field runoff. In the case of algae, the safest fungicide of those tested was tebuconazole, whereas the least safe was azoxystrobin. In the case of the invertebrate *Daphnia magna*, the safest fungicide was trifloxystrobin because of its expected low runoff concentration. The least safe fungicide for invertebrates was propiconazole. It is important to note that these "least safe" fungicides are expected to reach concentrations in runoff that exceed concentrations associated with mortality of more than 50% of exposed organisms. In addition, our study described for the first time in an freshwater invertebrate a synergistic response between the fungicide propiconazole and the insecticide parathion. The study demonstrated that this enhancement of toxicity when organisms are exposed to these two chemicals sequentially or simultaneously is mediated by an enzyme systems similar to that implicated in this interaction in vertebrates. This is a very significant result because this synergism is likely to also occur among other conazole fungicides and organophosphorous insecticides. In addition, propiconazole by itself was proven to be significantly toxic to invertebrates at concentration expected in runoff so any increase in toxicity due to coeexposure to other chemicals is of significance.

Summary

We determined the potential environmental effects of fungicides to be used in fighting soybean rust. The EPA issued emergency exemption registrations for use of these fungicides to combat this newly introduced soybean disease. It is expected that combating this disease will require the use of large amounts of fungicides within Indiana, even though their safety to humans and aquatic organisms has not been adequately documented. Annual mean and 4-day peak surface water concentrations for fungicides predicted with a model using spatially overlaid data on fungicide and land use, soil characteristics, and weather were compared to effects data from toxicity tests conducted on unicellular algae and the invertebrate *Daphnia magna*. We found that azoxystrobin can be expected to reach concentrations in runoff associated with significant toxicity to algae and that the same would occur for propiconazole in the case of invertebrates. Our study also described a synergistic response between the fungicide propiconazole and the insecticide parathion. This enhanced toxic response is likely to also occur among other conazole fungicides and organophosphorous insecticides. Our results show that further studies are needed to better characterize the environmental safety of these fungicides.

Results and Significance

a. Fungicide concentrations in runoff

Using the National Agricultural Pesticide Risk Analysis (NAPRA) for predicting runoff and leaching pesticide concentrations, we developed runoff concentration distributions for Indiana that would occur under normal conditions when the fungicides are applied per label recommendations. The following figures show the distribution of runoff concentrations likely to be observed across Indiana. The annual 5th percentile values represent the annual average runoff concentration likely to be observed during the worst (i.e., more prone to high fungicide runoff) five out of a hundred years being simulated. The 4-day maximum, 50th percentile values represent the most likely (i.e., median or 50th percentile) maximum concentration measured over a 4-day period during the year; whereas the 4-day maximum concentration, 5th percentile values are also calculated from the maximum concentration measured over a 4-day period during the year, but observed only during the worst (i.e., more prone to high fungicide runoff) five out of a hundred years being simulated. In the case of the toxicity evaluation conducted under the present funding, the most relevant distributions are those representing 4-day maxima. Chronic algal toxicity tests are conducted over a period of 3-4 days and the relevant toxicity endpoint is the IC_{50} over that period. Exceedance of an IC_{50} by the simulated 4-day maximum values would indicate that a significant decrease in algal biomass might be observed. On the other hand, acute toxicity tests using invertebrates also last 4 days, so again the most relevant simulated concentrations are those calculated as the 4-day maximum.

Figure 1

Distribution of Indiana lands receiving runoff concentrations of Azoxystrobin under per-label application conditions (annual values in µg/L; 4-day values in mg/L).

- **Annual, 5th Percentile**
  - Mean 18.88
  - SD 3.526
  - Median 19.26
- **4-day Max, 50th Percentile**
  - Mean 0.180
  - SD 0.051
  - Median 0.173
- **4-day Max, 5th Percentile**
  - Mean 0.249
  - SD 0.0626
  - Median 0.242

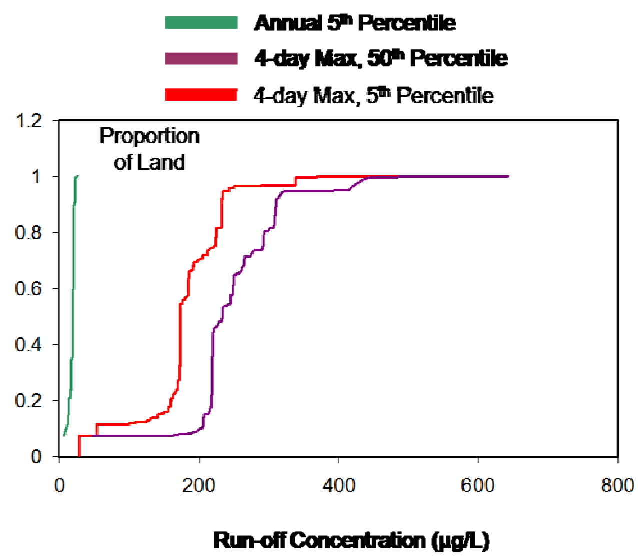


Figure 2

Distribution of Indiana lands receiving runoff concentrations of Myclobutanil under per-label application conditions (annual values in µg/L; 4-day values in mg/L).

- **Annual, 5th Percentile**
  - Mean 7.08
  - SD 2.139
  - Median 6.67
- **4-day Max, 50th Percentile**
  - Mean 0.098
  - SD 0.034
  - Median 0.103
- **4-day Max, 5th Percentile**
  - Mean 0.169
  - SD 0.0406
  - Median 0.173

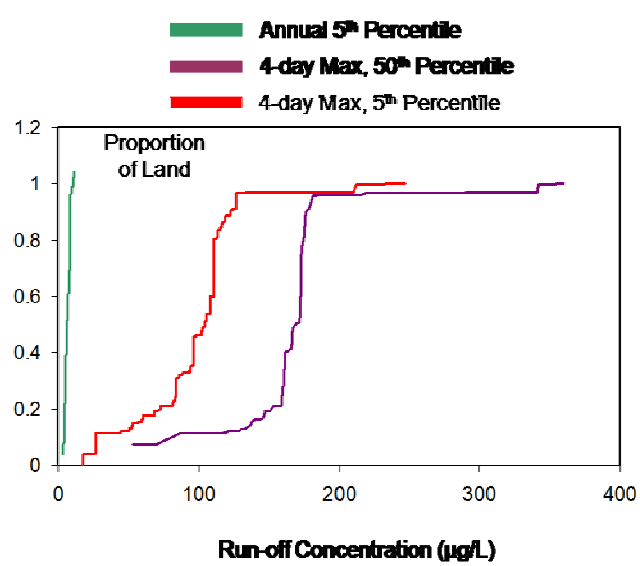


Figure 3

Distribution of Indiana lands receiving runoff concentrations of Propiconazole under per-label application conditions (annual values in µg/L; 4-day values in mg/L).

- **Annual, 5th Percentile**
  - Mean 26.9
  - SD 5.70
  - Median 26.16
- **4-day Max, 50th Percentile**
  - Mean 0.189
  - SD 0.0543
  - Median 0.192
- **4-day Max, 5th Percentile**
  - Mean 0.286
  - SD 0.0644
  - Median 0.278

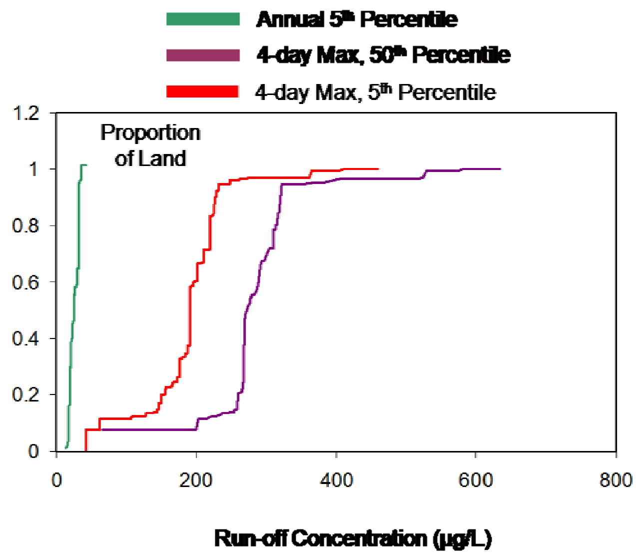


Figure 4

Distribution of Indiana lands receiving runoff concentrations of Tebuconazole under per-label application conditions (annual values in µg/L; 4-day values in mg/L).

- **Annual, 5th Percentile**
  - Mean 30.01
  - SD 6.30
  - Median 28.28
- **4-day Max, 50th Percentile**
  - Mean 0.106
  - SD 0.0279
  - Median 0.109
- **4-day Max, 5th Percentile**
  - Mean 0.153
  - SD 0.034
  - Median 0.149

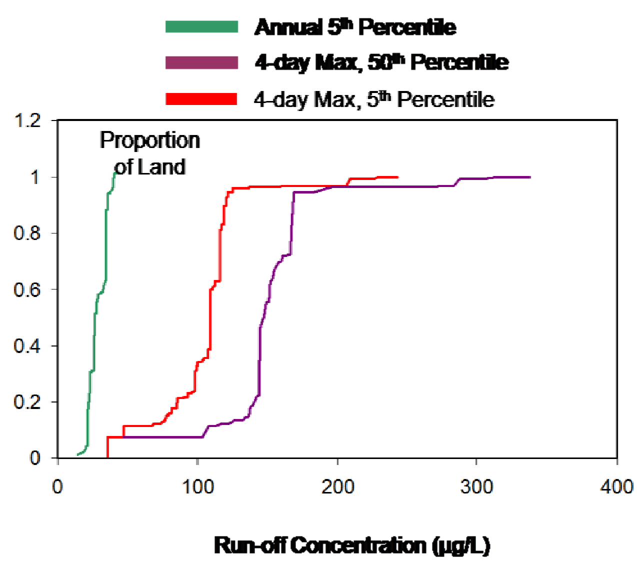
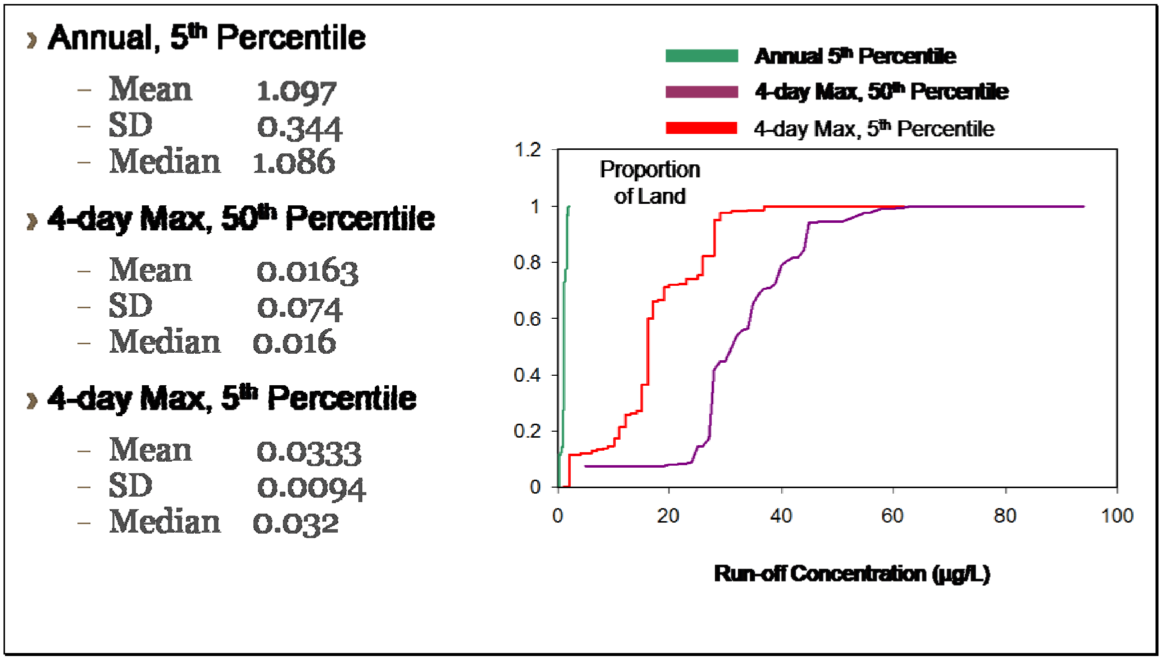


Figure 5
Distribution of Indiana lands receiving runoff concentrations of Trifloxystrobin under per-label application conditions (annual values in µg/L; 4-day values in mg/L).



b. <u>Toxicity of soybean rust fungicides to algae</u>

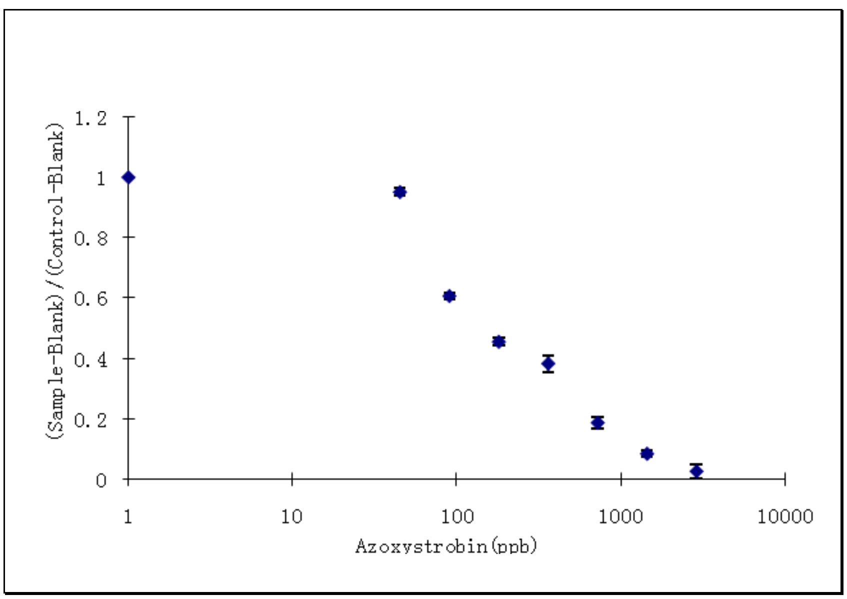
We tested the toxicity of some fungicide formulations to have a preliminary evaluation of the relative toxicity of soybean rust fungicides to algae. As can be seen in Table 1, the No Observe Effect Concentration (NOEC) and the IC_{50} varied by two orders of magnitude among the formulations tested. Although one of the fungicides tested seems unlikely to reach runoff concentrations that might have adverse effects on freshwater algae (e.g., tebuconazole), modeled concentrations for the other fungicides exceeded at least the NOEC after correction for the concentration of active ingredient in the formulation. Myclobutanil, azoxystrobin, and propiconazole all had 5th percentile 4-day maximum concentrations predicted to approach or exceed the IC_{50} over a significant proportion of Indiana's agricultural land.

Table 1
Fungicide formulations tested using freshwater algae, their calculated toxicity endpoints, and modeled runoff concentrations.

Fungicide Formulation	Active Ingredient	Active ingredient concentration	NOEC (µg/L)	IC_{50} (µg/L)	4-day Maximum Runoff (µg/L)	
					50th perct.	5th perct.
Domark®	tetraconazole	20.70%	16	800	NA	NA
Folicur®	tebuconazole	38.70%	1500	5200	106	150
Laredo®	myclobutanil	25%	60	880	100	170
Quadris®	azoxystrobin	22.9	18	55	**180**	**250**
Tilt®	propiconazole	41.80%	25	600	190	290

To confirm the results obtained for Quadris®, we conducted toxicity tests of algae using the pure active ingredient. As can be seen in Figure 6, azoxystrobin elicited toxicity below 45 µg/L, with a calculated IC_{50} of 200 µg/L. The higher toxicity of the formulation, as compared to the active ingredient alone may be due to enhanced toxicity due to the presence of surfactants or other compounds in the formulation. We are currently performing these tests for all the other active ingredients.

Figure 6
Dose response curve of *Selenastrum capricornutum* unicellular alga biomass to azoxystrobin.



c. Toxicity of fungicides to the invertebrate *Daphnia magna*

We selected *Daphnia magna* as our model organism because of its importance in aquatic food webs and also because is a species routinely used in toxicity testing of water contaminants. *Daphnia* are members of the crusteacean Class Branchiopoda (Thorp and Covich 2001). Branchiopods are planktonic and have flat, leaflike appendages (called phylopods) that are used to collect suspended food1. Functionally, this Class is involved in aquatic communities as prey items for fish, birds, and other aquatic predators.

Because of their potential toxicity to invertebrates, as suggested by the literature and our results using algae, we focused this part of the funded project on azoxystrobin, trifloxystrobin, and propiconazole. Figures 7 through 9 show the increase in mortality of *Daphnia* in response to increases in the concentration of the three fungicides tested. Table 2 presents the NOEC and LC_{50} calculated from the data, as well as the simulated runoff concentrations for Indiana. For azoxystrobin and propiconazole, toxicity to invertebrates is likely under the simulated scenario, as the projected runoff concentrations are very close or even exceed the LC_{50}.

Figure 7
Dose response curve of *Daphnia magna* 4-day mortality to azoxystrobin.

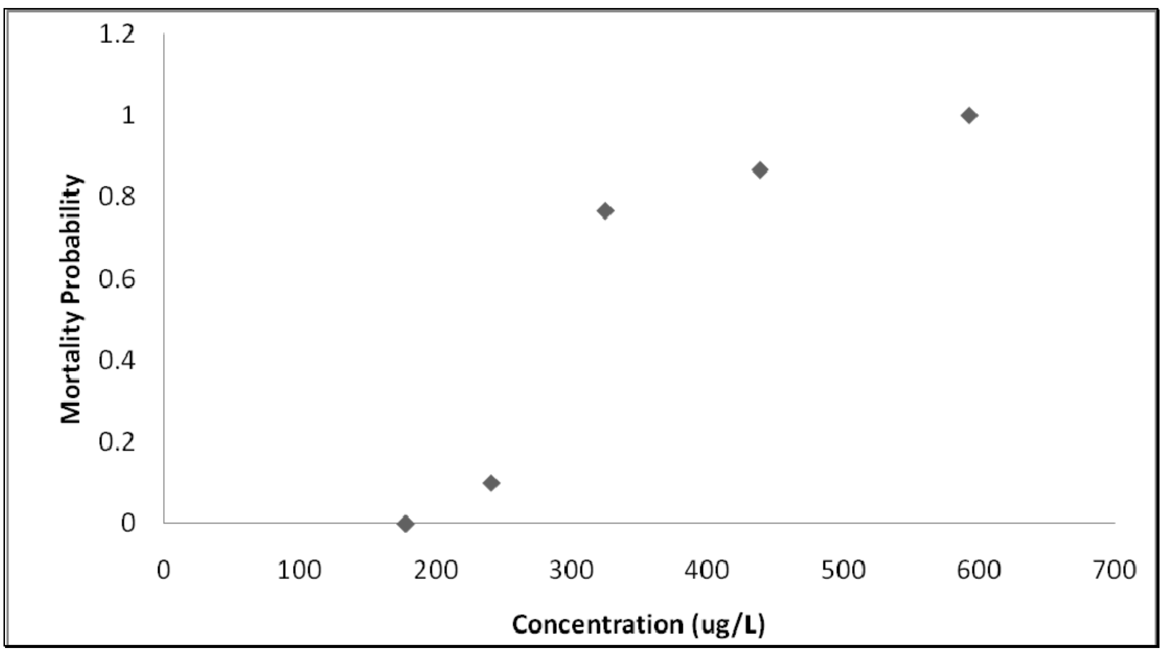


Figure 8
Dose response curve of *Daphnia magna* 4-day mortality to trifloxystrobin

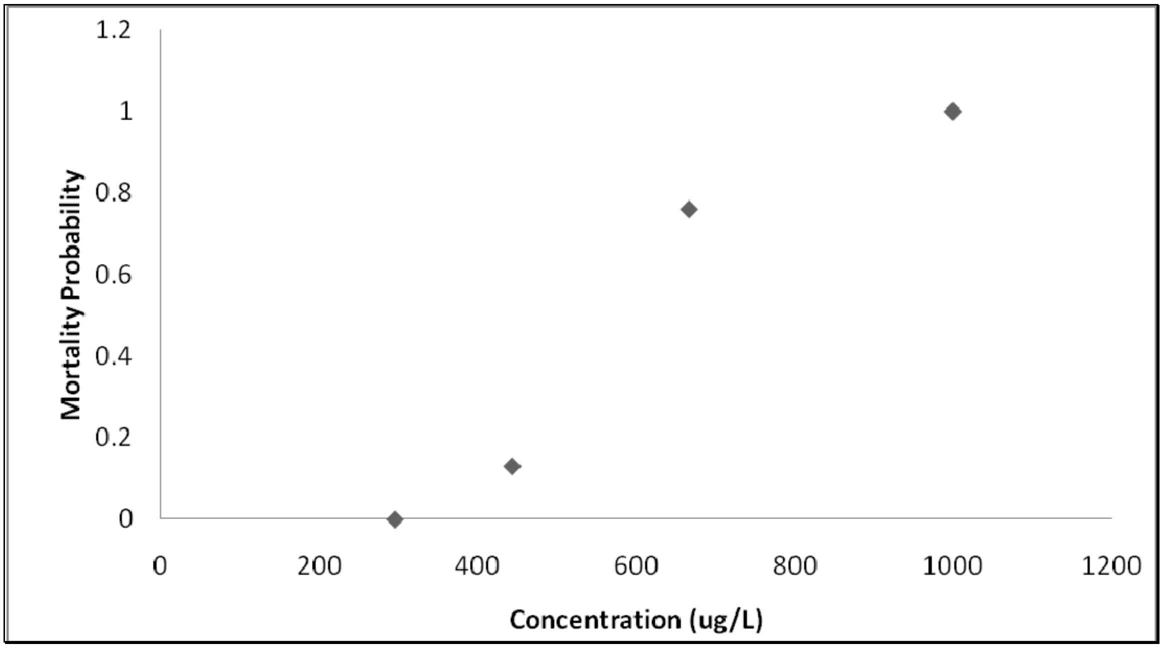


Figure 9
Dose response curve of *Daphnia magna* 4-day mortality to propiconazole

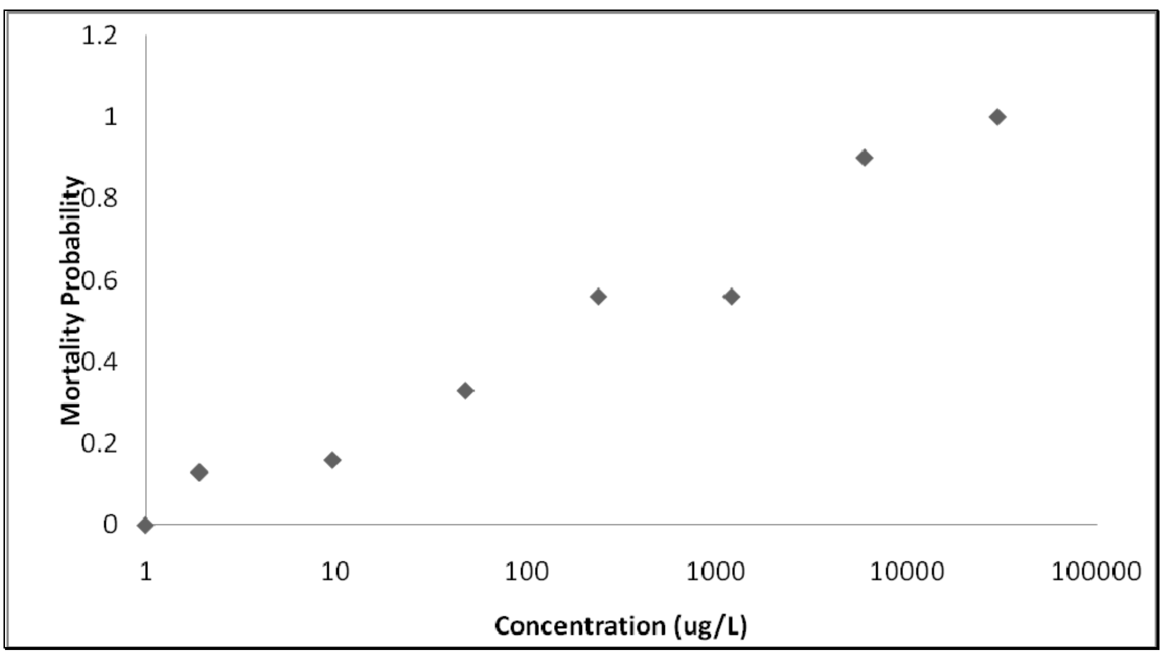


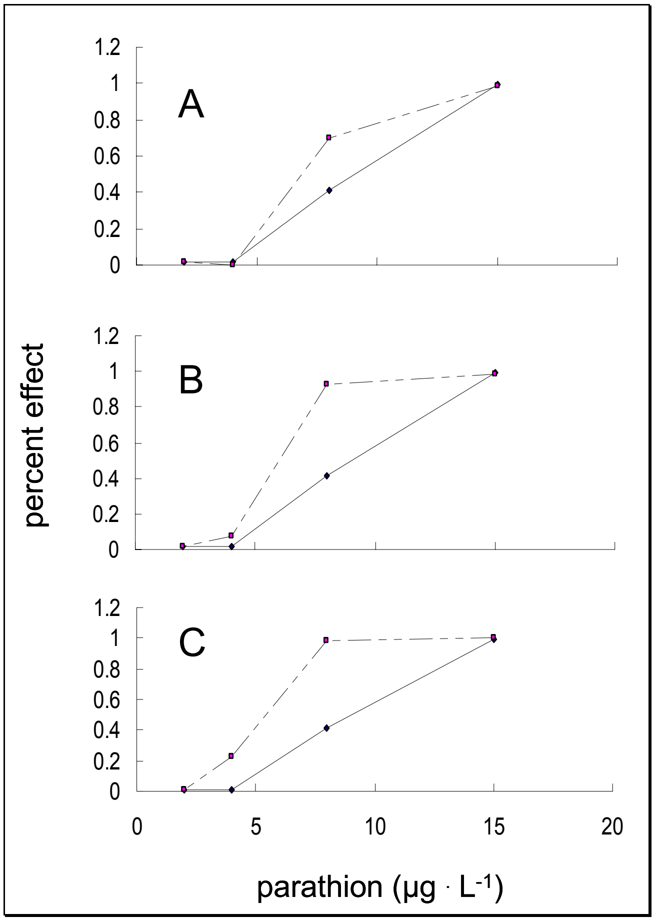
Table 2
Fungicide active ingredients tested using the invertebrate *Daphnia magna*, their calculated toxicity endpoints, and modeled runoff concentrations

Active Ingredient	**NOEC (µg/L)**	**LC_{50} (µg/L)**	**4-day Maximum Runoff (µg/L)**	
			50th perct.	**5th perct.**
azoxystrobin	180	310	180	250
trifloxystrobin	300	560	16	33
propiconazole	1	230	190	**290**

d. Synergistic action of propiconazole and parathion

Daphnia that received pretreatment with propiconazole for 24 h prior to 72 h exposure with 0, 4, 8, and 16 µg/L parathion demonstrated increased mortality relative to controls (i.e., groups exposed only to parathion) at several concentrations (Figure 10). These significant increases in toxicity are beyond what would be expected simply by exposing the animals to an additional chemical since the concentrations of propiconazole that elicited these responses were well below concentrations associated with direct toxicity.

Figure 10
Percent mortality of *Daphnia magna* exposed to 0, 4, 8, and 16 µg/L parathion. Solid lines indicate 24 h pretreatment with control solution; dashed lines represent 24 h pretreatment with 100 (A), 400 (B), and 1600 (C) µg/L propiconazole.



Piperonyl butoxide effectively eliminated the mortality observed in the 0, 100, and 400 µg/L propiconazole pretreated animals (Table 3). Treatment with 933 µg/L piperonyl butoxide alone had an effect on the survivability of *Daphnia* in one treatment (Table 3). This observation is most likely the result of random mortality events and is still below the observed mortality for the control. Two important observations were made: (1) animals receiving piperonyl butoxide in the pretreatment medium were less likely to die than animals treated with propiconazole alone, and, (2) pretreatment with piperonyl butoxide alone did not significantly affect the survivability of *Daphnia*. These results imply that by not causing mortality by itself, piperonyl butoxide is an effective cytochrome inhibitor for tests with *Daphnia*.

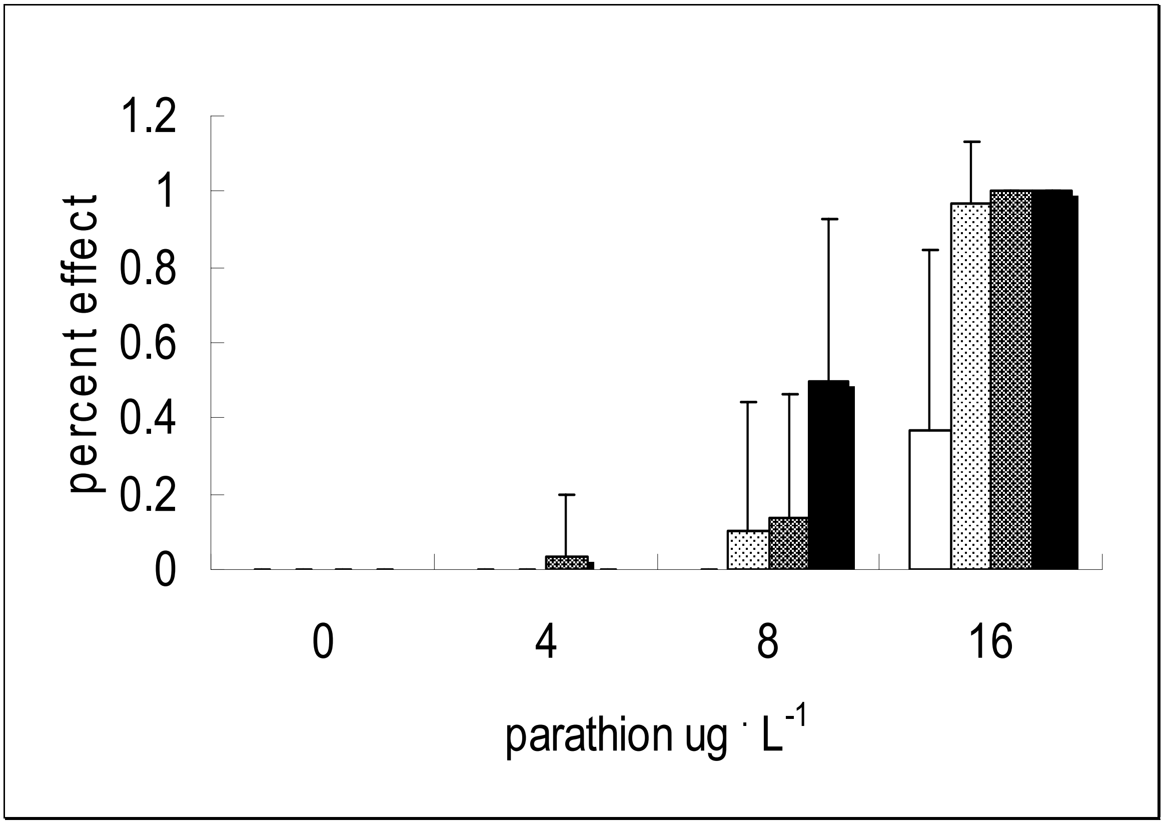
Table 3
Mortality probabilities for *Daphnia magna* exposed to 16 μg/L parathion following exposure with propiconazole pretreatments.

Mortality probabilities for *Daphnia magna* exposed to 16 μg/L parathion following exposure to various propiconazole pretreatments.			
		PBO[b]	
pretreatment duration[a]	propiconazole (μg/L)	absent	present
8	0	0	0.05
8	100	0.3	0.1
8	400	0.95[c]	0
16	0	0.85[c]	0
16	100	0.85[c]	0
16	400	1.0[c]	0
24	0	0.05	0
24	100	0.05	0
24	400	0.5	0

[a]Pretreatment exposure duration (h)
[b]933 μg/L piperonyl butoxide presence/absence in pretreatment
[c]P≤0.05 (T≤t) one-tailed paired differences of means

Daphnia magna exposed simultaneously to both propiconazole and parathion demonstrated similar dose-dependent responses as animals receiving pretreatments (Figure 11). No significant increases in mortality were observed for animals exposed in 0 or 4 μg/L parathion, regardless of the presence of propiconazole. However, significant increases were observed for animals exposed to 50 (P≤0.05), 100 (P≤0.05), and 400 μg/L (P≤0.001) propiconazole relative to controls at 8 μg/L. At 16 μg/L parathion all concentrations of propiconazole (50, 100, and 400 μg/L) significantly enhanced the toxic effect of parathion (P≤0.001).

Figure 11
Mortality probability of *Daphnia magna* simultaneously exposed to 0, 4, 8, and 16 µg/L parathion and to 0☐ 50: 100: and 400: µg/L propiconazole. Error bars represent standard errors.



Major Conclusions

The large acreage annually devoted to soybean production and the need to prevent and treat soybean rust may result in significant fungicide inputs to our freshwater systems. The results of this study suggest that at least some fungicides to be used against soybean rust could reach concentrations in runoff associated with significant adverse effects to aquatic organisms that are the basis for healthy fisheries and a stable and productive aquatic ecosystem.

Publications: Several publications are in preparation, with at least two being part of the MS thesis by Walter Bialkowski, due to graduate in July 2007. In addition, the following oral and poster presentations are a result of the funded work:

Cerbin S, Biallkowski W, Ochoa-Acuña H. 2006. Effect of Propiconazole and Strobilurin Fungicides alone or in combination on two algal species: Selenastrum

capricornutum and Chlorella vulgaris. Society for Environmental Toxicology and Chemistry North America 27th Annual Meeting, Montreal, Quebec, Canada.

Ochoa-Acuña H, Bialkowski W, Cerbin S, Hahn L, Engel B. 2006. Probabilistic Evaluation of Potential Environmental Effects Caused by Soybean Fungicides use in Indiana. Society for Environmental Toxicology and Chemistry North America 27th Annual Meeting, Montreal, Quebec, Canada.

Bialkowski W, Cerbin S, Ochoa-Acuña H. 2006. Acute and Chronic Toxicities of Parathion and Propiconazole Mixtures on the Aquatic Invertebrate, *Daphnia magna*. Society for Environmental Toxicology and Chemistry North America 27th Annual Meeting, Montreal, Quebec, Canada.

Hahn L, Zhai T, Lim K, Ochoa-Acuña H, and Engel B. 2006. Potential Water Quality Impacts of Asian Soybean Rust Fungicide Applications in Indiana. Society for Environmental Toxicology and Chemistry North America 27th Annual Meeting, Montreal, Quebec, Canada.

Students

Graduate Students: Walter Bialkowski, MS student, Comparative Pathobiology; Guo Yu, PhD student, Civil Engineering

Undergraduate Students: Stefan Cerbin, Shelby Koon,

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